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In vitro C3 deposition on *Cryptococcus* capsule occurs via multiple complement activation pathways

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ABSTRACT

Complement can be activated via three pathways: classical, alternative, and lectin. *Cryptococcus gattii* and *Cryptococcus neoformans* are closely related fungal pathogens possessing a polysaccharide capsule composed mainly of glucuronoxylomannan (GXM), which serves as a site for complement activation and deposition of complement components. We determined C3 deposition on *Cryptococcus* spp. by flow cytometry and confocal microscopy after incubation with serum from C57BL/6J mice as well as mice deficient in complement components C4, C3, factor B, and mannose binding lectin (MBL). *C. gattii* and *C. neoformans* activate complement in EGTA-treated serum indicating that they can activate the alternative pathway. However, complement activation was seen with factor $B^{-/-}$ serum suggesting activation could also take place in the absence of a functional alternative pathway. Furthermore, we uncovered a role for C4 in the alternative pathway activation by *Cryptococcus* spp. We also identified an unexpected and complex role for MBL in complement activation by *Cryptococcus* spp. No complement activation occurred in the absence of MBL-A and -C proteins although activation took place when the lectin binding activity of MBL was disrupted by calcium chelation. In addition, alternative pathway activation by *C. neoformans* required both MBL-A and -C, while either MBL-A or -C was sufficient for alternative pathway activation by *C. gattii*. Thus, complement activation by *Cryptococcus* spp. can take place through multiple pathways and complement activation via the alternative pathway requires the presence of C4 and MBL proteins.
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1. Introduction

The complement system consists of a cascade of serum proteins that contribute to opsonization, membrane lysis, and chemotaxis. There are three pathways through which complement can be activated: classical, alternative, and lectin. Complement components C3 and C5–9 are required in all three complement pathways. C1q and factor B are required only by the classical and the alternative pathway, respectively. C4 is used in both the classical and lectin pathways and mannose binding lectin (MBL) is used in both the lectin and alternative pathways (Shi et al., 2004). MBL exists in two forms in rodents (MBL-A and MBL-C) while only one form is found in humans. Murine MBL-A and MBL-C have 50% homology and slight differences in ligand recognition, especially D-glucose and α-methyl-D-glucose (Hansen et al., 2000), but neither of these sugars is part of the polysaccharide capsule of *Cryptococcus* spp. Ficolins have been shown to play a role in complement activation via the lectin pathway in humans (Runza et al., 2008).

C57BL/6J mice deficient in different components of the complement cascade can be used to determine the role of different pathways in complement activation. The classical pathway does not operate in $C1q^{-/-}$ (Botto et al., 1998) or $C4^{-/-}$ mice (Fischer et al., 1996); $C4^{-/-}$ mice are also deficient in the lectin pathway, and factor $B^{-/-}$ mice are deficient in the alternative pathway (Matsumoto et al., 1997). MBL-A or MBL-C deficient mice still have functional classical, lectin and alternative pathways but MBL-A/C double knockout mice completely lack the lectin pathway activated by MBL (Shi et al., 2004; Takahashi et al., 2002). C3 plays the central role in complement activation and all three complement pathways do not function in $C3^{-/-}$ mice (Wessels et al., 1995).

Abbreviations: GXM, glucuronoxylomannan; MBL, mannose binding lectin; EGTA, ethylene glycol tetraacetic acid.

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There are two major pathogenic species of *Cryptococcus*: *C. neoformans* and *C. gattii*. *C. neoformans* usually infects immunocompromised people, while *C. gattii* generally infects immunocompetent people; both can cause pneumonia and meningoencephalitis (Casadevall and Perfect, 1998). *C. neoformans* is responsible for most infections in people with T cell deficits, including HIV-infected individuals, while the ongoing Vancouver Island outbreak is attributed to *C. gattii* (Stephen et al., 2002).

Both pathogenic *Cryptococcus* spp. possess a polysaccharide capsule composed mainly of GXM. The Kozel laboratory and others have shown that *Cryptococcus* spp. strongly activate the alternative pathway of the complement cascade (Diamond et al., 1974; Kozel et al., 1991), while the polysaccharide capsule blocks activation of the classical pathway that can occur at the cell wall of non-encapsulated strains via anti-cell wall IgGs found in normal human serum (Kozel et al., 1991). No studies have assessed classical pathway activation using anti-capsule antibodies. Human MBL has been reported to not bind to *Cryptococcus* spp. (Panepinto et al., 2007; Schelenz et al., 1995), but no studies have been published characterizing the role of murine MBL in activation of complement by pathogenic cryptococcal species.

The capsule serves as a site for deposition of C3 fragments, mainly iC3b, which promote phagocytosis of the yeast (Kozel, 1993). While one study found that *C. gattii* bound less C3 and factor B than *C. neoformans* (Washburn et al., 1991), another study indicated the maximum amount of bound C3 did not differ significantly between species. Instead, the rate of C3 deposition was found to be species dependent, with faster accumulation on serotypes A and D (Young and Kozel, 1993). Capsule size is known to influence complement activation, with a larger capsule resulting in more C3 deposition, though the correlation is not perfectly proportional (Young and Kozel, 1993). Capsule size is dependent upon strain, but *Cryptococcus* spp. produce more capsule *in vivo*, as a result of changes in carbon dioxide concentration, osmolarity, and phenotypic switching (Jain and Fries, 2008). Capsule production can be induced *in vitro* using serum or carbon dioxide (Zaragoza et al., 2003). *Cryptococcus* spp. grown under capsule-inducing conditions may be more clinically relevant, since a larger capsule is correlated with increased virulence (Casadevall and Perfect, 1998).

