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MINIREVIEWS

Cryptococcal Interactions with the Host Immune System▽

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Opportunistic pathogens have become of increasing medical importance over the last decade due to the AIDS pandemic. Not only is cryptococcosis the fourth-most-common fatal infectious disease in sub-Saharan Africa, but also *Cryptococcus* is an emerging pathogen of immunocompetent individuals. The interaction between *Cryptococcus* and the host's immune system is a major determinant for the outcome of disease. Despite initial infection in early childhood with *Cryptococcus neoformans* and frequent exposure to *C. neoformans* within the environment, immunocompetent individuals are generally able to contain the fungus or maintain the yeast in a latent state. However, immune deficiencies lead to disseminating infections that are uniformly fatal without rapid clinical intervention. This review will discuss the innate and adaptive immune responses to *Cryptococcus* and cryptococcal strategies to evade the host's defense mechanisms. It will also address the importance of these strategies in pathogenesis and the potential of immunotherapy in cryptococcosis treatment.

The basidiomycetous yeast genus *Cryptococcus* includes the two medically important pathogens *C. neoformans* and *C. gattii*. These two species are further divided into *C. neoformans* serotypes A (*C. neoformans* var. *grubii*), D (*C. neoformans* var. *neoformans*), and A/D and *C. gattii* serotypes B and C (formerly *C. neoformans* var. *gattii*) based on differential antibody recognition of the polysaccharide capsule (135). The two pathogenic species show different geographical distributions. *C. neoformans* is globally distributed and has been isolated from various natural sources, with particularly high concentrations occurring in avian guano, rotting vegetables, and soil. In contrast, *C. gattii* is geographically restricted to tropical and subtropical regions, with the notable exception of British Columbia. In tropical and subtropical regions, it has been found to be associated with the eucalyptus species *Eucalyptus camaldulensis*, *Eucalyptus tereticornis*, *Eucalyptus rudis*, and *Eucalyptus gomphocephala* (64, 172). *C. neoformans* causes mainly opportunistic infections in immunocompromised patients with underlying conditions, such as HIV, leukemia, and other cancers, or in those taking corticosteroid medication (135). Serotype A is responsible for the majority of cryptococcosis cases in immunocompromised hosts (135). In contrast, *C. gattii* affects mainly immunocompetent individuals. The recent and spreading cryptococcosis outbreak in healthy individuals in British Columbia has highlighted the potential of *C. gattii* to act as an emerging pathogen (84, 85, 121). In addition, other non-*C. neoformans*/non-*C. gattii* species, such as *Cryptococcus laurentii* and *Cryptococcus albidus*, have recently started to emerge as potential human pathogens (83).

Cryptococcal infection can be asymptomatic, chronic, or acute. Typically, an initial pulmonary infection can spread systemically, with a particular predilection for the central nervous system. Pulmonary infections are in most cases asymptomatic. However, they can involve coughing, pleuritic chest pain, fever, dyspnoea, weight loss, and malaise. Pneumonia and acute respiratory distress syndrome have been reported mainly for immunocompromised patients (17, 141). Cryptococcosis of the central nervous system is life threatening and presents as meningitis or meningoencephalitis, with symptoms such as headache, increased intracranial pressure, fever, lethargy, coma, personality changes, and memory loss. Less common are secondary infections of the skin, lungs, prostate, and eye (135). A recent publication estimated 957,900 cases of cryptococcal meningitis resulting in 624,700 deaths globally each year (150). It is the leading cause of death in HIV-infected individuals, with an incidence of 30% and a mortality of 30 to 60%. The mortality rate in transplant patients is even higher (20 to 100%) (Centers for Disease Control and Prevention) (135).

The dramatic course of *Cryptococcus* infections in immunocompromised individuals shows the importance of an intact immune response to the pathogen. This review will consider both the host's innate and adaptive immune responses to *C. neoformans* and *C. gattii* together with the pathogens' strategy to undermine these defense mechanisms and how current knowledge might be applied to improve anticryptococcal therapy.

INNATE IMMUNE RESPONSE TO *CRYPTOCOCCUS*

A variety of innate factors interfere with the establishment of cryptococcal infection. Besides physical barriers, such as the skin and the nasal mucosa, the anticryptococcal activity of human serum and saliva has been described repeatedly (8–10, 65, 74, 143, 181). However, the complement system and phagocytic effector cells are the major players in the nonspecific host immune response to *Cryptococcus*.

Complement response to *Cryptococcus*. The complement system is an antipathogen cascade of serum proteins that can be activated by the classical (antibody-mediated), lectin, or alternative (microbial-surface-mediated) pathway. All three pathways eventually converge in the formation of the C3-conver-

