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Host immunity to *Cryptococcus neoformans*

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Abstract

Cryptococcosis is caused by the fungal genus *Cryptococcus*. Cryptococcosis, predominantly meningoencephalitis, emerged with the HIV pandemic, primarily afflicting HIV-infected patients with profound T-cell deficiency. Where in use, combination antiretroviral therapy has markedly reduced the incidence of and risk for disease, but cryptococcosis continues to afflict those without access to therapy, particularly in sub-Saharan Africa and Asia. However, cryptococcosis also occurs in solid organ transplant recipients and patients with other immunodeficiencies as well as those with no known immunodeficiency. This article reviews innate and adaptive immune responses to *C. neoformans*, with an emphasis on recent studies on the role of B cells, natural IgM and Fc gamma receptor polymorphisms in resistance to cryptococcosis.

Keywords

acquired immunity; antibodies; B cells; cryptococcosis; *Cryptococcus neoformans*; Fc receptors; host response; innate immunity; macrophages; polymorphism; T cells

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Cryptococcal disease, or cryptococcosis, is caused by a basidiomycetous yeast belonging to the genus *Cryptococcus*. This genus is unique among pathogenic fungi in having a polysaccharide capsule. Although the *Cryptococcus* genus contains many species, the majority of human infections are caused by two species: *Cryptococcus neoformans* and *Cryptococcus gattii* [1]. These species are further categorized based on the antigenic specificity of their capsular polysaccharide, with each variety constituting a separate serotype. *C. neoformans* is comprised of three varieties, *C. neoformans* var. *grubii* (serotype A), *C. neoformans* var. *neoformans* (serotype D) and a hybrid (serotype AD). *C. gattii* is comprised of two serotypes, B and C. *C. neoformans* predominantly causes disease in individuals with immune impairment, most commonly those with HIV/AIDS and CD4 T

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cells less than 50 cells/μl [2,3], whereas *C. gattii* predominantly causes disease in individuals without consistent or unifying immune defects [4]. However, the pathogenesis of *C. gattii* is an area of intense investigation as it is thought that as yet unidentified immune defects could enhance susceptibility to *C. gattii*-related cryptococcosis. This review will focus on the species *C. neoformans*.

Ecology

C. neoformans is an environmental microbe that is found in soil. Historically, the majority of human cases of cryptococcosis outside of Australia were attributed to *C. neoformans* var. *grubii* and var. *neoformans* [5]. However, a significant minority of cases in the world are due to *C. gattii* [6], which recent data show is an emerging cause of cryptococcosis in North America [7]. *C. neoformans* is a free-living microbe that does not require a host to reproduce or survive. As such, it is honed for survival, with its key virulence factors, including the polysaccharide capsule and cell wall associated melanin serving as protection from environmental assaults ranging from predators such as ameobae, UV irradiation and temperature [8–10]. Thus, *C. neoformans* is an accidental human pathogen. For a review of this topic, see reference [11].

Epidemiology

Cryptococcosis emerged as a global epidemic in patients with HIV/AIDS in the 1980s. Prior to the HIV/AIDS pandemic, there were fewer than 200 cases of cryptococcosis in the literature [12]. The occurrence of disease in HIV-infected individuals with profound CD4 T-cell deficiency highlights the central role of T-cell-mediated immunity in immunity to *C. neoformans*. However, loss of CD4 T cells alone is not sufficient for disease to occur. The use of fluconazole therapy in patients with profound CD4 T-cell deficiency and the introduction of combination antiretroviral therapy (cART) in the mid-1990s led to a marked decrease in cryptococcosis in those with access to cART [13,14]. However, cryptococcosis remains a catastrophe in under-resourced settings where cART is not available, such as sub-Saharan Africa and parts of Asia [15,16]. In 2009, Park *et al.* estimated the global burden of the disease to be close to 950,000 cases with approximately 625,000 deaths annually [16]. Cryptococcal meningitis also causes significant morbidity and mortality in the USA [17]. Compounding HIV-associated cryptococcosis-attributable morbidity and mortality is the immune reconstitution inflammatory syndrome (IRIS). There are two types of *C. neoformans*-associated IRIS; paradoxical IRIS, which is a recurrence of cryptococcosis that occurs after the initiation of cART, and unmasking IRIS, which occurs soon after the initiation of cART in patients with no prior diagnosis of cryptococcosis [18]. A recent study found that delaying the start of cART in patients presenting with HIV-associated cryptococcosis was associated with a significant reduction in mortality [19]. The occurrence of IRIS in HIV-associated cryptococcosis as well as in solid organ transplant recipients [20] reveals that excessive as well as insufficient inflammatory responses can result in disease, underscoring that the outcome of host–*C. neoformans* interaction is determined by the immune status of the patient. This fits with the paradigm put forth in the Damage response framework, whereby host damage and disease can stem from either insufficient or excessive immune responses [21].

