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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 *C. 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.

Keywords

Cryptococcus; complement

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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 -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).

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 ten-fold (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 ml Sabouraud's dextrose broth (Becton Dickinson, Franklin Lakes, New Jersey) 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 hemacytometer, and diluted to the desired concentration in cold PBS. To heat-kill *Cryptococcus*, organisms were incubated at 55°C for one hour.

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 MgSO₄•7H₂O, 6.5 g urea, 10 mg thiamine•HCl, 10 mg MnCl₂•4H₂O, 14 mg FeSO₄•7H₂O, 14 mg ZnSO₄•7H₂O, 1 mg CuSO₄•5H₂O, 74 mg CaCl₂•2H₂O, in 500 ml water, sterile-filtered), 10 X supplement solution (10.1 g sodium bicarbonate, 29.8 g HEPES, in 500 ml water, sterile-filtered), and 10X buffer solution (2.65 g KH₂PO₄, 5.3 g K₂HPO₄, 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, California). 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, New Jersey) and allowed to clot for thirty minutes at room temperature. Tubes were then spun in a microcentrifuge at 14,200 rpm for fifteen minutes at 4°C. Serum was collected from the tubes and stored at -20°C.

2.3. Detection of C3 binding by flow cytometry

Sixty μ l of *Cryptococcus* (1×10^7 CFU/ml) was incubated with 40 μ l serum in the presence of 1 mM MgCl₂ and 0.15 mM CaCl₂. To inhibit the classical and lectin pathways, EGTA was added to a final concentration of 40 mM in the presence of 5 mM MgCl₂. EDTA was added to a final concentration of 40 mM to inhibit all pathways (data not shown). After incubation at 37°C for 30 minutes, the reaction was stopped with 1 ml ice cold 10 mM EDTA in PBS. Cells were centrifuged (850 g for 5 minutes at 4°C) and washed two times with 1 ml PBS. Cells were stained with 50 μ l of 1:500 goat anti-mouse C3-FITC (ICN, Aurora, Ohio) and incubated on ice for 30 minutes. Cells were washed two times with 1 ml PBS and fixed with 200 μ l 2% paraformaldehyde. Cells were then washed two times with 1 ml PBS and resuspended in 300 μ l PBS with 1% bovine serum albumin (BSA, Sigma Aldrich, Saint Louis, Missouri) 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, Oregon).

2.4. Detection of C3 binding by fluorescence confocal microscopy

Binding of C3 to *C. neoformans* and *C. gattii* was also determined by confocal microscopy. Yeast cells were incubated with mouse serum with calcium and magnesium with or without EGTA (as described in 2.3). Cells were centrifuged (850 $\times$ g for 5 minutes at 4°C) and washed two times with 1 ml PBS. Cells were then stained with 50 μ l of 1:500 goat anti-mouse C3-FITC and incubated on ice for 30 minutes. After a final wash, yeast cells were resuspended in *SlowFade* Gold antifade mounting media (Invitrogen), placed on a glass slide and examined under oil immersion at 1000 $\times$. Acquisition of images was performed with a Zeiss LSM 510 laser-scanning confocal microscope using LSM 510 software. No differences in binding patterns were observed between live and heat-killed organisms (data not shown).

3. Results

3.1. The classical pathway does not play a direct role in complement activation by *Cryptococcus* spp

To examine complement pathways activated by *Cryptococcus* spp., we used flow cytometry to measure C3 deposition on live or heat-killed *C. gattii* and *C. neoformans* grown under normal or capsule-inducing conditions, incubated with mouse serum. Both *C. gattii* and *C. neoformans* had C3 deposition in the presence of wild type serum under all conditions (Figure 1A). There was decreased complement activation under all conditions in the presence of EGTA. EGTA has a higher affinity for calcium ions than magnesium ions. C1 and MBL require Ca²⁺ for activity and consequently do not function in the presence of EGTA, so only the alternative pathway remains functional. These results support a role for the classical and/or lectin pathways in activation of complement by *Cryptococcus* spp., although a functional alternative pathway is sufficient for some activation. As expected, there was no C3 deposition seen when C3-deficient serum was used (Figure 1B). Live *C. gattii* had C3 deposition using serum deficient in C1q, in which the classical pathway is non-functional, while EGTA inhibited activation in a similar fashion to wildtype serum (Figure 1C). Similar results were obtained for *C. neoformans* and C1q^{-/-} serum (data not shown).

This suggests the classical pathway does not play a role in complement activation by *Cryptococcus* spp. under these conditions using serum from non-immune mice.

