Journal of Microbiology (2016) Vol. 54, No. 3, pp. 202–211
DOI 10.1007/s12275-016-5625-7
eISSN 1976-3794
pISSN 1225-8873

REVIEW

Innate host defenses against *Cryptococcus neoformans*

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(Received Dec 16, 2015 / Revised Jan 11, 2016 / Accepted Jan 11, 2016)

***Cryptococcus neoformans,* the predominant etiological agent of cryptococcosis, can cause life-threatening infections of the central nervous system in immunocompromised and immunocompetent individuals. Cryptococcal meningoencephalitis is the most common disseminated fungal infection in AIDS patients, and remains the third most common invasive fungal infection among organ transplant recipients. The administration of highly active antiretroviral therapy (HAART) has resulted in a decrease in the number of cases of AIDS-related cryptococcosis in developed countries, but in developing countries where HAART is not readily available, *Cryptococcus* is still a major concern. Therefore, there is an urgent need for the development of novel therapies and/or vaccines to combat cryptococcosis. Understanding the protective immune responses against *Cryptococcus* is critical for development of vaccines and immunotherapies to combat cryptococcosis. Consequently, this review focuses on our current knowledge of protective immune responses to *C. neoformans*, with an emphasis on innate immune responses.**

***Keywords*:** *Cryptococcus neoformans*, *Cryptococcus*, fungal pathogenesis, host-fungal interactions, fungal immunity

Introduction

Cryptococcus neoformans is a ubiquitous encapsulated opportunistic fungal pathogen that causes fungal pneumonia and life-threatening infections of the central nervous system (CNS) in immunocompromised patients, especially in recipients of organ transplants or individuals with AIDS (Park *et al.*, 2009; Pappas *et al.*, 2010; Andama *et al.*, 2013). Cryptococcosis was previously thought to be a disease observed only in immunocompromised individuals, but increasing evidence now points to infection in immunocompetent hosts as well (Husain *et al.*, 2001; Chen *et al.*, 2008; Choi *et al.*, 2011; Panackal *et al.*, 2015). Although *C. neoformans* is most often acquired via the pulmonary route, few patients present with a clinical picture of pneumonia (Levitz, 1991; Powderly, 1993; Mitchell and Perfect, 1995; Baddley *et al.*, 2008; Wu *et al.*, 2009). Protection against *C. neoformans* is predominantly orchestrated by T helper(h)1-type $CD4^+$ T cell-mediated immune responses (Huffnagle *et al.*, 1991; Powderly, 1993; Huffnagle *et al.*, 1994; Murphy, 1998; Milam *et al.*, 2007; Zhang *et al.*, 2009; Leopold Wager *et al.*, 2014). However, the first line of defense against *C. neoformans* is the responsibility of the various components comprising the innate immune system. Thus, this review will primarily focus on the innate immune response to *C. neoformans.*

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Adaptive immunity

The patient populations most at risk for developing *C. neoformans* infection have severe defects in T cell function. Th1-type $CD4^+$ T cells direct the protective host immune response against *C. neoformans* (Casadevall, 1995; Zhang *et al.*, 2009), whereas a Th2-type immune response is non-protective (Arora *et al.*, 2005; Jain *et al.*, 2009). A Th1-type response is characterized by the production of interleukin (IL)-2, IL-12, interferon gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α) (Milam *et al.*, 2007; Jain *et al.*, 2009). Th1-type cytokines induce enhanced uptake and killing by neutrophils and dendritic cells, induce macrophages to polarize to a classically activated, M1 type, phenotype, and promote clearance of the organism (Wozniak *et al.*, 2009, 2011; Arora *et al.*, 2011; Hardison *et al.*, 2012; Leopold Wager and Wormley, 2014). A Th2-type response is characterized by the production of IL-4, IL-5, IL-9, IL-10, and IL-13 (Milam *et al.*, 2007; Jain *et al.*, 2009) and is associated with a significant chemotaxis of eosinophils to the lungs, the induction of alternately activated, M2 type, macrophages and dissemination of the pathogen (Olszewski *et al.*, 2001; Muller *et al.*, 2007; Arora *et al.*, 2011; Davis *et al.*, 2013). Th17-type responses consisting of IL-17A, IL-21, IL-22, IL-23, and TGF-β (reviewed in [Onishi and Gaffen, 2010]) have been suggested to be important in protection against *C. neoformans* (Murdock *et al.*, 2014). However, inhibition of IL-17A production and/or signaling has no effect on survival of mice with experimental pulmonary *C. neoformans* infection (Wozniak *et al.*, 2011), and IL-17A is not required for M1 macrophage activation (Hardison *et al.*, 2010b). Thus, Th17-type responses appear to play a secondary role to the protection orchestrated by the Th1-type response (Kleinschek *et al.*, 2010).