Other forms of immunodeficiency, including that due to drugs used to prevent organ rejection also pose a risk for cryptococcosis [22], which occurs in approximately 2.8% of solid organ transplant recipients [23]. Some biologics, such as the TNF-α inhibitor adalimumab have also been linked to an increased risk for cryptococcosis [24]. Others at increased risk for cryptococcosis are pregnant women [25], and those with X-linked immunodeficiency [26], liver disease [27], idiopathic CD4 T-cell deficiency [28] and apparently immune competent individuals [29,30]. Presence of anti-GM-CSF autoantibodies was also associated with some cases of cryptococcal meningitis in immunocompetent individuals [31]. Hence, conditions associated with cryptococcosis range from profound CD4 T-cell deficiency to none that can be identified.

Pathogenesis of human Cryptococcosis

Infection with *C. neoformans* occurs by inhalation of desiccated yeast cells or spores from the environment in early childhood, most likely at the time of acquisition of other encapsulated microbes [32]. This event is not thought to be associated with clinical manifestations, although an association between childhood asthma and serological evidence of cryptococcal infection has been noted [33]. Based on serological surveys of immunocompetent and immunocompromised adults and children, cryptococcal infection is common [32,34,35]. However, disease is rare. In most, infection leads to a state of latency, most likely in the lungs, where the yeast resides in granulomata usually without evidence of clinical disease. However, in some, predominantly those with underlying immune impairment, the state of latency transitions to a state of disease as the fungal burden rises [11,36]. Although reactivation is a major cause of disease due to *C. neoformans*, particularly in those with immune impairment, disease can also follow primary acquisition [22]. Cryptococcosis is most commonly a disseminated disease characterized by meningoencephalitis and/or fungemia, but pneumonia and skin lesions can also occur.

HIV-associated cryptococcosis can be heralded by identification of cryptococcal antigen (CrAg) in the blood. Monitoring serum CrAg levels in HIV-infected individuals with CD4 T-cell levels less than 100 cells/μl in resource-limited settings in regions of high HIV/AIDS prevalence has been extremely successful and cost-effective in identifying high-risk patients who are candidates for fluconazole treatment in sub-Saharan Africa [37–40]. The success of this approach underscores the importance of rapid, point-of-care diagnostic tests [41]. CrAg screening and fluconazole treatment are a goal of the Global Action Fund for Fungal Infections (GAFFI [42]).

Cryptococcal virulence

The central determinant of virulence for *C. neoformans* is its polysaccharide capsule. There is abundant experimental evidence that the capsule is required for cryptococcal virulence in immunologically normal hosts [43,44]. The capsule is comprised primarily of glucuronoxylomannan (GXM), which can have many deleterious effects on the host response including inhibition of phagocytosis [45,46]. Fungal containment is crucial for host resistance to cryptococcosis. Thus, impairment of the function of macrophages or phagocytes by GXM or other cryptococcal virulence factors, such as melanin, enhances

cryptococcal virulence. However, *C. neoformans* is also able to replicate intracellularly and can escape the intracellular state without being killed or killing the host cell [47,48]. The latter, which could contribute to intracellular or extracellular dissemination, is one of several mechanisms that have been implicated in how *C. neoformans* enters the bloodstream and invades the CNS. For a review of cryptococcal virulence, see reference [49].

Host response to *C. neoformans*

Fungal diseases in animals and humans are relatively rare. In fact, although there are more than 1.5 million fungal species, only 300 cause human disease [50]. One determinant of the latter is that many fungal species cannot survive at mammalian thermal temperatures [51]. Importantly, *C. neoformans* and other fungi that cause human mycotic disease are able to survive at mammalian temperatures. Nonetheless, most of these fungi are very rare causes of disease in immunologically intact individuals. This is exemplified by the fact that cryptococcosis was rare before the HIV/AIDS pandemic and frequent use of transplant drugs that induce immune suppression. Similarly, candidiasis was rare before the use of broad-spectrum antibiotics and intravenous catheters. Hence, the rise in fungal diseases in the last quarter of the 20th century paralleled the emergence of an expanded population of patients with immune impairment. The degree to which cryptococcosis and HIV-associated immune deficiency are intertwined is illustrated by a report in the 1950s of cryptococcosis in young people in sub-Saharan Africa, the area where the HIV/AIDS pandemic is thought to have begun [52].

The recognition that normal host defense is sufficient to confer resistance to cryptococcosis in most individuals led to intense study of the immune response to *C. neoformans*. This research led to a better understanding of factors that predispose to cryptococcosis and has informed strategies to devise new drugs, vaccines and immunotherapy. The immune response to *C. neoformans* is reviewed below. This is an enormous topic and we have tried to cite critical literature, but it is not possible to cite every study. With respect to our goal of reviewing immunity to *C. neoformans*, we have endeavored to provide an overview of the innate and acquired immune response, while focusing in more detail on new discoveries on the contribution of B cells, natural antibody and genetic polymorphisms to host defense against *C. neoformans*.

Innate immune & cellular responses to *C. neoformans*

The first line of defense against *C. neoformans* is the surface barrier(s) of the innate immune system (e.g., skin, nasal mucosa). In addition, human serum and saliva demonstrate anticryptococcal activity [53,54].