We also examined C3 deposition on yeast cells by fluorescence confocal microscopy (Figure 2). In both *C. neoformans* and *C. gattii*, C3 deposition occurred evenly around the capsule. Consistent with the flow cytometry analysis, there was decreased fluorescence observed when samples were treated with EGTA (Figure 2A). There was no fluorescence detected when examining yeast cells incubated with serum from mice deficient in C3, C4 (in presence of EGTA), or Factor B (in presence of EGTA), data not shown.

3.2. Complement activation by *Cryptococcus* spp. through the alternative pathway requires C4

C3 deposition was also decreased in the absence of C4, consistent with the classical and/or lectin pathway playing a role in complement activation (Figures 2B and 3). Surprisingly, complement activation by C4 deficient serum was completely inhibited when EGTA was added, indicating that the complement activation through the alternative pathway seen in Figure 1 required the presence of C4. The level of C3 deposition in the presence of C4^{-/-} serum was lower in large capsule *C. gattii* than in normal capsule *C. gattii*; enhanced complement activation was seen with heat-killed large capsule *C. neoformans*.

3.3. Complement activation may take place through the lectin pathways

The alternative pathway has previously been shown to play a major role in complement activation by *Cryptococcus* spp. We observed decreased but significant C3 deposition using factor B-deficient serum, which is deficient in complement activation via the alternative pathway (Figures 2B and 4). Thus, complement activation can take place through the lectin pathways in addition to the alternative pathway. Complement deposition using factor B-deficient serum was completely inhibited in the presence of EGTA, as expected.

3.4. MBL is essential for complement activation by *Cryptococcus* spp

To evaluate the role of the lectin pathway in complement activation, MBL-A^{-/-}/C^{-/-} serum was used (Figure 5). Complement activation did not occur under any of the conditions tested, suggesting that MBL is essential for complement activation by *Cryptococcus* spp., even through the alternative pathway.

3.5. Alternative pathway activation by *C. neoformans* requires both MBL-A and MBL-C, while either MBL-A or MBL-C is sufficient for alternative pathway activation by *C. gattii*

To further explore the role of MBL in complement activation, sera from MBL-A^{-/-} and MBL-C^{-/-} mice were used. Complement activation in the absence of MBL-A varied by *Cryptococcus* spp. *C. gattii* was able to activate complement in the absence of MBL-A, although not to the level observed using wild type serum (compare Figures 1 and 6). Using heat-killed *C. gattii* in the presence of only an active alternative pathway there was no (normal capsule) or little (large capsule) complement activation. This contrasts with what is observed when MBL-A protein is present and rendered non-functional by EGTA (Figure 1). Different results were seen with *C. neoformans*. Live normal capsule *C. neoformans* was unable to activate the complement cascade in the absence of MBL-A, while heat-killed normal capsule *C. neoformans* showed some activation. Both live and heat-killed large capsule *C. neoformans* were able to activate complement, though at a reduced level compared to C57BL/6J serum. Little to no activation via the alternative pathway was seen in the absence of MBL-A protein.

Complement activation occurred in all of the conditions tested when MBL-C^{-/-} serum was used, with a slight reduction in levels of C3 deposition in comparison to wild type serum

(Figure 7). The size of the small population of cells without C3 deposition varied from experiment to experiment. In the presence of EGTA, *C. gattii* was capable of activating complement, although at a reduced level, especially with heat-killed organism. Very little to no C3 deposition was observed with MBL-C^{-/-} serum in the presence of EGTA using *C. neoformans*.

4. Discussion

The complement system is important in immunoprotection against the pathogenic *Cryptococcus* spp. *C. neoformans* and *C. gattii*. There is strong evidence supporting a role for the alternative pathway in innate protection, since animals deficient in C3 but not C4 have reduced survival (Diamond et al., 1973; Mershon et al., 2009; Shapiro et al., 2002). In mouse strains B10.D2/oSn, DBA/2, and A/J, which are all deficient in C5, *C. neoformans* infection progresses to a fatal pneumonia with higher fungal burdens in multiple major organs compared to wild type animals (Dromer et al., 1989; Rhodes et al., 1980; Rhodes, 1985). *Cryptococcus* spp. strongly activate the alternative pathway of the complement cascade (Diamond et al., 1974; Laxalt and Kozel, 1979). Unencapsulated strains of *C. neoformans*, which are nonpathogenic, activate complement much in the same way as zymosan (*Saccharomyces cerevisiae*) granules (Kozel et al., 1992; Truelsen et al., 1992), and both activate classical and alternative pathways when incubated with serum. In pathogenic encapsulated strains, the polysaccharide capsule apparently blocks classical pathway activation (Kozel et al., 1991).