Patients with defects in antibody mediated immunity (AMI) such as hypogammaglobulinemia, hyper-IgM syndrome or X-linked immunodeficiency (XID) have an increased probability of developing cryptococcosis. Anti-cryptococcal antibodies can act as opsonins, participate in antibody-dependent cellular cytotoxicity, augment Th1-type polarization and help to eliminate immunosuppressive polysaccharide antigen from serum and tissues (Martinez and Casadevall, 2005; Casadevall and Pirofski, 2007; McClelland *et al.*, 2010; Brena *et al.*, 2011). Naturally occurring IgM (nIgM) is essential for the prevention of cryptococcosis in mice (Subramaniam *et al.*, 2010). Cryptococcal disease is more severe in mice lacking serum IgM (sIgM) and in sIgM knockout mice. Experimental pulmonary *C. neoformans* infection of sIgM knockout mice results in increased mortality and higher blood and brain fungal burden compared to that observed in infected wild-type mice (Subramaniam *et al.*, 2010). XID mice lack B-1 B cells and natural IgM, and these mice exhibited increased dissemination during a pulmonary *C. neoformans* infection compared to control mice (Szymczak *et al.*, 2013). Mice that were ablated of B-1 cells presented higher brain and lung fungal burdens and reduced phagocytosis of cryptococci by alveolar macrophages compared to control mice or mice reconstituted with B-1a B cells (Rohatgi and Pirofski, 2012). In line with the mouse studies, there also is evidence that HIV infected patients that have lower IgM memory B-cell levels are more susceptible to cryptococcosis (Subramaniam *et al.*, 2009). These data indicate a role for B-1 B cells and IgM in protection against *C. neoformans.*

Innate immune responses

While adaptive cell-mediated immunity (CMI) is universally regarded as needed for a protective immune response, the first line of defense against *C. neoformans* is, of course, the innate immune system. Cells of the monocyte lineage including macrophages and dendritic cells (DCs) are necessary to direct the protective immune response to *C. neoformans*. There is an increase in neutrophils and eosinophils in the pulmonary tissues during human infection (Yamaguchi *et al.*, 2008). As the patient populations most at risk for developing progressive *C. neoformans* disease have severe defects in their adaptive CMI, understanding how a protective innate immune response can be induced is critical for development of vaccines and immunotherapies to prevent and/or treat cryptococcosis.

Macrophages

Cells of the monocytic lineage have the ability to polarize to different activation phenotypes depending on their exposure to specific stimuli (Davis *et al.*, 2013; Leopold Wager and Wormley, 2014). When macrophages are exposed to stimuli that induce Th1-type responses, such as IFN-γ and/or microbial products like lipopolysaccharide (LPS), they differentiate into classically activated macrophages, also known as M1 macrophages (Leopold Wager and Wormley, 2014). These M1 macrophages produce high levels of reactive oxygen and nitrogen species and pro-inflammatory cytokines such as IL-12 and TNF-α. The hallmark marker of M1 activation is the production of inducible nitric oxide synthase (iNOS). iNOS acts on arginine reducing it to citrulline and nitric oxide (NO) (McNeill *et al.*, 2015). These M1 macrophages switch their metabolism from mitochondrial oxidative phosphorylation to glycolysis (Kramer *et al.*, 2014). M1 activated macrophages are efficient killers of intracellular pathogens. When macrophages are exposed to Th2-type cytokines such as IL-4 and IL-13 and/or parasitic products and allergens, they differentiate into alternatively activated macrophages, also referred to as M2 macrophages (Davis *et al.*, 2013; Leopold Wager and Wormley, 2014). These macrophages are anti-parasitic and have a role in tissue repair (Allen and Sutherland, 2014; Ruckerl and Allen, 2014). The markers of M2 activation are chitinase-like 3 (Ym1), found in inflammatory zone (FIZZ1), CD206 and arginase-1 (Arg1). In an M2 macrophage, Arg1 is produced and acts on arginine to produce urea, polyamines, and ornithine (Leopold Wager and Wormley, 2014). In mice, iNOS and arginase are hallmarks of M1 and M2 macrophage activation, respectively. These compete for the same substrate, L-arginine, thereby either reducing or increasing the amount of NO production in the macrophage. The activation state of the macrophage can determine the outcome of the host response. However, M1 and M2 macrophage activation phenotypes *in vivo* may not be as clear as it appears *in vitro* as it is common to find a mixed macrophage activation phenotype *in vivo*. Thus, it may be more appropriate to describe macrophage polarization *in vivo* as M1 or M2 skewed macrophages.

