Anti-Granulocyte-Macrophage Colony-Stimulating Factor Autoantibodies Are a Risk Factor for Central Nervous System Infection by *Cryptococcus gattii* in Otherwise Immunocompetent Patients

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ABSTRACT **Cryptococcosis is caused by either *Cryptococcus neoformans* or *C. gattii*. While cryptococcal meningoencephalitis is caused mostly by *C. neoformans* in immunocompromised patients, the risk factors remain unclear for patients with no known immune defect. Recently, anti-granulocyte-macrophage colony-stimulating factor (GM-CSF) autoantibodies were detected in the plasma of seven "immunocompetent" cryptococcosis patients, and the cryptococcal strains from these patients were reported as *C. neoformans* (three strains), *C. gattii* (one strain), and *Cryptococcus* (three strains not identified to the species level). We identified all three strains that had not been identified to the species level as *C. gattii*. Notably, the three strains that were reported as *C. neoformans* but were unavailable for species confirmation originated from Sothern California and Thailand where *C. gattii* is endemic. Most clinical laboratories designate *C. neoformans* without distinguishing between the two species; hence, these three strains could have been *C. gattii*. Since *C. gattii* infects more immunocompetent patients than *C. neoformans*, we pursued the possibility that this antibody may be more prevalent in patients infected with *C. gattii* than in those infected with *C. neoformans*. We screened the plasma of 20 healthy controls and 30 "immunocompetent" patients with cryptococcal meningoencephalitis from China and Australia (multiple ethnicities). Anti-GM-CSF autoantibodies were detected only in the plasma of seven patients infected by *C. gattii* and one healthy volunteer and in none infected by *C. neoformans*. While plasma from these *C. gattii* patients completely prevented GM-CSF-induced p-STAT5 in normal human peripheral blood mononuclear cells (PBMCs), plasma from one healthy volunteer positive for anti-GM-CSF autoantibodies caused only partial blockage. Our results suggest that anti-GM-CSF autoantibodies may predispose otherwise immunocompetent individuals to meningoencephalitis caused by *C. gattii* but not necessarily to that caused by *C. neoformans*.**

IMPORTANCE **Cryptococcal meningoencephalitis is the most serious central nervous system (CNS) infection caused by *Cryptococcus neoformans* or *C. gattii*. *Cryptococcus* primarily infects immunocopromised patients but is also sporadically encountered in otherwise "immunocompetent" patients with no known risk. In a recent study, anti-GM-CSF autoantibodies were detected in the plasma of seven otherwise immunocompetent patients with cryptococcal meningoencephalitis. Four of seven (57%) cryptococcal isolates from these patients were identified as *C. gattii*, while three strains were unavailable for species confirmation. We collected plasma from 30 otherwise healthy patients with CNS cryptococcosis in China and Australia (multiethnic) and analyzed the samples for the presence of anti-GM-CSF autoantibodies. The results suggest that anti-GM-CSF autoantibodies are a risk factor for CNS infection by *C. gattii* but not *C. neoformans*. GM-CSF may have a specific role in host defense against *C. gattii*, thereby elevating the importance of determining the level of anti-GM-CSF autoantibodies which can impact clinical management.**

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Cryptococcus neoformans and its closely related sibling species *C. gattii* are both environmental fungal pathogens that cause cryptococcosis in humans and a wide range of mammals (1, 2). Although *C. neoformans* is the most common cause of cryptococcosis in AIDS patients globally (3), epidemiological studies from far east Asian countries present a different picture regarding the risk for *C. neoformans* infection: the species infects mostly HIV-uninfected patients for whom a predisposing underlying factor may or may not be apparent (4–6). In Australia, approximately 20% of individuals with *C. neoformans* infection have been apparently healthy hosts (4).