We measured C3 deposition on the pathogenic *Cryptococcus* spp. *C. gattii* and *C. neoformans* using mouse serum and a fluorescently-labeled polyclonal antibody that recognizes C3 and its fragments. Thus, we were able to detect intact C3 or C3 that had been cleaved as part of the complement activation cascade that remained on the *Cryptococcus* cells. We grew *Cryptococcus* spp. under normal conditions as well as capsule-inducing conditions. Capsule size can influence complement deposition (Young and Kozel, 1993) and more capsule is produced during *in vivo* infections (Casadevall and Perfect, 1998). We examined both live and heat-killed *Cryptococcus* as heat-killing is often used when performing *in vitro* studies to decrease the risks of working with this biohazardous organism. However, heat-killing may compromise the capsule integrity, thus altering complement activation.

As expected, C3 was deposited on both *Cryptococcus* spp. in the presence of C57BL/6J serum, regardless of capsular growth conditions and heat exposure. Complement activation occurred in the presence of EGTA, indicating the alternative pathway was sufficient for C3 deposition, consistent with previous studies using human serum as the complement source (Diamond et al., 1974; Laxalt and Kozel, 1979). Heterogeneity in C3 deposition was observed in the presence of EGTA in the conditions where the alternative pathway remained functional. It is possible that heterogeneity in the capsule of *Cryptococcus* cells affects complement activation by the alternative pathway. There were no differences observed in complement activation using C1q-deficient serum in comparison to wild type serum, indicating that the classical pathway does not play a role in direct activation of complement by *Cryptococcus* spp.

C4 was found to play an unexpected role in complement activation by *Cryptococcus* spp. C4 is known to play a role in activation via both the classical and lectin pathways. C4-deficient serum showed a slight decrease in C3 deposition using both *Cryptococcus* spp., consistent with a role for the lectin and/or classical pathway in complement activation. This role was also supported by the results obtained with factor B-deficient serum, where significant complement activation was seen in the absence of functional factor B. Surprisingly, under

conditions in which alternative pathway activation should take place (treatment with EGTA), C3 deposition was completely absent with C4-deficient serum. This result indicated that complement activation via the alternative pathway required C4, which contradicts the current paradigm that C4 is not involved in alternative pathway activation. However, it has been reported that C4b binds to C3b and protects it from inactivation by factors H and I, resulting in a ten-fold greater stability compared to free C3b or C3b bound to a non-activating surface (Meri and Pangburn, 1990). While it has been shown that the lectin pathway can activate complement using components of the alternative pathway in the absence of C4 and C2 (Selander et al., 2006), our studies suggest an essential role for C4 in activation of the alternative pathway by *Cryptococcus* spp.

Human MBL only binds to acapsular strains, suggesting that the capsule inhibits MBL binding (Schelenz et al., 1995; Panepinto et al., 2007). Since acapsular strains are not pathogenic, the inability of MBL to bind to encapsulated strains suggests that MBL does not play a role in protection against cryptococcal infection. Takahashi et al recently reported a role for MASP-1, one of the enzymes in the lectin pathway, in activation of the alternative pathway (Takahashi et al., 2010). However, a direct role for MBL-A and MBL-C in alternative pathway activation by *Cryptococcus* spp. was unexpected. In the absence of both lectins, no complement activation took place by either organism. To date, there have been no studies that suggest a role for MBL-A or MBL-C in activation of the alternative pathway. However, our study shows that MBL contributed to the activation of the alternative complement pathway *in vitro*. Interestingly, the alternative pathway was activated when MBL was present, but rendered non-functional by the calcium chelator EGTA, but was not activated when the MBL proteins were absent. Perhaps a domain other than the calcium-dependent binding domain of MBL contributes to alternative pathway activation. The absence of both MBL lectins and the absence of C4 result in the same functional consequence, which was the inability of *Cryptococcus* spp. to activate complement through the alternative pathway. Although ficolins have been shown to play a role in complement activation, mouse ficolin A, the only ficolin present in mouse serum (Fujimori et al., 1998), was not sufficient for complement activation by *Cryptococcus* spp. since no complement activation was seen with MBL-deficient serum (Figure 4). This is not surprising given that mouse ficolin A is present at less than 10% of the serum concentration of MBL-A and MBL-C combined (Endo et al., 2009). Furthermore, mouse ficolin A binds to N-acetylglucosamine and N-acetylgalactosamine, which are not present in the polysaccharide capsule of *Cryptococcus* spp., though N-acetylglucosamine is present in the chitin of yeast cell walls. However, the capsule of *Cryptococcus* spp. inhibits binding of soluble molecules to cell wall components. We could find no reports of ficolins binding to *Cryptococcus* spp.