M1 skewed macrophages are associated with protection against *C. neoformans*, whereas M2 skewed macrophages are non-protective (Arora *et al.*, 2011; Hardison *et al.*, 2012; Leopold Wager *et al.*, 2014, 2015; Kushwah, 2011). Experimental pulmonary infection with *C. neoformans* results in the induction of strong Th2-type responses and induction of M2 skewed macrophages. Likewise, mice deficient in the Th1-type cytokine IFN-γ have increased Th2-type cytokine production and M2 skewed macrophage activation in the lungs during pulmonary *C. neoformans* infection (Arora *et al.*, 2005). The Th2-type cytokine IL-13 promotes M2 differentiation, Th2-type cytokine responses, and allergic inflammation during experimental pulmonary cryptococcosis in mice (Muller *et al.*, 2007). Arora *et al.* (2011) examined the relationship between Th1 and Th2 cytokines on macrophage activation in a chronic *C. neoformans* infection model using *C. neoformans* strain 52D in C57B6 mice. In this model, the immune response is not permanently Th2 biased but changes dynamically over time. Th2-type cytokines are expressed early during infection, and Th1-type cytokines appear later (week 5) (Arora *et al.*, 2011). However, this switch to a Th1-type response did not aid in clearance of the organism, indicating that an early Th1-type immune response is critical to control of cryptococcosis. Additionally, it was shown that the cytokines IFN-γ and IL-4 regulate the polarization state of the macrophage during cryptococcal infection. A higher IL-4/IFN-γ ratio leads to M2 skewed macrophage activation, while a higher IFN-γ/IL-4 ratio leads to M1 macrophage activation (Arora *et al.*, 2011). In addition, IL-4 and IFN-γ can have additive effects in the activation of

macrophages leading to an intermediate phenotype that expresses markers of both M1 and M2 macrophages (Arora *et al.*, 2011). It has been demonstrated that macrophage activation is highly plastic. Macrophages that were polarized to a M1 phenotype by IFN-γ could re-polarize to a M2 phenotype by switching to IL-4-stimulating conditions and vice versa (Davis *et al.*, 2013).

Macrophages within the lungs of mice infected with a strain of *C. neoformans* that was modified to produce murine IFN-γ (H99γ) are predominantly of the M1 macrophage phenotype (Hardison *et al.*, 2010a, 2010b, 2012; Leopold Wager *et al.*, 2014, 2015). M1 polarization was associated with increases in Signal Transducer and Activator of Transcription 1 (STAT1) transcripts as well as phosphorylation of the STAT1 protein (Hardison *et al.*, 2012). STAT1 KO mice infected with either H99γ or a moderately virulent WT strain (52D) exhibited a significant decrease in survival, defects in M1 macrophage activation, and reduced production of NO (Leopold Wager *et al.*, 2014). Investigation of the role of STAT1 specifically in macrophages revealed that during infection with H99γ in mice with macrophage-specific STAT1 ablation a dysregulated Th1/Th2 type immune response, increased pulmonary fungal burden and deficient M1 macrophage activation was observed (Leopold Wager *et al.*, 2015). These studies indicated a role for STAT1 signaling in the activation of M1 skewed macrophages and protection during a *C. neoformans* infection.

C. neoformans has the ability to grow and proliferate inside macrophages and escape from them through non-lytic exocytosis (Alvarez and Casadevall, 2006; Nicola *et al.*, 2011). This is advantageous to the fungus as an immune evasion strategy, and exit from the macrophage may facilitate *C. neoformans* dissemination from the lungs to other organs, including the CNS and brain. One of the proposed mechanisms for *C. neoformans* crossing the blood brain barrier is by hiding inside of macrophages, thereby acting as a "Trojan horse" (Charlier *et al.*, 2009; Sorrell *et al.*, 2015) leading to dissemination of the pathogen.