Complement

Complement is a vital component of the innate immune response and a key mediator of phagocytosis of *C. neoformans* [55,56]. Complement-deficient animals are more susceptible to *C. neoformans* infection than complement sufficient animals [57] and complement components can be depleted in patients with cryptococcal fungemia [58]. The cryptococcal capsule activates the complement system, mainly through the alternative pathway, leading to

C3 deposition on the fungal capsule [56]. Species differences have been shown to influence the site of C3 deposition on the cryptococcal capsule [59]. It was also observed that specific anticapsular antibodies could promote *C. neoformans* activation of the classical complement pathway [59,60]. Also, mannose-binding lectin (MBL) can bind to the *C. neoformans* cell wall and activate complement via the lectin pathway [61].

Phagocytosis

Containment of *C. neoformans* by phagocytic cells, including macrophages, dendritic cells and neutrophils, is crucial for natural host defense [46,62–63]. Once the fungus is inhaled, it travels to the lungs where it encounters various phagocytic effector cells. Phagocytosis can be mediated by complement receptors as well as Fc receptors. Complement-mediated phagocytosis of *C. neoformans* occurs via recognition of complement-opsonized yeast by complement receptors (CRs) CR1, CR3 and CR4 [64]. Fc-γ receptors on macrophages/ neutrophils/dendritic cells can bind and mediate phagocytosis of antibody- opsonized *C. neoformans* [65], although phagocytosis of nonopsonized *C. neoformans* can also occur [66]. Additionally, mannose receptors on macrophages and dendritic cells can bind cryptococcal mannoproteins and mediate phagocytosis of *C. neoformans* [67,68].

When *C. neoformans* reaches the brain, resident microglial cells act as the primary phagocytic cells. Microglial cells express Toll-like receptors (TLRs), which can identify pathogen-associated molecular patterns. TLR2, TLR4 and TLR9 have been shown to bind and interact with zymosan (yeast β-glucan), GXM and fungal DNA, respectively [69–71]. Redlich *et al.* demonstrated that stimulation of microglial cells by these TLR agonists enhanced *C. neoformans* phagocytosis [72].

Macrophages

Alveolar macrophages (AMs) internalize *C. neoformans* in the lungs where they can link innate and adaptive immunity, but *C. neoformans* can also survive and proliferate intracellularly [73]. In mice, depletion of AMs delays cryptococcal dissemination and improves survival, suggesting that AMs promote fungal growth and dissemination [74]. However, depletion is detrimental in rats [63], highlighting the importance of species differences in host–*C. neoformans* interaction. *C. neoformans* can also escape from host macrophages through nonlytic exocytosis [48,75]. It can also be taken up by and survive in amoebae [8], underscoring its ability to resist destruction by environmental predators. *C. neoformans* has been shown to be able to disseminate to the brain inside of monocytes, providing experimental support for a Trojan horse model to explain fungal invasion of CNS [76]. Nonetheless, monocytes and macrophages have a major role in controlling *C. neoformans* in the lungs [77]. Macrophages can promote Th1-like responses that induce fungal clearance, serve as APCs to T lymphocytes [78], and secrete cytokines that skew CD4 T cells toward Th1/ Th2 or Th17 pathways [79]. AMs can enhance or control *C. neoformans* pathogenesis depending on the immune status of the host [80]. The host response to *C. neoformans* in the lungs correlates with macrophage polarization [81], whereby M1 (classically activated) macrophages lead to Th1 responses (mainly IFN-γ dominated) and M2 (alternatively activated) macrophages lead to Th2 responses. M1 macrophages are more efficient fungicidal cells than M2 macrophages. Changes in the

cytokine environment can influence macrophage polarization, with M1 macrophages enhancing host defense against *C. neoformans* [81].

Dendritic cells

Dendritic cells (DCs) phagocytose *C. neoformans* via complement or antibody-mediated opsonization, leading to fungal internalization and killing [68,82]. DCs also induce T-cell responses following stimulation of pattern recognition receptors (e.g., TLR4 and TLR9) and secrete cytokines that are crucial for immunity to *C. neoformans*, including IL-12 and IL-23 [83]. *In vivo*, DCs internalize *C. neoformans* in the lungs of mice [84], and their lysosomal components can mediate fungal killing [85].

Polymorphonuclear cells

Neutrophils can enhance granuloma formation, by containing and killing *C. neoformans* in the lungs by oxidative and nonoxidative mechanisms [86–88]. They also contain antimicrobial peptides (defensins) that are cytotoxic to *C. neoformans* [89]. However, early depletion of neutrophils *in vivo* was protective against *C. neoformans* in a murine pulmonary infection model in which there was less inflammatory damage [90]. On the other hand, in a systemic infection model, survival of neutrophil-depleted mice was comparable to that of neutrophil sufficient mice, underscoring that regulation of the inflammatory response in the lungs is crucial for protection [90].

Eosinophils were associated with a lack of protection against *C. neoformans* in pulmonary infection model in which their presence was associated with excessive lung inflammation [91]. C57BL/6 mice develop eosinophilic pneumonia in response to pulmonary cryptococcal infection [92] and eosinophilia has also been observed in human cryptococcosis [93].