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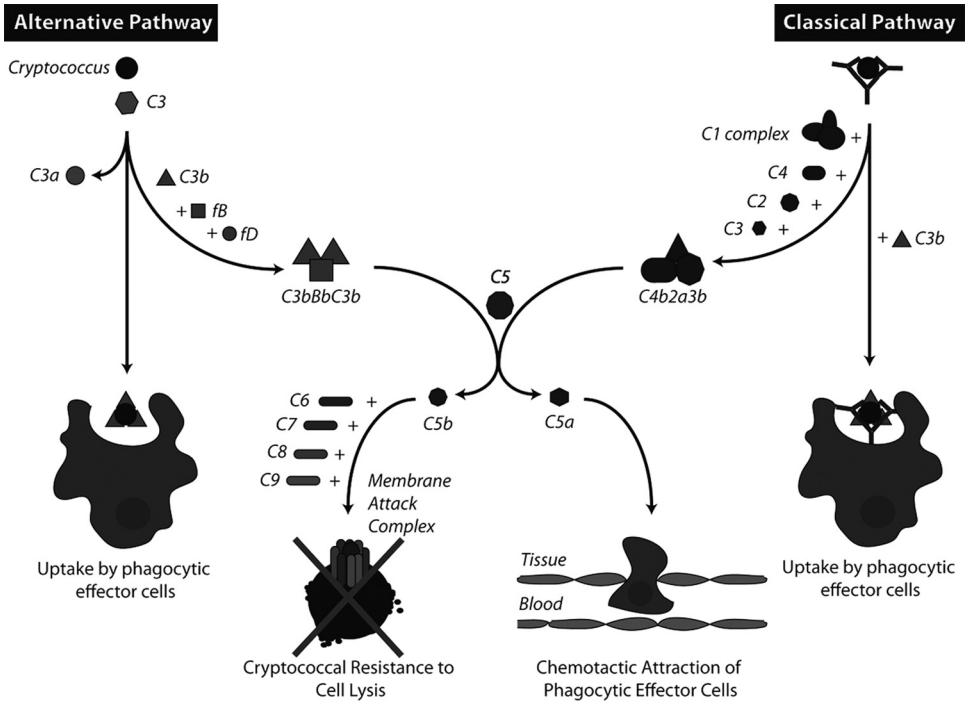

FIG. 1. Summary of the complement pathways activated upon infection with *Cryptococcus neoformans*. The yeast can activate the classical (antibody-mediated) and alternative (microbial-surface-mediated) pathways. Both pathways eventually converge in the formation of the C3-convertase and the cleavage of C3 into C3a and C3b. C3b either facilitates pathogen opsonization and enhances uptake by phagocytic cells or enables the cleavage of C5 into C5a and C5b. C5a functions as a mediator of inflammatory responses and attracts phagocytic effector cells, whereas C5b initiates the formation of the membrane attack complex (C5b, C6, C7, C8, C9). However, *C. neoformans* is resistant to pore formation and cell lysis by the membrane attack complex (165). fB, factor B; fD, factor D.

tase and the cleavage of C3 into C3a and C3b. C3b either facilitates pathogen opsonization and thus enhances uptake by phagocytic cells or enables cleavage of C5 into C5a and C5b. C5a functions, together with C3a, as a mediator of inflammatory responses and attracts phagocytic effector cells, whereas C5b initiates the formation of the membrane attack complex (C5b, C6, C7, C8, C9) (76).

Observations from animal model systems and human patients have repeatedly shown the importance of the complement system during cryptococcal infections (Fig. 1). The survival time of *C. neoformans*-infected guinea pigs and mice treated with cobra venom to deplete late complement components (C3 to C9) is shortened and the ability to clear *C. neoformans* from extraneural sites reduced (37). Mice deficient in C5 are more susceptible to intravenously injected *C. neoformans* and succumb three times quicker than C5-positive mice due to acute and fatal pneumonia (158, 159). Furthermore, patients presenting with cryptococcal fungemia show reduced levels of C3 and alternative complement factor B (122). Brain sections from patients with cryptococcal meningitis do not show C3 binding to the yeast (186). In contrast, however, the survival time of C4-deficient guinea pigs is similar to that of normal guinea pigs after infection with *C. neoformans*, indicating that the alternative activation pathway is the major protective complement pathway during infections with *C. neoformans* (37).

This finding is supported by results from *in vitro* analysis of the complement binding dynamics of *C. neoformans*. Diamond et al. (38) reported the consumption of complement components by *C. neoformans* when it was incubated with normal or C4-deficient guinea pig serum, while *C. neoformans*-dependent activation of the complement cascade can be reconstituted from six proteins (factor D, factor B, factor H, factor I, C3, and properdin) belonging to the alternative pathway (96). It was estimated that approximately 10^7 to 10^8 C3 fragments bind to an encapsulated cryptococcal cell and, dependent on the source of serum, localize inside and at the outer edge of the capsule (56, 88, 93, 203). The binding of C3 starts characteristically with a lag phase of 4 to 6 min, followed by rapid binding of C3 fragments to encapsulated yeast cells when they are incubated in normal human serum (95). Incubation with Mg-EGTA to chelate calcium required for classical pathway activation did not change the C3 binding dynamics, supporting the idea of a dominant role for the alternative complement pathway during cryptococcosis (95). According to the complement model, the activation of the alternative pathway relies on the spontaneous decomposition of C3 to C3b and Bb upon pathogen interaction. Similarly, closer examination of the binding process of C3 to yeast cells by immunofluorescence revealed an initial slow C3 deposition in small loci with subsequent expansion to larger areas after a lag (95). Investigation into the molecular form of bound C3 by SDS-PAGE of eluted radioactively labeled fragments revealed a rapid decay of the C3 hydrolysis product C3b into iC3b, which in turn is the dominant fragment bound to the cryptococcal capsule (92, 153).