Dendritic cells

In early studies examining DCs during cryptococcal infection, Bauman *et al.* (2002) classified the DC subset needed for the protective immune response to *Cryptococcus* (Bauman *et al.*, 2000). Mice were immunized with cryptococcal culture filtrate and complete freund's adjuvant (CFA) (protective) or heat-killed (HK) *C. neoformans* with CFA and the DC profile was assessed in the draining lymph nodes. These studies revealed that Langerhans cells and myeloid DCs appeared to be needed for the induction of the protective immune response against *C. neoformans* (Bauman *et al.*, 2000). DCs and alveolar macrophages are needed in the early host defense (Osterholzer *et al.*, 2009b). Depletion of $CD11c^+$ cells in CD11cDTR mice during cryptococcal infection with a *C. neoformans* serotype D strain which is non-lethal in many mouse strains led to mortality within 6 days post-infection. (Osterholzer *et al.*, 2009b). The chemokine receptor CCR2 has been shown to be important in recruiting monocyte-derived DCs. CCR2 KO mice infected with *C. neoformans* show impairment in DC recruitment and the mice developed features of a Th2-type response (Osterholzer *et al.*, 2008). It was further shown that the CCR2-dependent recruitment of DCs into *C. neoformans* infected lungs was due to increased recruitment of Ly-$6C^{high}$ monocytes that differentiate into $CD11b^+$ DCs in the lungs (Osterholzer *et al.*, 2009a) suggesting that CCR2 is required for the recruitment of DCs to the lungs during a cryptococcal infection.

One of the main virulence factors of *Cryptococcus* is the anti-phagocytic polysaccharide capsule that is comprised primarily of the polysaccharides glucuronoxylomannan (GXM) and galactoxylomannan (GalXM) and to a much lesser extent, <1%, mannoproteins (MP) (Zaragoza *et al.*, 2009). Cryptococcal polysaccharides such as GXM have profound suppressive effects on immune responses (Yauch *et al.*, 2006; Zaragoza *et al.*, 2009). Cryptococcal capsule interferes with DC activation and maturation (Vecchiarelli *et al.*, 2003; Lupo *et al.*, 2008; Grijpstra *et al.*, 2009). Acapsular strains are phagocytosed by DCs and induce surface expression of MHC class II and other costimulatory molecules, whereas the encapsulated strains do not induce activation unless opsonized by an anti-GXM antibody which is recognized by CD32 and CD16 (Vecchiarelli *et al.*, 2003). DCs were shown to be the most efficient antigen presenting cell type to present *C. neoformans* mitogen to T cells (Syme *et al.*, 2002). Only a small number of DCs were needed for antigen presentation to T cells, and these DCs were also able to internalize the acapsular cryptococci. This is particularly important considering that DCs are the bridge between innate and adaptive immunity and their activation is critical to host survival. Acapsular mutant strains *cap56*Δ, but not *cap10*Δ, induce human DC activation as seen by increased CD80 and CD86 surface expression (Grijpstra *et al.*, 2009). In addition to affecting DC maturation, capsular material can influence DC gene expression. The encapsulated WT *C. neoformans* strain induced a down-regulation of cytokine genes and inhibited the induction of the genes noted above (Lupo *et al.*, 2008). However, DCs incubated with *cap59*Δ show an increase in DC maturation genes as well as the cytokines IL-12, IL-2, IL-1α, IL-1β, IL-6 IL-10, TNF-α, and the chemokines CCR7, CCL17, CCL22, CCL3, CCL4, CCL7, and CXCL10 as well as genes associated with antigen presentation (Lupo *et al.*, 2008).

In addition to its immunosuppressive effects, the polysaccharide capsule is anti-phagocytic. Opsonins are required for cryptococcal binding and uptake by both human and murine DCs (Kelly *et al.*, 2005). Binding of unopsonized cryptococcal cells to DCs is inefficient. Antibody opsonization can induce cryptococcal uptake and prominent anti-cryptococcal activity. Antifungal activity to opsonized *C. neoformans* was reduced when DCs were treated with inhibitors of the respiratory burst response (Kelly *et al.*, 2005). During a cryptococcal infection, within 2 h post-inoculation, fluorescently labeled cryptococci were internalized by DCs in the lungs (Wozniak *et al.*, 2006). There was an increase in CD80, CD86, and MHC class II surface expression on DCs 7 days post-infection. Culture of the DCs *ex vivo* with cryptococcal-specific T cells, led to T cell proliferation measured by IL-2 production, indicating that these DCs were capable of presenting cryptococcal antigens to the T cells (Wozniak *et al.*, 2006). Following DC phagocytosis of cryptococci, the yeast translocate to the endosomal