C. gattii causes disease mainly in otherwise immunocompetent hosts and only rarely in those with AIDS (2). Although it has been speculated that *C. gattii* infection is due to increased environmental exposure to the fungus because of the overrepresentation of *C. gattii* infection in Australian Aboriginal peoples living in rural areas (2), the specific mechanisms explaining this susceptibility have not been evaluated. Recently, Rosen et al. detected anti-granulocyte-macrophage colony-stimulating factor (GM-CSF) autoantibodies in HIV-uninfected otherwise immunocompetent patients with cryptococcal meningitis and postulated that this antibody may have preceded and predisposed patients to this mycosis (7). Interestingly, anti-GM-CSF antibodies have been recognized as causal for most cases of pulmonary alveolar proteinosis (PAP), a severe lung disease that results as a failure of GM-CSF-induced alveolar macrophage differentiation and subsequent ineffective clearance of pulmonary surfactant (8). While cryptococcal infection has been recognized under this condition since its original description (9), it has been postulated only recently that anti-GM-CSF autoantibodies may contribute to susceptibility to infections without manifestations of PAP (7). We hypothesized that anti-GM-CSF autoantibodies might also explain some of the cryptococcosis observed in otherwise healthy patients from Far East Asia and Australia. To investigate the possibility that anti-GM-CSF autoantibodies may heighten susceptibility to cryptococcal disease, we collected blood from 41 Chinese patients and nine Australian patients of various ethnicities with central nervous system (CNS) cryptococcosis who had been categorized as immunocompetent as well as healthy volunteers and tested their plasma for the presence of anti-GM-CSF autoantibodies. We attempted to confirm the species status of the cryptococcal strains in these patients and in the seven previously reported cases of anti-GM-CSF autoantibody-positive cryptococcosis patients, excluding the four strains (three *C. neoformans* strains and one *C. gattii* strain) that were no longer available. We report here a clear association between the presence of anti-GM-CSF autoantibodies in the blood and CNS infection caused by *C. gattii* in patients previously considered immunocompetent.

TABLE 1 Each patient's gender and age, the presence of anti-GM-CSF autoantibodies in plasma, and species and molecular types of cryptococcal strains isolated from cerebrospinal fluid[a]

Patient	Race	Gender	Age (yr)	Aab	Cryptococcal strain	
					Species	Molecular type
C1	Chinese	Female	37	−	*C. neoformans*	NA
C2	Chinese	Male	55	−	*C. neoformans*	NA
C3	Chinese	Female	46	−	*C. neoformans*	VNI
C4	Chinese	Male	28	−	*C. neoformans*	NA
C5	Chinese	Female	37	−	*C. neoformans*	NA
C6	Chinese	Female	32	−	*C. neoformans*	NA
C7	Chinese	Male	22	−	*C. neoformans*	VNI
C8	Chinese	Male	10	−	*C. neoformans*	NA
C9	Chinese	Male	45	−	*C. neoformans*	VNI
C10	Chinese	Male	57	−	*C. neoformans*	VNI
C11	Chinese	Female	40	−	*C. neoformans*	NA
C12	Chinese	Male	4	−	*C. neoformans*	NA
C13	Chinese	Male	61	−	*C. neoformans*	NA
C14	Chinese	Male	42	−	*C. neoformans*	VNIII
C15	Chinese	Male	55	−	*C. neoformans*	VNI
C16	Chinese	Male	32	−	*C. neoformans*	NA
C17	Chinese	Female	49	+	*C. gattii*	VGI
C18	Chinese	Male	40	−	*C. neoformans*	NA
C19	Chinese	Male	56	−	*C. neoformans*	NA
C20	Chinese	Female	40	−	*C. neoformans*	NA
C21	Chinese	Male	43	−	*C. neoformans*	NA
A1	Caucasian	Female	NA	+	*C. gattii*	VGI
A2	Caucasian	Female	NA	+	*C. gattii*	VGI
A3	Caucasian	Male	NA	+	*C. gattii*	VGI
A4	Aborigine	Female	NA	+	*C. gattii*	VGI
A5	Caucasian	Female	NA	−	*C. gattii*	VGI
A6	Asian (Indian)	Female	NA	−	*C. neoformans*	NA
A7	Asian	Female	NA	+	*C. gattii*	VGI
A8	Caucasian	Male	NA	+	*C. gattii*	VGII
A9	Asian (Filipino)	Male	NA	−	*C. gattii*	VGI

[a] Aab, anti-GM-CSF autoantibody; C, patients in China; A, patients in Australia; NA, not available for this study.