The two major functions of the complement system during cryptococcosis are to stimulate the chemotaxis of phagocytic

effector cells and enhance the uptake of cryptococcal cells by these phagocytes. Early evidence for the involvement of complement in the opsonization of *C. neoformans* was drawn from phagocytosis assays with heat-inactivated serum (39). Assays with phagocytic cells and serum, depleted of specific components of the complement pathways, revealed the requirement of the complement pathway for phagocytosis of cryptococci by neutrophils (38), polymorphonuclear leukocytes, and monocytes in an antibody-free situation (30). However, although the alternative pathway is sufficient for yeast opsonization, the classical pathway is required for optimal opsonization kinetics (38). Laxalt and Kozel (100) and Diamond and Erickson (36) observed the chemotactic potential of serum; both serum-opsonized encapsulated and nonencapsulated *C. neoformans* cells are able to chemotactically attract neutrophils and monocytes *in vitro*. As C5-deficient mice are more susceptible to cryptococcosis (158, 159) and closer investigations revealed a lack of neutrophil accumulation in pulmonary vessels, C5a, the C5 cleavage product, seems to be the chemotactic active component (Fig. 1) (113).

The cryptococcal polysaccharide capsule is a well-known factor required for the pathogen's virulence, e.g., by inhibiting phagocytosis (reviewed in reference 202). *C. neoformans* mutants with a capsule-deficient phenotype are avirulent in mice (19, 98). Several studies with encapsulated and nonencapsulated *C. neoformans* strains also showed a difference in complement activation dependent on capsulation. The capsule inhibits the binding of mannan-binding lectin and thus the activation of the complement system via the lectin pathway in *C. neoformans* (149). The total numbers of bound C3 molecules are similar in different cryptococcal strains, independent of the capsule size (93). However, a comparison of the depositions of C3 on *C. neoformans* strain 145 grown under capsule-inducing and non-capsule-inducing conditions indicates a relationship between capsule diameter and C3 density under high yeast cell concentrations. The small-capsule variant bound more C3 molecules per cubic micrometer of capsule than the population with a large capsule (94). In contrast, the noncapsular strain 602 accumulates significantly less C3 on its cellular surface than capsulated strains (88, 95, 97). The decay rate of C3b to iC3b is lower in nonencapsulated *C. neoformans* than in capsulated forms (70% versus 100%, respectively, after 20 min of incubation with factors H and I) (92, 153). Furthermore, the acapsular strain 602 activates not only the alternative but also the classical complement pathway (95). C3 binding to the acapsular strain occurs immediately, without any lag phase, and rather than the characteristic small C3 deposition sites in encapsulated strains, sudden and rapid binding of C3 to the entire cell surface can be observed. This accumulation is ongoing and does not stop after 8 min, as seen in encapsulated *C. neoformans*. However, the dynamics described for capsular strains can be reinduced by treatment with Mg-EGTA (95). Interestingly, there seems to be a species-specific difference in activation of the complement system. Although the binding efficiency of C3 to *C. gattii* serotype B and C strains is higher than to *C. neoformans* serotype A and D strains (161), the total accumulation of C3 on the cellular surface of *C. gattii* serotype B and C strains is only half of that of the latter (194, 198).

The complement system is the first line of defense against *Cryptococcus* in the bloodstream and, by opsonizing the pathogen and attracting immune effector cells, performs important preparations for the subsequent host defense response. The capsule probably functions as an inhibitor of complement-related host responses, such as uptake by phagocytosis, by inhibiting the classical pathway and constricting C3-convertase activity by the efficient removal of C3b, an essential part of the alternative C3-convertase, and thus restricting activity amplification. In addition, the differences in complement activation between *C. neoformans* and *C. gattii* might be important for the increased virulence of certain serotype B strains. Recent data from survival assays of factor B- or C3-deficient mice after *C. gattii* infection have suggested that complement pathways other than the alternative activation pathway contribute to the host's protection (129).

Phagocytic effector cells in the host's immune response to *Cryptococcus*. Research over the last few decades has shown the importance of phagocytic effector cells during a host's immune response to cryptococcal infections. This section will describe how the yeast cells are taken up and discuss the interaction with the different types of phagocytes. A particular focus will be given to the interaction between cryptococcal cells and macrophages.

(i) Phagocytosis. Uptake of cryptococcal cells has been shown repeatedly by a variety of leukocytes (15), such as rat and mouse peritoneal macrophages (62, 89, 134), guinea pig pulmonary macrophages (16), human neutrophils and macrophages (40), and swine microglia (110). Phagocytosis is triggered by direct recognition of the yeast or by receptor-mediated recognition via complement or antibodies (135). Conserved structures, such as the components of the cryptococcal capsule, can be directly recognized by pattern recognition receptors. *C. neoformans* glucuronoxylomannan (GXM) can bind to Toll-like receptor 4 (167), and the mannose receptor of dendritic cells (DCs) binds to mannoproteins expressed on the yeast's surface (154) and, in the absence of complement to CD18, to a subunit of the complement receptors (40). Serum-opsonized (i3Cb) *C. neoformans* is recognized either by the complement receptor CR1 (CD35) or by the heteromeric β2-integrins CR3 (CD11b/CD18) and CR4 (CD11c/CD35) (108, 203). Studies with the receptors heterologously expressed in Chinese hamster ovary cells indicate that binding to any of the receptors occurs independently, with the greatest binding avidity being shown for CR3, followed by CR1 and CR4 (109). Antibody-opsonized yeast cells are recognized by Fcγ receptor molecules expressed on the surfaces of macrophages, neutrophils, and dendritic cells (62, 115, 136, 180).