compartment within 10 min, fuse to the DC lysosomal compartment within 30 min, and are killed by oxidative and non-oxidative mechanisms (Wozniak and Levitz, 2008). Using a DC lysosomal extract made from bone marrow derived dendritic cell (BMDC) lysosomes, it was shown that DC lysosomal extracts kill cryptococci *in vitro* (Wozniak and Levitz, 2008). DC lysosomes exhibit fungicidal activity against all cryptococcal serotypes (Hole *et al.*, 2012). Purified lysosomal enzymes, specifically cathepsin B, inhibit cryptococcal growth. Interestingly, cathepsin B combined with its enzymatic inhibitors enhanced cryptococcal killing. Electron microscopy revealed structural changes and ruptured cryptococcal cell walls following treatment. It was further demonstrated that osmotic lysis was responsible for cryptococcal death (Hole *et al.*, 2012).

Mannoproteins (MPs) found in the cryptococcal polysaccharide capsule and fungal cell wall have been shown to induce Th1-type responses in immunized mice upon infection with *C. neoformans* (Mansour *et al.*, 2002). Cryptococcal MPs can induce DC activation and maturation (Pietrella *et al.*, 2005; Dan *et al.*, 2008a, 2008b) and induce the expression of MHC class I and MHC class II as well as CD40, CD80, and CD86 in DCs treated with purified MP (Pietrella *et al.*, 2005). Human DCs treated with MPs secrete IL-12 and TNF-α as well as lead to Iκβα phosphorylation (Pietrella *et al.*, 2005). MP loaded DCs are efficient stimulators of T cells resulting in $CD4^+$ and $CD8^+$ T cell proliferation (Pietrella *et al.*, 2005). Human and murine DCs are able to recognize and capture cryptococcal MPs by a mannose receptor (CD206) mediated process (Mansour *et al.*, 2006). Another C-type lectin receptor (CLR) DC-SIGN (CD209) was also shown to have an affinity for mannoproteins, suggesting that multiple mannose receptors on DCs are able to recognize MPs (Mansour *et al.*, 2006). Cryptococcal MPs in combination with Toll-like receptor (TLR) ligands enhanced production of proinflammatory cytokines and chemokines from DCs as well as heightened MP-specific MHC class II-restricted $CD4^+$ T-cell responses (Dan *et al.*, 2008a). The CLR Dectin-2 has been shown to recognize mannan from multiple fungal organisms. Dectin-2 KO BMDCs infected with *C. neoformans* failed to produce IL-12p40 and TNF-α as well as failed to express CD86 and MHC class II on the cell surface when compared to WT BMDCs (Nakamura *et al.*, 2015).

In addition to MPs, there are many other cryptococcal products that are recognized by DCs. TLR9 recognition of cryptococcal DNA can activate DCs to produce IL-12p40 and express CD40 (Nakamura *et al.*, 2008). The DNA methylation pattern is necessary for DC TLR9 activity as treatment of the DNA with methylase led to a reduction of IL-12p40 produced by the DCs. In addition to TLR9, DNA recognition and activation by the DCs was also dependent on MyD88 as KO of TLR9 or MyD88 completely nullified the effect of cryptococcal DNA on the DCs (Nakamura *et al.*, 2008). Culture supernatants from *C. neoformans* are able to dampen the DC response to cryptococcal DNA (Yamamoto *et al.*, 2011). The inhibitor effects of the supernatants were reduced by heat or trypsin treatment indicating that *C. neoformans* secretes proteinaceous molecules that suppress activation of DCs by cryptococcal DNA (Yamamoto *et al.*, 2011). It was shown that the cryptococcal URA5 gene specifically activates DCs through a TLR9-mediated signaling pathway using a mechanism that is different from the canonical CpG motif that is associated with TLR9 signaling (Tanaka *et al.*, 2012). TLR9 was also shown to be important in a chronic model of *C. neoformans* infection. Ablation of TLR9 lead to reduction of $CD11b^+$ DCs and CCL7 during the afferent phase (week 1) and reduced the pulmonary accumulation of $CD11b^+$ DCs during the efferent phase (week 3) (Qiu *et al.*, 2012).