Natural killer cells

Natural killer (NK) cells bind and inhibit *C. neoformans* growth *in vitro* [94] and induce fungal clearance in mice [95]. Human NK cells are able to kill *C. neoformans* [96] with direct killing being mediated by perforin [97,98]. A recent study showed that in HIV-infected patients, reduced expression of an NK-cell receptor that mediates direct fungal recognition, (NKp30), led to significantly reduced anticryptococcal cytotoxicity [99].

NK T cells

NK T cells (NKT cells) play an important role in the development of Th1 responses and host resistance to *C. neoformans*. In mice, NKT cells increased in the lungs after intratracheal infection with *C. neoformans*, and the chemokine MCP-1 was implicated in NKT-cell migration and accumulation [100]. Compared with NKT-cell-deficient mice, activation of NKT cells with a synthetic glycolipid (α-galactosylceramide) resulted in increased IFN-γ production and improved host defense [101]. Along with IFN-γ, NKT cells also secrete IL-4, suggesting that this subset regulates both Th1- and Th2-mediated immune responses [102].

Adaptive immunity

CD4 T cells

CD4 T cells are a crucial component of cell-mediated immunity to *C. neoformans* in mice [103,104], as they mediate fungal clearance [105,106] and confer protection upon adoptive transfer to naïve mice [103,107]. CD4 T cells also play a dominant role in recruiting macrophages and granulocytes to the lungs in pulmonary cryptococcal infection [108]. Cryptococcal mannoproteins stimulate a protective CD4 T-cell response to *C. neoformans*, with glycosylation of mannoproteins being essential for an optimal response [109]. However, the specific epitopes recognized by T cells have not been definitively identified.

In humans, CD4 T-cell deficiency is a major predisposing factor for cryptococcosis [110,111], whereby a CD4 T-cell count less than 100 cells/μl and detectable serum CrAg portend high risk for HIV-associated cryptococcosis [37,112]. Cryptococcosis has also been reported in idiopathic CD4 lymphocytopenia, which is characterized by reduced levels of CD4 T cells without evidence of HIV infection [28,113].

CD8 T cells

CD8 T cells also play an important role in the host immune response to *C. neoformans* [114]. CD8 T cells mediate killing of *C. neoformans* [115], whereby killing requires direct cell contact thought to be mediated by granulysin. *In vivo* depletion of murine CD8 T cells reduced survival in a lethal cryptococcal infection model [116]. CD8 T cells also limit growth and survival of *C. neoformans* in macrophages by means of IFN-γ production independent of CD4 T cells [117]. For a complete review on this topic, see reference [114].

Gamma delta (γδ) T cells

γδ T cells are known to regulate Th1-Th2 responses to *C. neoformans*. They secrete anti-inflammatory Th2 cytokines to balance exaggerated Th1 response caused by NKT cells. Depletion of γδ T cells resulted in increased IFN-γ synthesis and promoted cryptococcal clearance via Th1-mediated responses in lungs of mice [118]. Of note, increased production of IL-17A by γδ T cells was observed in neutrophil- depleted mice during pulmonary cryptococcal infection, suggesting that γδ T cells can induce protective response to *C. neoformans* in the absence of traditional adaptive immune response [119].

T-cell-derived cytokines

Th1-type CD4 T cells orchestrate host immunity to *C. neoformans* [103]. Th1-type responses are characterized by production of IL-2, IL-12, IFN-γ and TNF-α. IL-12, IFN-γ and TNF-α, protect against cryptococcosis in experimental models [120,121]. *C. neoformans* also induces secretion of other pro-inflammatory cytokines, including Type-I IFN (IFN-I), IL-1β and IL-6 from innate immune cells [122]. IL-1β and IL-6 induce the development of T-helper Th17 cells in the presence of IL-23. IL-17 and IL-22 are the major cytokines secreted by Th17 cells. While Th17 immunity was required for vaccine-mediated protection against *C. neoformans* in mice [123], Th17-mediated responses were not required to protect naïve mice [124]. Of note, IL-17A produced by neutrophils rather than T cells, was implicated in optimal vaccine-mediated protection against *C. neoformans* in mice [125].

TNF-α production is required for the development of protective T-cell immunity to *C. neoformans* in mice [120]. TNF-α is crucial for induction of IL-12 and IFN-γ in the lungs following *C. neoformans* infection, which then promotes Th1-cell-mediated immunity [121].

Analysis of *C. neoformans*-specific CD4 T-cell responses in patients with HIV-associated cryptococcal meningitis revealed that the presence of an IFN-γ/TNF-α CD4 T-cell response correlated with survival [126]. In other studies, fungal clearance was positively associated with cerebrospinal fluid (CSF) IFN-γ levels, which were in turn positively correlated with the CD4 T-cell count [127,128]. The latter provided the rationale for IFN-γ as adjunctive therapy for HIV-associated cryptococcosis. The addition of a short course of IFN-γ to standard treatment increased the rate of cryptococcal clearance from the cerebrospinal fluid [129]. The importance of IFN-γ in resistance to cryptococcosis has been demonstrated experimentally with a *C. neoformans*- based vaccine that produces IFN-γ [130]. Thus, adjunctive IFN-γ and vaccines that induce tissue-specific IFN-γ production hold promise as immunotherapeutic interventions for cryptococcosis.