(ii) Dendritic cells. *Cryptococcus neoformans* is phagocytosed by human primary DCs *in vitro* (82, 180) and also in the mouse model (197). Dendritic cells function as major antigen-presenting cells that constantly monitor the current antigen population and modulate adaptive immune responses accordingly (28). During cryptococcal infections, DCs are thought to be the major initiators of protective cell-mediated immunity (11, 147). Not only are major antigens (e.g., mannoproteins and glycoantigens) for the activation of anticryptococcal T-cell responses dominantly presented by DCs (107, 125), but also the induction of T-cell responses by DCs is much more efficient than by alveolar or peritoneal macrophages (125, 180).

(iii) Neutrophils. Neutrophils are thought to contribute strongly to the innate immune response to cryptococcosis. Upon

cryptococcal challenge, the number of polymorphonuclear cells increases at the site of infection in animal models (54, 152). *In vitro*, the oxidative burst exerted by neutrophils effectively kills *C. neoformans* (21, 124, 131). However, despite rapid antimicrobial activity *in vivo*, cryptococci are only partially cleared from the infected site (54, 152). Interestingly, induced neutropenia in mice increases survival after pulmonary challenge with *C. neoformans* (128), a counterintuitive finding that may be due to the absence of neutrophils, resulting in more interleukin 4 (IL-4) and IL-10 signaling, which, in turn, modifies the inflammatory status and reduces tissue damage due to the aggressive oxidative burst (128). Together with data showing neutrophils being present in infected tissues only in low numbers and at early stages of infection (46), this might suggest a more immune-regulatory than antimicrobial role for neutrophils.

Besides killing pathogens by means of respiratory bursts, neutrophils also produce antimicrobial peptides and proteins as part of the antimicrobial response (101). One such family of antimicrobial peptides—the defensins—is found in abundance in human, rat, rabbit, and guinea pig neutrophils; however, expression is lacking in mouse neutrophils (43). As mice are routinely used as a system to model cryptococcal disease, this finding needs to be considered when assessing the role of neutrophils in the anticryptococcal immune defense. The lack of defensins might also be a reason for the high susceptibly of mice to challenge with the yeast.

(iv) Macrophages. The importance of macrophages in cryptococcal infections has become more and more obvious in the last decade. Research has revealed an intriguing interaction between the phagocytic effectors and yeast cells that revealed *C. neoformans* as an intracellular parasite (48). *Cryptococcus* has developed a unique method to manipulate host macrophages. After phagocytosis, *C. neoformans* can survive and proliferate within these infected host cells, eventually leading to host cell lysis (5, 33, 46, 48, 118, 187). Intracellular proliferation occurs despite the harsh environment within macrophages, and the yeast does not seem to utilize strategies that are known from other intracellular pathogens to manipulate the host cell. In contrast to pathogens such as *Listeria monocytogenes* or *Shigella flexneri*, *C. neoformans* can reside in the phagosome and does not have to escape into the cytoplasm to establish the intracellular niche (106, 168, 174). Moreover, *C. neoformans* does not inhibit phagosome-lysosome fusion (106), as has been shown for *Legionella pneumophila* (70), nor does the yeast interfere with phagosome maturation or acidification, as occurs during infections with *Histoplasma capsulatum* or *Mycobacterium* species (106, 168, 178, 179). Instead, *C. neoformans* survives and replicates in the acidic phagolysosome, and in fact, any increase in phagosomal pH (e.g., by experimental addition of chloroquine or ammonium chloride) leads to reduced intracellular proliferation (105). Host cell lysis is a common escape route for intracellular pathogens. To date, there is not much information on the mechanisms of cryptococcal lytic escape (201). However, given its documented role as a virulence factor (25), phospholipase B (*plb1*) is a potential candidate molecule that may mediate the permeabilization of *C. neoformans*-containing phagosomes (47, 187).

Besides lytic escape, a novel expulsive mechanism (Fig. 2) by which the yeast can exit macrophages without killing the host cell, thus avoiding a local inflammatory response, has recently been described (4, 117). In contrast to other expulsive mechanisms, such as the actin-based protrusion shown by *Listera monocytogenes*, *Rickettsia* spp., *Shigella flexneria*, and *Burkholderia pseudomallei* (177), cryptococcal expulsion occurs without obvious involvement of the actin cytoskeleton or damage to either the pathogen or the host (4, 117). The process is dependent on live cryptococci and occurs very rapidly, requiring less than 60 s, with events being randomly distributed over time (4, 6, 117). Expulsion events are independent of the route of uptake (117), although antibody-opsonized yeast cells tend to be expelled as a clump of cells that subsequently continue to replicate as a biofilm (6), whereas complement-opsonized yeast cells are released individually (6). In addition to having the ability to extrude from host cells, *Cryptococcus* can be laterally transferred from one macrophage to another (5, 118), an event which, like expulsion, does not occur with heat-killed cryptococci or latex beads (5) and is independent of the route of uptake or cryptococcal strain (118). In contrast to extrusion, however, lateral transfer is an actin-dependent process that can be inhibited by treatment with the actin depolymerization drug cytochalasin D (118).