Cryptococcal infection with wild-type *C. neoformans* induces a Th2-type immune response, which is generally detrimental during cryptococcal infections. Urease produced by *C. neoformans* induces a strong Th2-type immune response and leads to significantly increased immature DCs in the lung-associated lymph nodes (Osterholzer *et al.*, 2009c). Furthermore, a tetramer for CDA2 induced *Cryptococcus*-specific Th2 $CD4^+$ T cells. Use of this tetramer showed that chitin recognition via chitotriosidase leads to the induction of Th2-type T cells by lung-resident $CD11b^+$ IRF4-dependent conventional DCs, indicating that DCs can have either a protective or non-protective role against cryptococcal infections (Wiesner *et al.*, 2015). Although the Th2-type response is considered detrimental, infection of IL-4Ra KO mice led to increased pulmonary fungal burden, reduced IFN-γ and NO production, and defective macrophage and DC recruitment to the lungs compared to control mice, suggesting that IL-4Ra signaling may be important during early cryptococcal infection (Grahnert *et al.*, 2014). Additionally, DCs cultured with *C. neoformans* and IL-4 produced increased IL-12 and reduced IL-10 compared to untreated DCs, showing that IL-4 (a Th2-type cytokine) may induce DCs to produce Th1-type cytokines (Grahnert *et al.*, 2014).

Neutrophils

Neutrophils are phagocytic cells that traffic to the site of infection to kill and degrade pathogens, but do not present antigen. Neutrophils have been shown to be important in defenses against many fungal pathogens, including *Aspergillus* and *Candida,* as patients that are neutropenic are at a high risk of developing certain fungal diseases (Walsh and Gamaletsou, 2013; Gedik *et al.*, 2014; Kosmidis and Denning, 2015; Loschi *et al.*, 2015; Oz *et al.*, 2015). *In vitro*, human polymorphonuclear leukocytes (PMNs) have been shown to have anti-cryptococcal activity by oxidative and non-oxidative mechanisms including hydrogen peroxide, hypochlorous acid, hydroxide, calprotectin, and defensins (Chaturvedi *et al.*, 1996; Mambula *et al.*, 2000). Myeloperoxidase (MPO) KO mice infected with *C. neoformans* either intranasally or intravenously succumb to the infection faster than WT mice (Aratani *et al.*, 2006). In addition to opsonizing *C. neoformans,* complement, specifically C5b, can act as a chemoattractant causing neutrophils to move towards the C5b coated *C. neoformans* as observed *in vitro* (Sun *et al.*, 2015). However, even though neutrophils can kill *C. neoformans,* the organism can inhibit neutrophil migration, neutrophil extracellular trap (NET) formation, killing, and respiratory burst (Chaturvedi *et al.*, 1996; Coenjaerts *et al.*, 2001; Ellerbroek *et al.*, 2004; Qureshi *et al.*, 2011; Rocha *et al.*, 2015).

Neutrophils were found to be the predominant source of IL-17A in mice infected with a strain of *C. neoformans* that was modified to produce murine IFN-γ (H99γ) (Wozniak *et al.*, 2011). However, neutrophil depletion in protectively immunized mice did not affect pulmonary fungal burden, indicating that neutrophils are not required for clearance (Wozniak *et al.*, 2012). These data corroborate earlier studies showing increased survival of neutropenic mice compared to control mice during pulmonary *C. neoformans* infection, suggesting that neutrophils are not required for protective anti-cryptococcal responses in the mouse model of infection (Mednick *et al.*, 2003)

Natural killer cells

Primary human NK cells possess anti-cryptococcal activity. They constitutively express both granulysin and perforin, but only perforin is needed for NK cell anti-cryptococcal activity (Ma *et al.*, 2004). Inhibiting granulysin had no effect on NK cell antifungal activity, whereas inhibiting perforin abrogated NK cell anti-cryptococcal activity (Ma *et al.*, 2004). Perforin release by NK cells is dependent on the PI3K-ERK1/2 signaling pathway (Wiseman *et al.*, 2007). Inhibition of PI3K significantly blocked NK cell killing of *C. neoformans* (Wiseman *et al.*, 2007). After perforin release and degranulation, NK cells can rearm, or recover their lytic activity. Degranulation alone was not enough to induce rearming; however exposure to *C. neoformans* provided the activation signals necessary to activate the rearming process as seen by an increase in perforin mRNA levels (Marr *et al.*, 2009). PI3K-ERK1/2 signaling has been shown to be crucial for the NK cell anti-cryptococcal activity and recently it was shown that the Src family kinases Fyn and Lyn were needed up-stream of PI3K-ERK1/2 in NK cells (Oykhman *et al.*, 2013). Inhibiting Fyn and Lyn blocked cryptococcal killing by failing to induce the polarization of perforin-containing granules to the NK cell-cryptococcal immunologic synapse (Oykhman *et al.*, 2013).