The role of B cells in immunity to *C. neoformans*

Numerous studies have now shown that B cells play a crucial role in protection against experimental cryptococcosis [131–133]. Prior to these reports, very few studies directly examined the role of B cells in host defense against *C. neoformans*. An early study utilizing an intravenous infection model reported no difference in *C. neoformans* lethality in B-cell-depleted and B-cell-sufficient mice [134]. However, another study linked B cells with resistance to *C. neoformans* in SCID mice [135], whereby SCID recipients of T cells from B-cell-deficient mice failed to express the adoptive immunity seen in recipients of T cells from B-cell-sufficient mice. Of note, B cells were the predominant cell type in the lungs of *C. neoformans*-infected A/JCr mice [136] and cryptococcal infection was more lethal and associated with more pulmonary immunopathology and inflammation in B-cell-deficient (uMT mice) than B-cell-sufficient mice [137], linking B cells to control of lung inflammation.

Mature B cells are subdivided into B-1 and B-2 cells. B-1 cells can be further classified into two populations based on expression of CD5, $CD5^+$ B-1a cells and $CD5^-$ B-1b cells, whereas conventional B-2 cells are composed of follicular and marginal zone B-cell subsets. B-1 cells enhance resistance to and prevent dissemination of *C. neoformans* in several different mouse models. X-linked immunodeficient (XID) mice, which lack B-1 cells and natural IgM, were more susceptible to intravenous infection with *C. neoformans* than CBA/Ca control mice [138]. In pulmonary infection models, B-1a cells were associated with fungal containment during the early immune response [132], and XID mice, which lack B-1a cells, are less able to contain *C. neoformans* in the lungs than control mice and develop more fungal dissemination to the brain [133]. This phenotype is associated with a virtual absence of serum IgM (see below), reduced yeast uptake by macrophages, an aberrant tissue inflammatory response and enlargement of yeast cells in the lungs [133]. These studies implicate B-1a cells and IgM in cryptococcosis, with the caveat that XID mice have other defects including T-cell and NK-cell immunodeficiency. In *C. neoformans* infected C57BL/6 mice, $CD5^+$B-1a cells exhibited more binding to *C. neoformans* than B-1b and B-2

cells and directly mediated lung and brain fungal clearance during early pulmonary cryptococcal infection [132]. Reconstitution of B-1 cells in B-1-cell-depleted mice increased AM phagocytosis of *C. neoformans* and reduced lung and brain fungal burdens. Several reports have demonstrated that B-1-cell-derived mono-nuclear phagocytes have fungicidal activity against *C. neoformans* [139,140].

In humans, IgM memory B cells are considered the main homolog of mouse $CD5^+$ B-1 cells. These cells, which are identified by their expression of CD27 and IgM, produce naturally occurring IgM that binds conserved microbial determinants and carbohydrates [141]. In one study, peripheral blood IgM memory B-cell levels were lower in HIV-infected individuals with a history of cryptococcosis than in those with no history of cryptococcosis and were predictive of cryptococcal disease status [142]. In this study, a predisease cohort had CD4 T-cell levels greater than 400 cells/μl, suggesting that IgM memory B-cell levels could hold promise as a biomarker of risk for human cryptococcosis.

Natural antibodies to *C. neoformans*

IgM memory B cells produce ‘naturally occurring’ IgM that binds conserved microbial determinants [143]. Because natural IgM is produced in the absence of antigen stimulation, it is a part of the innate immune system and considered to provide ready-made pathogen defense. In mice, IgM deficiency was associated with increased susceptibility to pulmonary *C. neoformans* infection and reduced AM phagocytosis of *C. neoformans*, which was restored by reconstitution with natural mouse (nonimmune) IgM [144]. B-1 cells from *C. neoformans*-infected mice secreted *C. neoformans*-binding IgM and depletion of these cells resulted in reduced AM phagocytosis and increased fungal dissemination to the brain [132]. These findings support the hypothesis that B-1 cells enhance innate antifungal immunity via natural IgM, which promotes fungal containment in the lungs [132,133]. This is consistent with a report that an antibody to laminarin, a fungal cell wall determinant recognized by natural IgM, bound to and was protective against *C. neoformans* in mice [145,146].

The natural antibody response to *C. neoformans* in humans has been studied in detail. IgM and IgG that bind cryptococcal polysaccharides (capsule and cell wall) are present in normal human serum, although they are generally not opsonic for macrophage phagocytosis of *C. neoformans* [147,148]. Serum GXM-binding antibodies are virtually ubiquitous in adults and can be detected early in childhood [32,34–35,149]. Like other capsular polysaccharides, IgG2 is the predominant GXM-binding IgG subclass. Notably, IgG2 is decreased in individuals with HIV/AIDS [34,35]. HIV-infected individuals have higher levels of GXM-IgG1 and lower levels of IgG2 than HIV-uninfected individuals [94].