Recently, mice deficient in complement components C1q, C4, C3, factor B, and MBL have been generated on the C57BL/6J background. In this study, we used serum from these mice to investigate complement activation by the pathogenic cryptococcal species *C. gattii* and *C. neoformans*. Complement activation was followed by detecting C3 deposition using a fluorescent antibody against C3 and flow cytometry or confocal microscopy. We found that both *C. neoformans* and *C. gattii* were opsonized by C3 in the presence of factor B-deficient serum, indicating *in vitro* complement activation does not require a fully intact alternative pathway. Additionally, we found a dependence of the alternative pathway on C4, consistent with a previous report in which C4b was shown to increase the stability of C3b tenfold (Meri and Pangburn, 1990). We also found that activation of the alternative pathway by *C. neoformans*, but not *C. gattii*, depended on both MBL-A and MBL-C. Furthermore, *C. neoformans* did not activate complement in the absence of MBL-A, while either isoform was sufficient for complement activation by *C. gattii*.

2. Materials and methods

2.1. Cryptococcus

Three milliliters Sabouraud's dextrose broth (Becton Dickinson, Franklin Lakes, NJ) was inoculated with a clinical isolate of *C. gattii* (strain A1MR265, provided by Dr. James Kronstad, University of British Columbia) or *C. neoformans* (strain H99, provided by Dr. Arturo Casadevall, Albert Einstein College of Medicine) and incubated at 37 °C with shaking for 48 h. *Cryptococcus* was pelleted by centrifugation (1950 × *g* for 10 min at 4 °C), counted using a haemocytometer, and diluted to the desired concentration in cold PBS. To heat-kill *Cryptococcus*, organisms were incubated at 55 °C for 1 h.

To induce large capsule formation, 0.1 ml of a two-day *Cryptococcus* culture was added to large capsule medium, consisting of 21 ml deionized water, and 3 ml each of 10× nutrient solution (100 g dextrose, 4.35 g L-glutamine, 1.5 g $MgSO_4 \cdot 7H_2O$, 6.5 g urea, 10 mg thiamine·HCl, 10 mg $MnCl_2 \cdot 4H_2O$, 14 mg $FeSO_4 \cdot 7H_2O$, 14 mg $ZnSO_4 \cdot 7H_2O$, 1 mg $CuSO_4 \cdot 5H_2O$, 74 mg $CaCl_2 \cdot 2H_2O$, in 500 ml water, sterile-filtered), 10× supplement solution (10.1 g sodium bicarbonate, 29.8 g HEPES, in 500 ml water, sterile-filtered), and 10× buffer solution (2.65 g KH_2PO_4, 5.3 g K_2HPO_4, in 500 ml water, sterile-filtered). Culture was gassed with carbon dioxide and incubated for three days at 37 °C with shaking. The cultures were centrifuged and washed twice in cold PBS. Large capsule size was verified by India ink staining.

2.2. Mouse sera

C57BL/6J mice were purchased from Charles River Laboratories. $C3^{-/-}$ and $C4^{-/-}$ mice were a gift from Dr. Michael Carroll (Harvard Medical School) (Fischer et al., 1996; Wessels et al., 1995). Factor $B^{-/-}$ mice were provided by Dr. Rick Wetsel (University of Texas, Houston) (Matsumoto et al., 1997). MBL-deficient mice were generated by Dr. Kazue Takahashi (Shi et al., 2004; Takahashi et al., 2002). $C1q^{-/-}$ mice were made by Dr. Mark Walport (The Wellcome Trust, London, United Kingdom) (Botto et al., 1998) and provided by Dr. Andrea Tenner (University of California, Irvine, CA). All animals were maintained in accordance with the Chancellor's Animal Research Committee at UCLA. Animals were anesthetized and bled retroorbitally, or euthanized and blood collected by cardiac puncture immediately after death. Blood was collected in Capiject gel clot activator tubes (Terumo Medical Corporation, Somerset, NJ) and allowed to clot for 30 min at room temperature. Tubes were then spun in a microcentrifuge at 14,200 rpm for 15 min at 4 °C. Serum was collected from the tubes and stored at −20 °C.

2.3. Detection of C3 binding by flow cytometry

Sixty microliters of *Cryptococcus* (1×10^7 CFU/ml) was incubated with 40 μl serum in the presence of 1 mM $MgCl_2$ and 0.15 mM $CaCl_2$. To inhibit the classical and lectin pathways, EGTA was added to a final concentration of 40 mM in the presence of 5 mM $MgCl_2$. EDTA was added to a final concentration of 40 mM to inhibit all pathways (data not shown). After incubation at 37 °C for 30 min, the reaction was stopped with 1 ml ice cold 10 mM EDTA in PBS. Cells were centrifuged (850 × *g* for 5 min at 4 °C) and washed two times with 1 ml PBS. Cells were stained with 50 μl of 1:500 goat anti-mouse C3-FITC (ICN, Aurora, OH) and incubated on ice for 30 min. Cells were washed two times with 1 ml PBS and fixed with 200 μl 2% paraformaldehyde. Cells were then washed two times with 1 ml PBS and resuspended in 300 μl PBS with 1% bovine serum albumin (BSA, Sigma Aldrich, Saint Louis, MO) and 0.02% sodium azide. In some experiments, all volumes were cut in half except for the volume of anti-mouse C3 and the final suspension in 1% BSA. Flow cytometry was conducted on a Becton Dickinson FACScan and a Becton Dickinson FACSCalibur using Cell Quest Pro software. Data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).