Leukocyte function-associated antigen (LFA)-1 is needed for NK cell anti-tumor activity and the β2 chain of LFA-1, CD18, can also bind to cryptococcal capsular components GXM and GalXM. However, LFA-1 is not required for NK cell anti-cryptococcal activity (Jones *et al.*, 2009). NK cells were able to bind to cryptococci, form an immunologic synapse and release perforin independently of LFA-1 (Jones *et al.*, 2009). Recently, the NK receptor NKp30 was determined to be responsible for NK cell recognition of *C. neoformans* and anti-cryptococcal activity (Li *et al.*, 2013). NKp30 expression was needed to bind to cryptococci, form an immu-

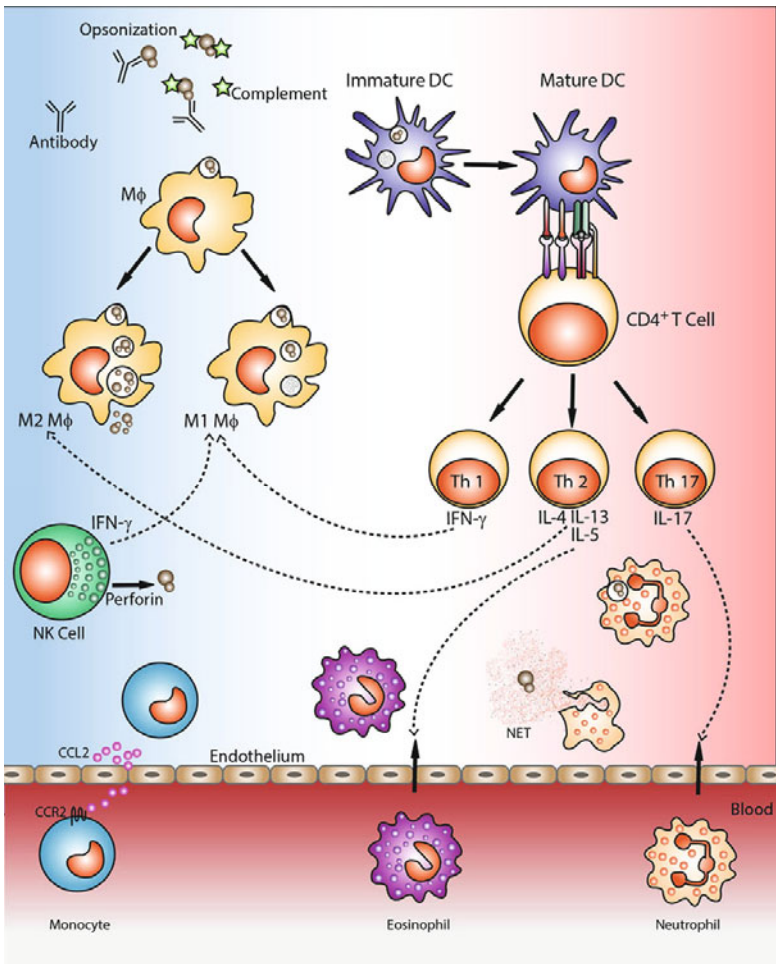

Fig. 1. The immune response to *Cryptococcus neoformans*. Opsonized *C. neoformans* is phagocytosed by macrophages and DCs in the lungs. DCs will kill *C. neoformans* by oxidative and non-oxidative mechanisms, mature, and present cryptococcal antigens to $CD4^+$ T cells initiating a T helper response. A Th1 response direct the protective host immune responses against *C. neoformans.* Th1-type cytokines induce enhanced uptake and killing by neutrophils and dendritic cells, induce macrophages to polarize to a classically activated, M1 type, phenotype, recruits monocytes to the site of infection and promote clearance of the organism. A Th2 response is associated with a significant chemotaxis of eosinophils to the lungs, the induction of alternately activated, M2 type, macrophages and dissemination of the pathogen. A Th17 response may be important in protection against *C. neoformans* and can induce neutrophil recruitment where they can kill by oxidative and non-oxidative mechanisms and/or produce NETs. DC, dendritic cell; MΦ, macrophage; IFN, interferon; IL, interleukin; Th, T helper; CCL2, monocyte chemotactic protein 1; NET, neutrophil extracellular traps.