A number of studies have shown that levels of GXM-binding IgM are lower in HIV-infected than HIV-uninfected individuals [34,142,150–151], including one in which levels were lower in HIV-infected individuals who developed cryptococcosis than those who did not [142]. GXM-binding IgM levels were also lower in HIV-uninfected solid organ transplant recipients who developed cryptococcosis post-transplant than those who did not [152]. Given that IgM memory B cells are a major source of serum IgM, reduced levels of GXM-binding IgM could stem from a loss of IgM-producing B cells. Consistent with this

hypothesis, IgM memory B cells are depleted in HIV/AIDS [153,154]. In fact, loss of these cells begins early in HIV infection before severe CD4 T-cell deficiency is manifest and unlike other B-cell subsets, IgM memory B cells are not fully reconstituted by cART [155]. The effect of drugs used in solid organ transplant recipients on B-cell and antibody levels has not been studied, but mycophenolate, prednisone and cyclophosphamide each depleted B-1a cells in mice [156]. Whether or not there is a loss of IgM memory B cells in solid organ transplant recipients and apparently normal individuals with cryptococcosis requires investigation.

Adaptive antibody response to *C. neoformans*

The acquired antibody response to various cryptococcal antigens, namely, GXM capsule, cell wall polysaccharides and cryptococcal proteins has been studied extensively. The antibody response to GXM is characteristic of antibody responses to other encapsulated microbes, such as *Streptococcus pneumoniae*. As T-independent type 2 antigens, capsular polysaccharides elicit restricted antibody responses notable for the use of a limited number of antibody variable region genes and an absence of class switching and recall or memory responses. GXM is considered a rational vaccine candidate for cryptococcosis, but similar to bacterial capsular polysaccharide-based vaccines, it was necessary to convert it to a T-dependent antigen by conjugating it to a protein carrier to enhance its immunogenicity. An investigational GXM-tetanus toxoid (GXM-TT) [157] vaccine was promising in preclinical trials [158], but was not developed further due to nonscientific reasons [159]. Extensive work with GXM-TT in mice led to a paradigm shift in our understanding of antibody immunity to capsular polysaccharides stemming from the observations that GXM elicited protective as well as a nonprotective antibodies and that antibody action was a function of idiotype, isotype and specificity. This is reviewed in [160].

When the antibody response to GXM-TT was examined in human volunteers the GXM response was predominantly IgG2, which was also the main mediator of *C. neoformans* phagocytosis by human mononuclear cells [161]. A human monoclonal antibody derived from a GXM-TT vaccinated donor protected mice against experimental cryptococcosis [162] as did human monoclonal IgMs produced from GXM-diphtheria toxoid (GXM-DT) vaccinated Xeno-mouse™ mice [163] and a peptide mimotope- vaccine-derived from a protective human monoclonal antibody [164].

Studies of GXM-TT (and DT) elicited mouse and human monoclonal antibodies revealed that they are derived from a restricted B-cell repertoire [165,166]. The immunoglobulin VH3 family genes encoded the human anti-GXM antibodies, and this family shares structural homologies with the VH5 (7183) family genes used for encoding mouse anti-GXM antibodies [163,167]. VH3-expressing B cells dominate the human response to bacterial polysaccharide antigens and are depleted in HIV infection. Thus, it has been hypothesized that a decrease in VH3-encoded antibodies could contribute to susceptibility for cryptococcosis [168].

A mouse monoclonal GXM IgG1 showed promise as adjunctive therapy for cryptococcosis in HIV-infected patients in a Phase I trial [169,170], but was not advanced further due to a

lack of resources. Nonetheless, there is extensive preclinical data on this antibody, including its efficacy as radioimmunotherapy [171].

Genetic susceptibility to cryptococcal disease

Given that not all HIV-infected individuals with CD4 T-cell deficiency develop cryptococcosis and cryptococcosis occurs in HIV-uninfected patients without CD4 T-cell deficiency, other risk factors are under investigation. The idea that genetic factors could impact susceptibility seems plausible because polymorphisms can affect the expression of a multitude of host response genes. However, to date, only a few studies have addressed this possibility [172].

MBL polymorphisms

MBL is a circulating C-type lectin that plays an important role in innate immunity as a first line of pathogen defense. It selectively recognizes the pattern of glycans displayed on certain microbial surfaces, leading to opsonization and subsequent activation of the lectin pathway of the complement system. Complement activation results in further opsonization and induction of inflammatory reactions. Human MBL is encoded by a single gene (*MBL2*), encoding six common single-nucleotide polymorphisms (SNPs). These polymorphisms have a major effect on serum MBL levels. MBL deficiency caused by polymorphisms in the *MBL2* gene was associated with increased susceptibility to cryptococcosis in HIV-uninfected Chinese patients [173].

Fc-γ receptor polymorphisms

Fc-γ receptors (FCGR) are present on certain immune cells (macrophages, monocytes, neutrophils, natural killer cells, B cells and mast cells), and bind IgGs connecting the humoral response to cellular effector mechanisms. FCGRs contribute to regulation of a multitude of immune and inflammatory responses [174]. FCGR polymorphisms are associated with certain autoimmune diseases as well as increased susceptibility to certain infections [175,176], including cryptococcosis [94,177,178].