RESULTS

Subjects, cryptococcal strains, and anti-GM-CSF autoantibodies. All patients with CNS cryptococcosis studied in this work were previously healthy HIV-uninfected individuals with no known predisposing factor. Tables 1 and 2 show the information available on gender, age, and ethnic background of the patients (Table 1) and of the healthy controls (Table 2), anti-GM-CSF autoantibody status, causative *Cryptococcus* species, and the results of molecular typing of the several selected *C. neoformans* strains and of all *C. gattii* strains recovered from the patients. One *C. gattii* strain isolated from a patient in China and all *C. gattii* strain isolates from Australian patients were of the VGI molecular type except for one which was VGII type. All three strains isolated from the anti-GM-CSF autoantibody-positive patients described in a previous work (7) and available for this study (one VGI type and two VGIII types) were serotype B strains of *C. gattii* (Table 3). Of the six randomly chosen *C. neoformans* strains isolated from CNS cryptococcosis patients in China, five were of VNI type and one was VNIII (Table 1). One *C. neoformans* strain isolated from an Australian patient was not available for molecular typing.

Anti-GM-CSF autoantibodies were detected in only 1 of 21 cryptococcosis patients and in only 1 member of the healthy control population in China. Among the Australian patients, samples were positive for anti-GM-CSF autoantibodies from six of nine patients (67%) (Fig. 1), and all the autoantibody-positive patients in both countries were infected by *C. gattii* (six VGI type and one VGII type). No anti-GM-CSF autoantibodies were detected from any patient who suffered from *C. neoformans* CNS infection either in China or in Australia (Table 1).

GM-CSF-induced p-STAT5 in normal PBMCs. Anti-GM-CSF autoantibody-containing plasma from Australian and Chinese patients with *C. gattii* CNS cryptococcosis prevented GM-CSF (10 ng/ml) induction of p-STAT5 production in normal

TABLE 2 Each healthy individual's gender and age and the presence of anti-GM-CSF autoantibodies in plasma[a]

Patient	Race	Gender	Age (yr)	Aab
H1	Chinese	Male	44	−
H2	Chinese	Female	40	−
H3	Chinese	Female	48	−
H4	Chinese	Female	23	−
H5	Chinese	Female	44	−
H6	Chinese	Male	40	−
H7	Chinese	Male	40	−
H8	Chinese	Female	40	−
H9	Chinese	Male	36	−
H10	Chinese	Male	36	−
H11	Chinese	Male	37	−
H12	Chinese	Male	35	+
H13	Chinese	Male	40	−
H14	Chinese	Female	30	−
H15	Chinese	Female	30	−
H16	Chinese	Male	36	−
H17	Chinese	Male	40	−
H18	Chinese	Female	34	−
H19	Chinese	Female	40	−
H20	Chinese	Female	41	−

[a] Aab, anti-GM-CSF autoantibody.

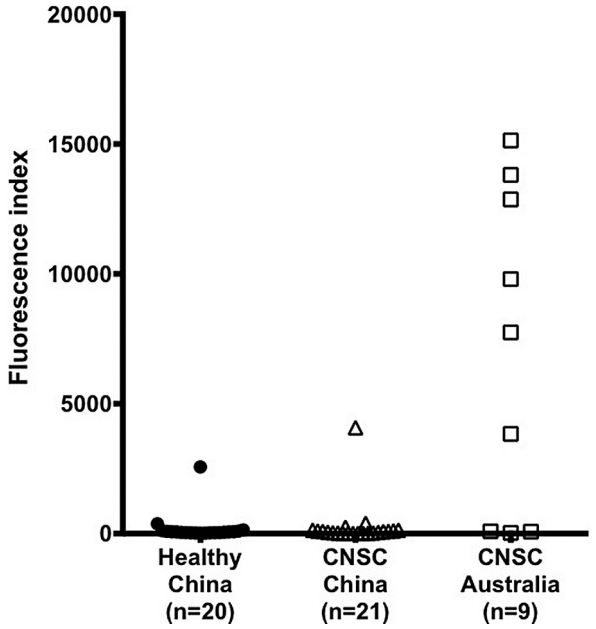

FIG 1 Anti-GM-CSF autoantibodies in plasma (1:100 dilution). The fluorescence intensity of the anti-GM-CSF autoantibodies in each healthy individual ($n = 20$), each CNS cryptococcosis (CNSC) patient ($n = 21$) in China, and each CNS cryptococcosis patient ($n = 9$) in Australia was plotted as a function of antibody concentration.