nologic synapse, and initiate PI3K-ERK1/2 signaling for perforin release (Li *et al.*, 2013). NK cells isolated from HIV-infected patients demonstrated reduced NKp30 expression and defects in perforin release (Li *et al.*, 2013). Interestingly, IL-12 was able to restore NKp30 expression and anti-cryptococcal activity (Li *et al.*, 2013).

Eosinophils

Eosinophils are granulocytic cells that are generally thought to be associated with allergy and protective immune responses to helminth infections. In cryptococcal infection, eosinophils have been shown to interact directly with the fungus, however, pulmonary eosinophilia is associated with poor clinical outcome (Yamaguchi *et al.*, 2008). Eosinophils are able to phagocytose *C. neoformans* both *in vitro* and *in vivo* which is dependent on opsonization (Feldmesser *et al.*, 1997). Rat peritoneal eosinophils phagocytose opsonized *C. neoformans* through FcγRII and CD18 (Garro *et al.*, 2011b). While not traditionally thought to be antigen presenting cells, *C. neoformans* loaded rat eosinophils were able to activate $CD4^+$ and $CD8^+$ T cells both *in vitro* and *in vivo* through the upregulation of surface MHC class I, MHC class II, and costimulatory molecules (Garro *et al.*, 2011a, 2011b).

The inability to clear a *C. neoformans* infection has been shown to correlate with eosinophil infiltration into the lungs (Huffnagle *et al.*, 1998). In C57BL/6 mice during peak fungal burden, up to 40% of the airway leukocytes are eosinophils (Humphreys *et al.*, 2003). Eosinophils produce significant amounts of IL-4 during a chronic murine model of *C. neoformans* infection. In mice deficient in eosinophils, there is a reduction in the Th2-type response during infection and enhanced Th1 and Th17-type responses (Piehler *et al.*, 2011). Eosinophil recruitment during *C. neoformans* infection is dependent on IL-5 production (Huffnagle *et al.*, 1998; Holmer *et al.*, 2014). Mice that over-express IL-5 have increased pulmonary eosinophilia and are more susceptible to *C. neoformans* infection compared to WT mice (Holmer *et al.*, 2014). Treating mice with anti-IL-5 mAb led to a significant reduction in eosinophil recruitment, however it did not alter fungal clearance (Huffnagle *et al.*, 1998). These data suggest a detrimental role for eosinophils in the protective immune response to *C. neoformans.*

Conclusions

Understanding the protective immune response to *C. neoformans* is a daunting, yet medically important task. The acute mortality rate of patients with cryptococcal meningitis is between 10–25% in medically-advanced countries (Pyrgos *et al.*, 2013; Jarvis *et al.*, 2014), and at least one third of patients with cryptococcal meningitis who receive appropriate therapy will still have *Cryptococcus* positive CSF cultures and exhibit symptoms associated with cryptococcal meningitis (i.e. fever, headache, and meningismus) (van der Horst *et al.*, 1997; Saag *et al.*, 2000; Jarvis *et al.*, 2014). As the patient populations most at risk to developing *C. neoformans* infection have severe defects in their adaptive cell mediated immunity or are immunosuppressed due to autoimmune diseases or organ transplantation, understanding the protective innate immune response is critical for development of vaccines and immunotherapies (Fig. 1). Recent evidence has shown that natural killer (NK) cells possess adaptive immune characteristics, undergo a secondary expansion and induce a protective immune response (Sun *et al.*, 2009), and studies with monocytes has shown innate protection that is not dependent on traditional "memory" cells (Netea *et al.*, 2011; Kleinnijenhuis *et al.*, 2012; Quintin *et al.*, 2012). Cells normally regarded as only participating in the non-specific/non-adaptive immune response may have some memory-like activity. Thus the role of innate cells (NK cells, macrophages, and DCs) may possibly be modulated to provide greater protection in immune suppressed patient populations.

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