FCGR2A 131H/R polymorphism involves the substitution of arginine (R) to histidine (H) at the 131 amino acid position in the ligand binding domain of the receptor. FCGR2A is unique in its ability to bind human IgG2 and is crucial for clearance of encapsulated pathogens. As noted above, IgG2 is the main subclass of antibodies to microbial polysaccharides [175]. The FCGR2A 131H allele displays higher IgG (including IgG2) binding than the 131R allele. FCGR2A 131H-expressing effector cells have a higher capacity for phagocytosis [179,180], as exemplified by a study showing that monocytes from FCGR2A 131HH donors internalized immune complexes more efficiently than monocytes from 131RR donors [181]. Meletiadis *et al.* reported an association of FCGR2A 131R with cryptococcosis in HIV-uninfected patients, including solid organ transplant recipients [177]. Although this study did not examine IgG2 levels, it speculated that increased susceptibility to cryptococcosis in individuals with FCGR2A 131RR could be explained by inefficient phagocytosis of IgG2-opsonized *C. neoformans* [177,182]. However, FCGR2A 131H/R polymorphism was not associated with risk for cryptococcosis

in an HIV-infected cohort that had lower levels of IgG2 than HIV-uninfected participants [94]. Given previous data showing that IgG2 is the main isotype responsible for phagocytosis of *C. neoformans* in immune sera [161], it is possible that FCGR2A 131R confers risk in those with normal levels of IgG2 [94]. This requires further study. Of note, FCGR2A 131R was not associated with cryptococcosis in an HIV-uninfected Chinese cohort [178].

FCGR3A 158F/V polymorphism results in substitution of phenylalanine (F) for valine (V) at amino acid position 158. Compared with FF homozygous donors, FCGR3A expressed on NK cells and monocytes in VV homozygotes bound more IgG1 and IgG3 despite identical levels of receptor expression [183]. The FCGR3A 158V polymorphism was first reported to be associated with risk for cryptococcosis among HIV-uninfected patients by Meletiadis *et al.* [177]. Recently, an association between FCGR3A 158V and risk for cryptococcosis was also reported in HIV-infected individuals [94]. This study also showed that the FCGR3A 158V allele exhibited more binding of *C. neoformans*–Ig complexes and when expressed by NK cells, it induced more antibody-dependent cellular cytotoxicity (ADCC) against *C. neoformans*-infected monocytes than FCGR3A 158F-expressing cells [94]. These findings provide a possible mechanistic explanation for how the 158V polymorphism could enhance *C. neoformans* virulence. It could increase phagocyte cargo (due to increased binding and uptake of *C. neoformans*–immune complexes), thereby increasing fungal burden, and/or it could increase immune activation via ADCC, thereby leading to host damage and fungal dissemination [94,172]. Again of note, FCGR3A 158F/V polymorphism was not associated with cryptococcosis in the aforementioned Chinese cohort [178]. Given that the cohorts in which this polymorphism was associated with cryptococcosis (Meletiadis *et al.* [177] and Rohatgi *et al.* [94]) were both predominantly Caucasian, further studies are needed to determine whether it affects risk in other racial groups.

FCGR2B is the only inhibitory receptor to suppress downstream events such as cellular proliferation, phagocytosis and inflammatory cytokine release. In addition to its expression on B cells, FCGR2B is expressed on macrophages, neutrophils and mast cells, but not on T or NK cells. FCGR2B receptors contain a polymorphism at position 232, in which an isoleucine (I) is replaced by a threonine (T). Receptors encoded by FCGR2B 232T lack inhibitory activity, as they are unable to interact with activating receptors. FCGRR2B 232T/T genotype was associated with increased risk for SLE and protection against malaria [184]. In a case-control genetic association study in the aforementioned study Chinese cohort, FCGR2B 232I/I was associated with cryptococcal meningitis [178].

Although more work and studies in larger, more diverse cohorts must be done, the discovery of SNPs of FCGRs that are associated with risk for cryptococcosis suggests that these polymorphisms, which are likely to contribute to dysregulation of inflammatory responses to *C. neoformans*, could serve as potential biomarkers of risk for disease and enable the identification of those who might benefit from prophylaxis.

Conclusion & future perspective

C. neoformans is ubiquitous in the environment and exposure is very common, yet clinically apparent disease is rare, except in patients with immune impairment. HIV/AIDS is the most common predisposing condition for disseminated cryptococcal disease, but disease also occurs in patients with other types of immunodeficiency, including that due to the immunosuppressive drugs used in solid organ transplantation. However, some patients with cryptococcosis have no known underlying condition.

Many different innate and acquired immune constituents and functions, including B cells, T cells, macrophages, cytokines and phagocytosis contribute to host defense against *C. neoformans*, but the loss or absence of a single constituent, (e.g., CD4 T cells), appears to be insufficient to cause human cryptococcosis. Recent studies reveal associations between FCGR polymorphisms and HIV-associated and HIV-unassociated cryptococcosis and roles for B cells, natural and *C. neoformans*-specific antibodies in resistance to disease. Current data suggest that B- and T-cell deficiency, absence of natural IgM, reductions in tissue-specific IFN-γ and FCGR polymorphisms could all promote *C. neoformans* growth and dissemination. However, more studies are needed to validate this hypothesis.