Unexpectedly, complement activation could also take place in the absence of the alternative pathway, as demonstrated by C3 deposition with factor B-deficient serum, indicating the lectin pathway is also sufficient for complement activation. C3 deposition was abrogated when factor B-deficient serum was treated with EGTA, which chelates only calcium thereby inhibiting the classical and lectin pathways, but not the alternative pathway. This confirmed that the lectin pathway plays a role in complement activation, since classical pathway activation is not required. The assay was specific, as no C3 deposition was seen when C3-deficient serum was used.

The role of MBL-A and MBL-C in complement activation was found to be complex. Either MBL-A or MBL-C was sufficient for complement activation by *C. gattii* under all conditions examined, consistent with a previous study showing either isoform of murine MBL can activate the complement cascade (Hansen et al., 2000). In the absence of either MBL-A or MBL-C, normal capsule and large capsule *C. gattii* could activate the alternative pathway. However, heat-killed *C. gattii* showed a reduced ability to activate the alternative

pathway in the absence of MBL-A or MBL-C; indeed, heat-killed normal capsule *C. gattii* required the presence of both lectins to activate the alternative pathway. Perhaps heat-killing alters the capsule in such a way that increased MBL-binding is required for alternative pathway activation. A somewhat different pattern was seen with *C. neoformans*. In the absence of MBL-A but not MBL-C, live normal capsule *C. neoformans* failed to activate complement, indicating that activation by live *C. neoformans* requires functional MBL-A. In contrast, activation by heat-killed normal capsule, as well as live and heat-killed large capsule *C. neoformans* could take place in the absence of MBL-A. Activation by all forms of *C. neoformans* could take place in the absence of MBL-C. While MBL-C is present at approximately six-fold higher concentration in serum than MBL-A (Liu et al., 2001), MBL-A has a higher affinity than MBL-C for D-glucose and α-methyl-D glucose, but neither of these sugars is found on the cryptococcal capsule. Importantly, alternative pathway activation by *C. neoformans* did not take place when either lectin was absent, indicating that alternative pathway activation by *C. neoformans* requires the presence of both MBL proteins. This contrasts with what was seen for live *C. gattii*, where the presence of either lectin was sufficient for complement activation in the presence of EGTA.

Our results suggest that *C. gattii* and *C. neoformans* are equally proficient in complement activation, which is contrary to previous reports that *C. neoformans* activates complement better than *C. gattii* (Washburn et al., 1991; Young and Kozel, 1993). These differences may be attributable to the different strains used. Using serum from wild-type mice, *C. neoformans* and *C. gattii* grown under capsule-inducing conditions showed similar levels of C3 deposition as organisms grown under normal capsule conditions. This contrasts with a previous study that showed larger capsule results in more C3 deposition (Young and Kozel, 1993), however that earlier study used human serum, while mouse serum was used in the present study. Heat-killed *C. gattii* had lower levels of C3 deposition than live *C. gattii* in the presence of serum deficient in MBL-A or MBL-C and treated with EGTA, but these differences were not observed with wild-type serum, suggesting that heat killing may decrease the ability of *C. gattii* to activate complement via the lectin pathway but not the alternative pathway.

In summary, our studies expand upon previous findings and show that complement activation is dependent upon *Cryptococcus* spp., capsule size, and whether organisms have been heat-killed. We have shown that complement activation by *Cryptococcus* spp. can take place *in vitro* in the absence of a functional alternative pathway. For *C. neoformans*, complement activation by normal capsule yeast required functional MBL-A, while the presence of either MBL isoform was sufficient for complement activation by normal capsule *C. gattii*. Effective activation of the alternative pathway by *C. neoformans* required that functional MBL-A and MBL-C both be present, illuminating a previously unknown dependency of alternative pathway activation on MBL. We have also confirmed a dependency of the alternative pathway on functional C4. Thus, our studies have revealed unexpected and complex interactions among the different activation pathways.

It remains to be determined how the *in vitro* complement activation we characterized relates to *in vivo* complement activation during murine infection with *C. neoformans* or *C. gattii*. The role of complement activation in providing protection against infection is likely to depend upon the strain of *Cryptococcus* as well as host background, and may reflect complex interactions between different effectors of the innate immune system.