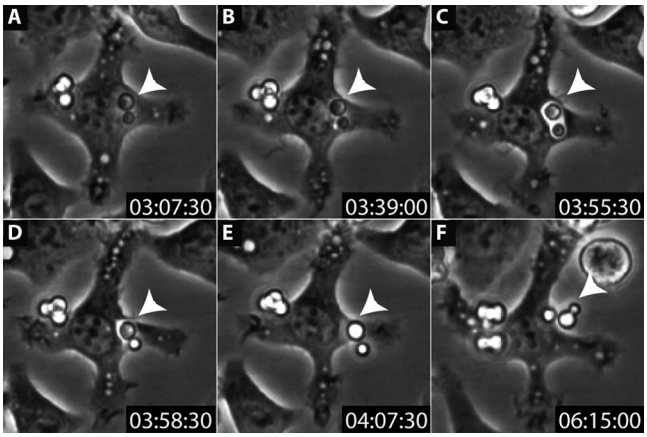

FIG. 2. Cryptococcal expulsion from within a macrophage. *Cryptococcus* can exit macrophages in a novel nonlytic way that does not involve the killing of the host cell or the yeast. (A to C) Time-lapse images of two intracellular yeasts within a macrophage. (D) Four hours into the experiment, the yeast is suddenly expelled. (E to F) Both the macrophage and the yeast remain alive after this process, as shown by continuing proliferation.

Results from restriction fragment length polymorphism analysis suggest that initial infection with *C. neoformans* often occurs in early childhood and can be followed by a long latent phase (55, 58). However, *C. neoformans* is generally capable of dissemination to other organs within the human body and shows a predilection for the central nervous system (73). The interaction between *Cryptococcus* and macrophages might be the key to explaining not only how cryptococcal infection remains latent but also how dissemination within the host is achieved. The intracellular environment is clearly beneficial to the pathogen, as it offers protection from the immune system and thus undisturbed proliferation. However, the purpose of expulsion might be more subtle. Although expulsion from macrophages subjects the yeast cells to a greater immune attack within the extracellular environment, after sufficient intracel-

lular replication it might also lead to fungemia and thus a general breakdown in host immunity. In addition, the so-called "Trojan horse" model suggests that replication within, lateral transfer between, and eventual expulsion from macrophages might offer a potential explanation for how *C. neoformans* stays latent and spreads within the host without triggering immediate immune responses (20, 22, 162). Expulsion and lateral transfer, in particular, might be involved in allowing the yeast to cross the blood-brain barrier either by using macrophages as a trafficking vehicle or by becoming directly transferred to the endothelial layer of the barrier and then expelled into the central nervous system.

Taken together, these possibilities raise the issue of whether macrophages exert a beneficial or deleterious effect during infection. In this context, two recent studies have demonstrated that the absence of macrophages or monocytes was associated with prolonged survival (20, 81). These finding suggest that in certain situations, macrophages are in fact responsible for the development of the disease. In addition, differences in the activities of macrophages have been correlated with the differential susceptibilities of different hosts to infection, highlighting the importance of macrophages in determining the outcome of the disease (164, 200).

ADAPTIVE IMMUNE RESPONSE TO *CRYPTOCOCCUS*

The development of an adaptive immune response is essential to overcoming cryptococcal infection. This section will concentrate on the antibody and cell-mediated immune responses to *Cryptococcus*.

Antibody-mediated immune response to *Cryptococcus*. With *Cryptococcus* being a facultative intracellular pathogen, there is some controversy about the importance of antibody-mediated immune mechanisms for effective microbial clearance. Several cases of cryptococcosis have been reported in patients with primary or acquired B-cell, antibody, or lymphoproliferative deficiencies (18), and antibodies against cryptococcal proteins and capsular polysaccharides are routinely found in individuals without an apparent infection (1, 51, 71), indicating either latent or asymptomatic cleared infections. Passive administration of capsule-binding antibody prolongs survival and/or reduces fungal burdens in experimental cryptococcosis (52, 139). Anticryptococcal antibodies elicit their protective function by opsonizing pathogens for Fc receptor-dependent phagocytosis and by activating the classical complement pathway. In addition, direct antibody opsonization of cryptococci, leading to complement-independent (but complement receptor-dependent via CD18) uptake into macrophages, has been described (145, 183). The specificity of antibodies seems to be of great importance for protective efficacy. Generally, it is thought that the two domains of antibodies fulfill two different functions: the variable region is responsible for antigen binding and the constant region for the effector functions. In studies with *C. neoformans*, it has been shown that this classical view might have to be rethought, since antibodies identical in their variable regions but differing in their constant regions show diverse binding affinity and specificity to a univalent peptide antigen (29, 184, 185). In addition, antibody subgroups developed from the same B cell can be either protective or nonprotective according to their staining patterns (annular or punctuate). Although derived from the same B cell, the two subtypes recognize spatially distinct areas of the cryptococcal capsule, and only a punctuate opsonization pattern can induce a protective immune response in mice. The preponderance of protective compared to nonprotective antibodies determines the efficacy of the antibody-mediated response to *C. neoformans* infection. It appears that small differences in antibody epitope specificities may have a large impact on the protective effect afforded by anticryptococcal antibodies. Those that are protective show a punctuate distribution within the capsule, and those with an annular pattern of capsular distribution are nonprotective (138). Furthermore, enhancement of cryptococcal disease has been described due to excess antibodies triggering immune unresponsiveness (90, 163). The concentration of antibody plays an important role for its protective functions. In mouse models, administration of antibody in higher concentrations can be less effective than in lower concentrations when mice are subsequently challenged with *C. neoformans*. This so-called prozone-like activity suggests that antibodies during cryptococcosis can be protective, nonprotective, or even disease enhancing depending on the antibody isotype and dose (182, 183).