New insights into susceptibility and resistance to cryptococcosis are likely to inform the development of novel agents to treat and prevent disease. However, a major challenge over the next 5–10 years will be to ensure the availability of cART and antifungal agents in all areas of the world, particularly those where HIV/ AIDS remains epidemic, such as sub-Saharan Africa and parts of Asia. Fluconazole therapy and initiation of cART can reduce the burden of HIV-associated cryptococcosis, but access to these agents and point-of-care rapid diagnostics, which are priorities for GAFFI [42], are needed to conquer cryptococcosis in under-resourced settings. Triumph over cryptococcosis in the resourced world will require clinical biomarkers that can identify high-risk patients who would benefit from antifungal prophylaxis, such as those with acquired and primary immunodeficiencies. Although levels of IgM memory B cells, GXM-IgM and FCGR polymorphisms have been associated with risk for disease in some studies, much work remains to be done to identify and validate biomarkers as clinical tools.

Given that the immune status of the patient is the main determinant of the outcome of host–*C. neoformans* interaction, therapies that augment host immunity are logical, particularly for patients with known defects. Immunotherapy, such as passive antibody-based therapy and prophylaxis, vaccines and selected mediators such as IFN-γ, are potential modalities for treating and preventing cryptococcosis. Development of a vaccine is a high priority for the cryptococcal field. Given that *C. neoformans* is ubiquitous in the environment, yet disease is rare, the ideal vaccine for *C. neoformans* would prevent reactivation as well as acute disease. However, because cryptococcosis may occur in the setting of either insufficient or excessive immune responses [21], more than one type of vaccine might be required. The vast amount of knowledge about the host response to *C. neoformans* gained over the past two decades is poised to accelerate vaccine development. In this regard, a number of vaccine candidates and platforms have been developed over the past two decades [185]. Although the most promising vaccines developed so far are GXM-protein conjugates and whole cell vaccines

that enhance IFN-γ production, vaccines that bolster innate immune responses and leverage the immunogenicity of cell wall determinants are in development [186]. Over the past two and a half decades, immense progress has been made in our understanding of immunity to *C. neoformans*. However, the challenge to the field will be to obtain sufficient resources to translate this knowledge to identify biomarkers of disease risk and treat and prevent cryptococcosis with development and clinical trials of therapies and vaccines that bolster immunity to *C. neoformans*.

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• of interest;

•• of considerable interest

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Executive summary

Background

- Cryptococcosis occurs predominantly in immunocompromised patients, most commonly those with HIV/AIDS.
- *Cryptococcus neoformans* (variety neoformans and variety grubii) and *Cryptococcus gattii* cause majority of disease in humans.

Ecology

- Cryptococcal species are ubiquitous environmental microbes, differing in geographic distribution.

Epidemiology

- Incidence of HIV-associated cryptococcosis has been reduced by combination antiretroviral therapy (cART) but remains a threat to those not on cART, especially in underresourced areas.
- Cryptococcosis also occurs in solid organ transplant recipients, and in people with no apparent immune defects.

Pathogenesis

- *C. neoformans* is acquired by inhalation followed by a state of latency in the lungs.
- Cryptococcosis occurs during latency breakdown in the setting of immune deficiency.
- HIV-associated cryptococcosis is heralded by CD4 T-cell counts less than 100 cells/μl and detectable serum cryptococcal antigen (CrAg).

Cryptococcal virulence

- The central virulence factor of *C. neoformans* is its polysaccharide capsule.
- Other virulence determinants include capacity to grow at mammalian temperatures as well as intracellular replication.

Host response to *C. neoformans*

- Innate and acquired immune mechanisms contribute to resistance to cryptococcosis.
- CD4 T cells and cytokines enhance phagocytosis and cryptococcal containment.
- CD8 T cells enhance cryptococcal killing.
- Macrophage phagocytosis promotes cryptococcal containment, but *C. neoformans* can replicate in macrophages.
- B cells enhance resistance to *C. neoformans* in experimental models.

- Natural IgM promotes containment of *C. neoformans* in murine lungs, preventing dissemination to brain.
- HIV-associated cryptococcosis was linked to reduced levels of IgM memory B cells and lower levels of IgM.

Genetic susceptibility to cryptococcosis

- MBL and Fc-γ receptor polymorphisms have been associated with cryptococcosis.
- Genetic factors influence susceptibility and resistance to *C. neoformans*.

Conclusion & future perspective

- Victory against cryptococcosis will require access to antifungal drugs, cART and administration of fluconazole based on pre-emptive CrAg screening of patients with CD4 T-cell count less than 100 cells/μl.
- Clinical biomarkers are needed to assess risk for cryptococcosis in high-risk populations and to enable antifungal prophylaxis and therapy.
- Novel adjunctive immunotherapies, (monoclonal antibodies and/or IFN-γ) and immunomodulators should be explored.
- Need for vaccine development which can prevent acute as well as reactivated disease.