peripheral blood mononuclear cells (PBMCs), whereas plasma from randomly chosen healthy Chinese volunteers without anti-GM-CSF autoantibodies showed no inhibition of p-STAT5 induction (Fig. 2A). One healthy donor possessed anti-GM-CSF autoantibodies, and his plasma caused partial inhibition of GM-CSF induction of p-STAT5 but to a far lesser degree (Fig. 2B). To evaluate the avidity of anti-GM-CSF autoantibodies in patients' plasma samples, we generated each dose-response curve by stimulating normal PBMCs with increasing amounts of GM-CSF in a fixed concentration (10% of plasma in each reaction mixture) of anti-GM-CSF autoantibody-containing patient's plasma, of autoantibody-negative normal plasma, or of autoantibody-positive plasma from a healthy volunteer and evaluated p-STAT5 production (Fig. 3A). The amount of GM-CSF required to achieve 50% of the maximum p-STAT5 production (half-maximal effective concentration = EC_{50}) was determined from each dose-response curve. PBMCs incubated with plasma positive for anti-GM-CSF autoantibodies from each CNS cryptococcosis patient required concentrations of GM-CSF that were about 2 to 3 log higher (114.2 ng/ml to 4,190 ng/ml) than the concentrations required for PBMCs incubated with anti-GM-CSF autoantibody-negative plasma from each healthy individual (0.66 ng/ml to 1.43 ng/ml) (Fig. 3B). The EC_{50} of GM-CSF for the healthy control positive for anti-GM-CSF autoantibodies was 11.7 ng/ml (Fig. 3B).

DISCUSSION

This study shows that all of the otherwise healthy CNS cryptococcosis patients we tested who were positive for anti-GM-CSF autoantibodies were infected by *C. gattii*. A previous study reported that of the seven patients who had anti GM-CSF autoantibodies (7), three were infected by *C. neoformans*, one by *C. gattii*, and three by *Cryptococcus* species not identified to the species level. We have identified the three *Cryptococcus* strains not previously identified to the species level as *C. gattii* (two VGIII molecular type and one VGI molecular type). This indicated that at least four (57%) of the seven strains belonged to *C. gattii*. Although the remaining three strains were initially identified as *C. neoformans* and were

TABLE 3 Each patient's origin and the cryptococcal strains isolated from the patients described in a previous study[a]

Patient	Race	Gender	Age (yr)	Aab	Cryptococcal species			
					Identification in previous study	Identification in this study		
						Species	Molecular type	Patient's origin
P1	Caucasian	Female	20	+	*C. neoformans*	NA	NA	S. California
P2	Caucasian	Female	31	+	*C. gattii*	NA	NA	S. California
P3	Asian (Thai)	Male	48	+	*C. neoformans*	NA	NA	Thailand
P4	Mexican	Male	47	+	*C. neoformans*	NA	NA	S. California
P5	African American	Male	26	+	*Crptococcus*	*C. gattii*	VGIII	NA
P6	Caucasian	Male	34	+	*Crptococcus*	*C. gattii*	VGIII	New Jersey
P7	Caucasian	Male	32	+	*Crptococcus*	*C. gattii*	VGI	New Jersey

[a] Aab, anti-GM-CSF autoantibody; S. California, Southern California; NA, not available for this study. Each patient's identification number corresponds to that used in Table 1 of the previous article (7).