Evidence from mouse models suggests that the protective effect of antibodies is at least partly due to an interaction with cell-mediated immunity. Mice defective in CD4, gamma interferon (IFN-γ), and Th1/Th2-associated cytokines cannot be protected by passive administration of IgG1 antibodies, whereas mice deficient in CD8, natural killer (NK) cells, or complement factor C3 can (12, 165, 199). Thus, antibody-mediated immunity is a significant part of the host defense that integrates into a complex network of different elements of protective anticryptococcal immunity.

Cell-mediated immune response to *Cryptococcus*. The number of cryptococcosis cases drastically increased with the onset of the AIDS pandemic, and to date the highest incidence is still found in HIV-stricken sub-Saharan Africa (150). Besides HIV patients, individuals with extensive corticosteroid treatment, organ transplantation, leukemia, or lymphoma and sarcoidosis belong to a group at high risk for cryptococcal infections (135). The common feature of all of these predispositions is a defect in cell-mediated immunity (CMI). This part of the host defense contributes to cryptococcal killing either directly by cytotoxic effects or indirectly by regulatory functions of natural killer cells or T lymphocytes. NK, $CD4^+$, and $CD8^+$ cells all exhibit direct antimicrobial activity to *C. neoformans* (103, 206), and the secreted proteins granulysin and perforin are able to induce both cryptococcal permeabilization and lysis (44, 191). Although NK cells express both proteins, perforin is the main mediator of anticryptococcal killing via a PI3K-dependent ERK1/2 signaling pathway (120, 126, 195). In contrast, $CD4^+$ and $CD8^+$ lymphocytes utilize the anticryptococcal function of granulysin that is expressed upon activation of STAT5 and PI3K in the presence of IL-2/IL-15 and IL-15, respectively (119, 205, 206). In HIV patients, these two pathways are defective, resulting in inefficient killing of *C. neoformans* (206).

The regulatory arm of CMI seems to be an even more important part of fungal clearance than the ability to directly lyse cryptococci. The outcome of cryptococcosis depends on the immune status of the infected individual and the expression of host cytokines generated in response to the pathogen. Both Th1 and Th2 cytokines are involved in protection against

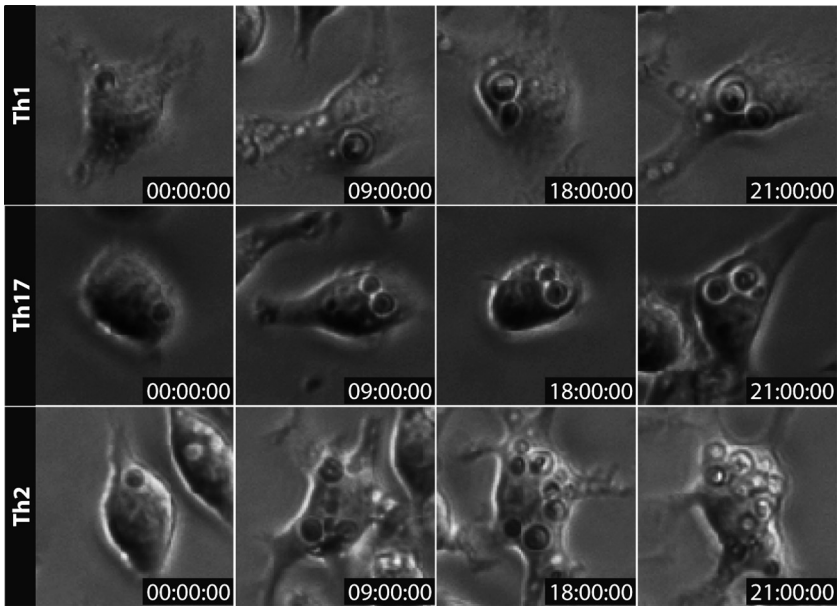

FIG. 3. Th1-Th2-Th17 balance during cryptococcosis. The ability of macrophages to inhibit cryptococcal growth is strongly dependent on cytokine balance. A Th1 and/or Th17 cytokine profile leads to less intracellular *C. neoformans* and *C. gattii* proliferation, whereas a dominant Th2 cytokine profile increases cryptococcal proliferative potential. The three rows show intracellular yeast proliferation at 0, 9, 18, and 21 h after treatment with the Th1 cytokine TNF-α, the Th17 cytokine IL-17, and the Th2 cytokine IL-13.

C. neoformans, but whereas Th1-associated cytokines are essential for natural immunity, Th2-associated immunity is not protective in mice (12, 68, 72). Increased expression of Th1 cytokines, such as tumor necrosis factor alpha (TNF-α) and IFN-γ, results in improved fungal control (50, 80, 130, 196), while IFN-γ knockout mice show an increased fungal burden (7). In addition, Müller et al. have recently demonstrated a significant role for IL-17 and the associated Th17 response in modulating the survival of *C. neoformans*-infected mice (86, 140). In contrast, Th2 cytokines, such as IL-4 and IL-13, reduce the host's ability to deal with *C. neoformans in vivo* (13, 31, 78, 140). The incidence of cryptococcosis increases throughout the course of HIV infection and correlates with the loss of the Th1 response in HIV-infected patients (3) and with a Th2 cytokine profile in transplant patients (171). Thus, the Th1-Th2-Th17 balance is essential for the survival of cryptococci.