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Abbreviations

GXM	Glucuronoxylomannan
MBL	mannose binding lectin
EGTA	ethylene glycol tetraacetic acid

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Highlights

- We determined complement deposition on the polysaccharide capsule of *Cryptococcus* spp.
- *C. gattii* and *C. neoformans* activate the alternative pathway.
- We found a role for C4 in the alternative pathway activation by *Cryptococcus* spp.
- Alternative pathway activation by *C. neoformans* required both MBL-A and -C.
- Either MBL-A or -C was sufficient for alternative pathway activation by *C. gattii*.

Figure 1. C3 deposition in the presence of C57BL/6J or C3^{-/-} serum

Live and heat-killed *Cryptococcus* spp. grown under normal and capsule-inducing conditions were incubated with (A) wild type serum with or without 40 mM EGTA to inhibit the classical and lectin pathways so that activation could take place only through the alternative pathway, (B) C3^{-/-} serum, or (C) C1q^{-/-} serum. Samples were analyzed by flow cytometry. Unstained *Cryptococcus* is shown in gray and staining with FITC-labeled goat anti-mouse C3 is shown in black. Results are representative of two or more independent experiments.

Figure 2. C3 deposition by fluorescence confocal microscopy

(A) Live *C. neoformans* and *C. gattii* were incubated with wild type serum with (bottom) or without (top) 40 mM EGTA to inhibit the classical and lectin pathways so that activation would take place only through the alternative pathway. (B) Live *C. neoformans* were incubated with C4^{-/-} or factor B^{-/-} serum. FITC-labeled goat anti-mouse C3 was added and samples then resuspended in mounting medium, placed on glass slides and examined under oil immersion at 1000 $\times$.

Figure 3. C3 deposition in the presence of $C4^{-/-}$ serum

Live and heat-killed *Cryptococcus* spp. grown under normal and capsule-inducing conditions were incubated with serum from $C4^{-/-}$ mice with or without 40 mM EGTA to inhibit the classical and lectin pathways so that activation could take place only through the alternative pathway. Samples were analyzed by flow cytometry. Unstained *Cryptococcus* is shown in gray and staining with FITC-labeled goat anti-mouse C3 is shown in black. Results are representative of two or more independent experiments.

Figure 4. C3 deposition in the presence of factor B^{-/-} serum

Live and heat-killed *Cryptococcus spp.* grown under normal and capsule-inducing conditions were incubated with serum from factor B^{-/-} mice with or without 40 mM EGTA to inhibit the classical and lectin pathways so that activation could take place only through the alternative pathway. Samples were analyzed by flow cytometry. Unstained *Cryptococcus* is shown in gray and staining with FITC-labeled goat anti-mouse C3 is shown in black. Results are representative of two or more independent experiments.

Figure 5. C3 deposition in the presence of MBL-A^{-/-}/C^{-/-} serum

Live and heat-killed *Cryptococcus* spp. grown under normal and capsule-inducing conditions were incubated with serum from MBL-A^{-/-}/C^{-/-} mice with or without 40 mM EGTA to inhibit the classical and lectin pathways so that activation could take place only through the alternative pathway. Samples were analyzed by flow cytometry. Unstained *Cryptococcus* is shown in gray and staining with FITC-labeled goat anti-mouse C3 is shown in black. Results are representative of two or more independent experiments.

Figure 6. C3 deposition in the presence of MBL-A^{-/-} serum

Live and heat-killed *Cryptococcus* spp. grown under normal and capsule-inducing conditions were incubated with serum from MBL-A^{-/-} mice with or without 40 mM EGTA to inhibit the classical and lectin pathways so that activation could take place only through the alternative pathway. Samples were analyzed by flow cytometry. Unstained *Cryptococcus* is shown in gray and staining with FITC-labeled goat anti-mouse C3 is shown in black. Results are representative of two or more independent experiments.

Figure 7. C3 deposition in the presence of MBL- $C^{-/-}$ serum

Live and heat-killed *Cryptococcus* spp. grown under normal and capsule-inducing conditions were incubated with serum from MBL- $C^{-/-}$ mice with or without 40 mM EGTA to inhibit the classical and lectin pathways so that activation could take place only through the alternative pathway. Samples were analyzed by flow cytometry. Unstained *Cryptococcus* is shown in gray and staining with FITC-labeled goat anti-mouse C3 is shown in black. Results are representative of two or more independent experiments.