In addition to DCs and neutrophils, primary lymphocytes, NK cells, and γδ T cells are involved in the maintenance of this cytokine balance during infection (128, 142, 188, 197). NK cells produce high concentrations of IFN-γ and IL-4 that trigger Th1-mediated immunity but not Th2-related immune responses (79). This activation is opposed by the function of γδ T cells; depletion of γδ T cells in a mouse model leads to a decreased fungal burden and reduced IFN-γ levels (188). Since Th1 immunity is proinflammatory, exaggerated Th1 activation during infection might have negative consequences for individuals, and thus γδ T cells might function as downregulators of Th1 responses to sustain a healthy Th1-Th2 balance (77). However, *C. neoformans* is able to actively change the Th1-Th2 balance toward a Th2 profile by expressing eicosanoids (e.g., prostaglandins and leukotrienes), which are potent inhibitors of Th1-type immunity (146). In addition, expression of the virulence factor urease promotes a Th2 immune response within the lungs via an unknown mechanism (26).

Cytokine signaling also leads to downstream activation or inhibition of antimicrobial effects in other immune cells, such as phagocytic effector cells; Th1 cytokines activate macrophages to create an oxidative and nitrosative burst as microbicidal mechanisms (classically activated macrophages) (72, 168), whereas Th2-polarized host responses lead to inhibition of phagocyte activity (alternatively activated macrophages) and enhanced susceptibility to *C. neoformans* (87). This alternative activation is associated with upregulated expression of genes involved in tissue repair, such as arginase-1 and the mannose receptor (reviewed in reference 59). Arginase-1 competes with inducible nitric oxidase for the substrate L-arginine and so decreases the synthesis of nitric oxide (66). In fact, intracellular cryptococcal proliferation is significantly higher, and the occurrence of expulsion significantly lower, in macrophages activated by the Th2 cytokine IL-4 or IL-13 than in cells stimulated by Th1 cytokines, such as IFN-γ and TNF-α (Fig. 3) (190). Likewise, the number of intracellular cryptococci is increased in alveolar macrophages isolated from IFN-γ knockout mice (7). It is therefore likely that a shift to a Th2 environment results in activation changes in phagocytic effector cells and hence to a change in the composition of phagolysosomes. However, IFN-γ treatment increases the intracellular growth of *C. neoformans* in human macrophages (104, 157), suggesting that aspects of this response may be host species specific.

CRYPTOCOCCAL IMMUNE EVASION STRATEGIES IN HEALTHY AND IMMUNOCOMPROMISED PATIENTS

Pathogens have evolved a great variety of strategies to gain advantage within the host's environment and to successfully undermine the host's defense mechanisms. This section will focus on strategies applied by *Cryptococcus* that counteract the host's immune responses and then discuss how this scientific

knowledge may contribute to the development of more efficient therapeutic regimes.

Cryptococcal immune evasion strategies. Infections are associated with a continuous struggle between the pathogen and the host. To achieve an advantage in the host cell environment, *Cryptococcus* expresses a wide range of virulence factors (e.g., capsule, melanin, secreted enzymes) that can modify the host's immune response to improve pathogen survival.

The cryptococcal polysaccharide capsule is the best-studied virulence factor (reviewed in reference 202). It inhibits phagocytosis by macrophages (14, 60, 102), dendritic cells (189), and neutrophils (39, 40, 91), and consequently, nonencapsulated strains are phagocytosed three times more effectively by human leukocytes (15, 27). This effect seems to result from a masking mechanism of opsonins by which the polysaccharides form a barrier between the opsonins and their receptors (127). Once infection is established, high concentrations of free glucuronoxylomannan, the major component of the cryptococcal capsule, are found in the patient's bodily fluids, making it likely that many antibodies form immune complexes before they can efficiently opsonize yeast cells (155). In addition, *C. neoformans* expresses the factor antiphagocytic protein 1 (APP1), which inhibits uptake through a complement-mediated mechanism of binding to complement receptors 2 and 3 (114, 176).

Besides having antiphagocytic properties, the cryptococcal capsule provides protection against reactive oxygen species (ROS) and nitrogen species within host cells. Capsule enlargement upon interaction with phagocytic effector cells *in vivo* and *in vitro* (45) correlates with cryptococcal susceptibility to ROS; ROS kill cells with larger capsules less efficiently than they kill cells with smaller capsules (201). This effect may be due to the action of glucuronoxylomannan; this major component of the cryptococcal capsule reduces cryptococcal killing and the production of superoxide in primary human neutrophils (137). In fact, *C. neoformans* shows high phenotypic plasticity; a process called phenotypic switching enables a switch in cryptococcal morphology between smooth and mucoid, with the latter cell type showing increased survival in murine macrophages (53, 63, 75). In addition, several secreted enzymes are also involved in detoxifying oxygen and nitrogen radicals: superoxide dismutase (SOD) (24), an alternative oxidase (AOX1) (2), a flavinhemoglobin denitrosylase (FHB1) (32), urease (URA1) (26), glutathione peroxidase (GLR1) (132), and thiol peroxidase (TSA1) (132). Finally, the enzyme laccase appears to protect *C. neoformans* in multiple ways from oxidative and nitrosative stresses; it is involved in the production of the antioxidant pigment melanin via the sphingolipid pathway (67, 192, 193) and also protects *C. neoformans* in a melanin-independent manner by an iron oxidase function that may maintain iron in an oxidized form, thereby inhibiting production of hydroxyl radicals (111).

Upon uptake, phagocytic effector cells decrease the phagolysosomal pH to below 5.5 to improve microbial killing (179). However, *C. neoformans* can survive in these low-pH conditions; indeed, artificially increasing the pH, by adding agents such as chloroquine, actually inhibits cryptococcal survival (106). The enzyme inositol phosphosphingolipid-phospholipase C (ISC1) is important for adaptation to the acidic environment, and deletion of *isc1* renders cryptococcal cells more susceptible to acidic, oxidative, and nitrosative stresses (166). ISC1 generates phytoceramides that are known to play an important role in regulating PMA1 (49, 57), an ATPase that is involved in the regulation of intracellular pH (69, 173, 175), as well as oxidative (170) and nitrosative (204) stresses in *Saccharomyces cerevisiae*.

Glucosylceramide (GlcCer), a glycosphingolipid found at the surfaces of *C. neoformans* cells, has been identified as a new regulator of fungal virulence in recent years (160). Knockout of the GlcCer synthase 1 (GCS1) gene results in a very interesting phenotype in mouse models where the mutant is rendered avirulent following nasal inhalation yet causes fatal disease when injected intravenously (160). The Δ*gcs1* mutant strain also shows a specific growth defect under high CO_2 and at neutral pH and grows well within macrophages (160). Within tissues, the CO_2 concentration is relatively high, at 5%, compared to 0.04% in the atmosphere, suggesting that the sphingolipid might be involved in adaptation to the conditions within the host environment and that the mutant is impaired in traversing the lung tissue to reach the intracellular niche (133).

The functions of many of the cryptococcal virulence factors mentioned above depend on the availability of metal ions and thus cation homeostasis. A recent study has suggested that anti-inflammatory cytokines might enhance iron uptake and storage by macrophages by suppressing the activation of iron regulatory proteins 1 and 2, leading to translational repression of the iron storage protein ferritin or translational activation of the membrane receptor for iron uptake (148). This would result in increased metal ion availability and thus increased activity of virulence factors.

Immunotherapy in cryptococcal disease. The new generation of antifungals, echinocandins, are not active against *C. neoformans* and in consequence are not used in clinical practice, thus limiting the range of antifungals available to treat the disease (123). Current antifungal treatment regimes involve combination therapy of amphotericin B, flucytosine, and fluconazole (151). The introduction of highly active antiretroviral therapy (HAART) has reduced the incidence of cryptococcosis in developed countries but not the short-term mortality, and hence HAART has not improved the clinical outcome of cryptococcosis. Despite rapid clinical intervention, the 3-month mortality of HIV patients with acute cryptococcal meningoencephalitis is as high as 20% (42, 112). This poor prognosis has resulted in the need for exploration of alternative treatment regimes, such as immunotherapy, passive immunization, and cytokine-based treatment strategies. One promising approach might be the use of adjunctive passive immunotherapy with monoclonal antibodies. As the administration of anticapsular antibodies is protective in infected mice (41, 61), a phase I trial with the monoclonal antibody 18B7 was conducted with HIV patients who had recovered from cryptococcal meningitis (99). Therapy was well tolerated for antibody concentrations up to 1 mg/kg of body weight, with higher doses showing pharmacological effects (99). A second antibody, 2G8, targeting the cell wall glucan exerts remarkable anticryptococcal activity *in vitro* and *in vivo* and might be a good candidate as a new therapeutic agent (156). Thus, there is clear potential for anticryptococcal-antibody-mediated therapy. However, it remains a significant challenge to design treatment strategies considering the complex pharmacodynamics of administrated antibodies and antigens within the host as well as drug-associated toxicity. Vacci-

nation with GXM-tetanus toxoid conjugates in mouse model systems has also shown promise (34, 35) but is not yet approved for clinical use.

Cytokine-based treatments have also been proposed for cryptococcosis treatment. IFN-γ levels at the site of infection correlate with fungal burden (169), and administration of IFN-γ has successfully improved the outcome of systemic cryptococcosis in mouse model systems (23, 116) and in one human patient (144). However, although IFN-γ seems to have a protective role in mice (7, 68) and to increase fungicidal activity of murine macrophages (50), the same treatment reduces anticryptococcal activity in human macrophages (104, 157), suggesting that this line of treatment may be less advantageous for human patients.

CONCLUSIONS

C. neoformans is now recognized to be a facultative intracellular pathogen of host cells. This intracellular location provides a niche to escape host immune mechanisms (e.g., complement and antibodies) and also reduces the exposure to antifungal agents. *C. neoformans* has developed a wide range of mechanisms to adapt to the intracellular niche and counteract the host's immune response. Future research will need to consider the ability to parasitize host cells in order to advance therapeutic schemes. Targeting intracellular survival and growth and/or cryptococcal virulence factors expressed during intracellular parasitism might offer new strategies to improve anticryptococcal treatment. Finally, since *C. neoformans* is a major pathogen of immunocompromised patients, new strategies need to consider targeted modification of the patient's immune system, for instance by the selective administration of proinflammatory cytokines, to encourage the expression of a protective immune profile.

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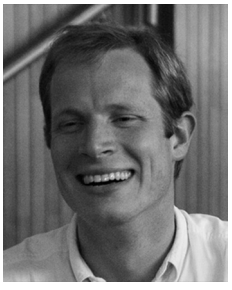

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