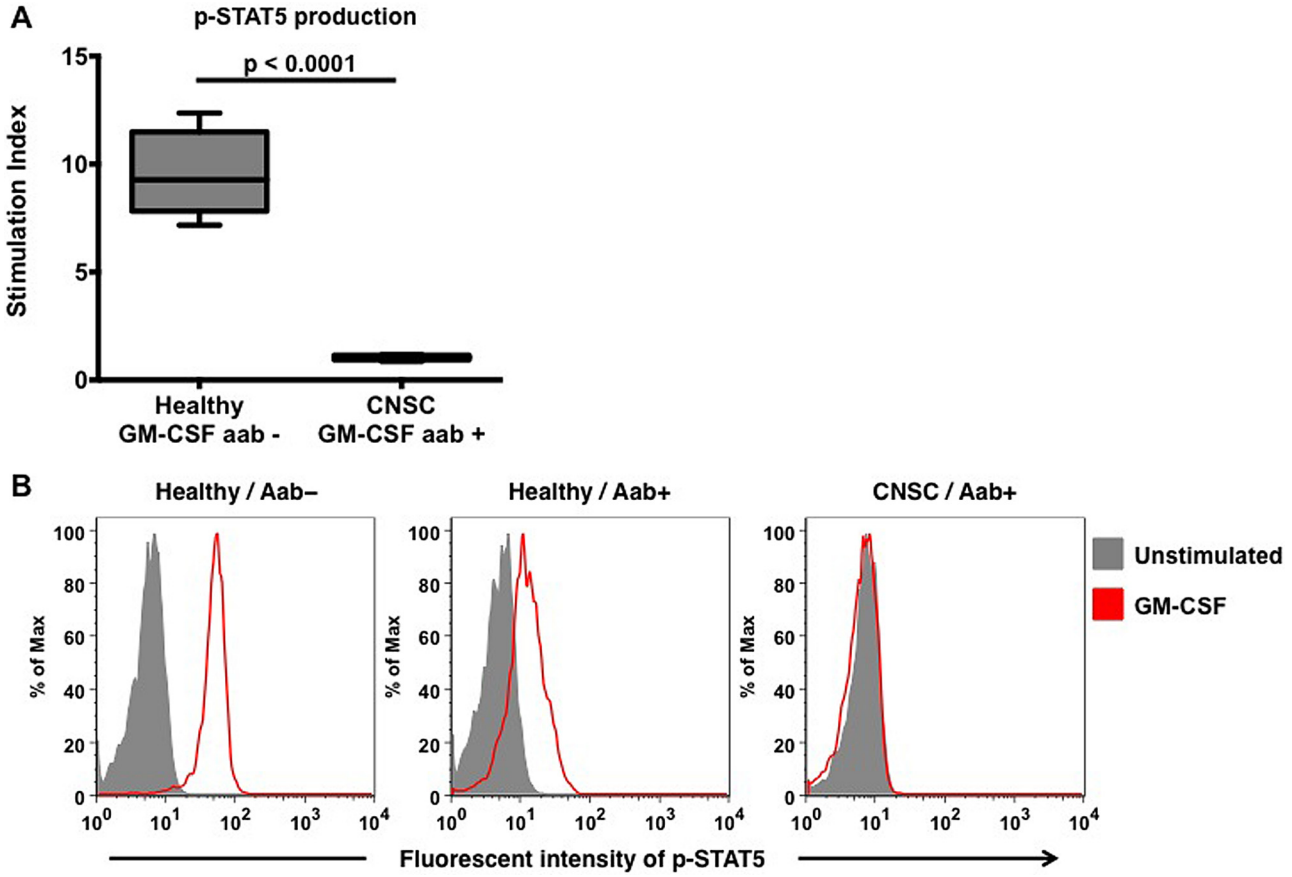

FIG 2 Inhibitory function of anti-GM-CSF autoantibodies. (A) Normal peripheral blood mononuclear cells (PBMCs) were incubated with 10% plasma of each anti-GM-CSF autoantibody-positive CNS cryptococcosis (CNSC) patient ($n = 7$) or of each anti-GM-CSF autoantibody-negative healthy volunteer ($n = 5$) in the presence or absence of GM-CSF (10 ng/ml) for 30 min and production of p-STAT5 was evaluated by flow cytometry. The stimulation index (ratio of stimulated/unstimulated geometric mean channels) was calculated. aab, autoantibodies. (B) Evaluation of the inhibition of p-STAT5 production by the plasma from one healthy control in China who was positive for anti-GM-CSF autoantibodies. Normal PBMCs were left unstimulated or were stimulated by 10 ng/ml·GM-CSF (cerebrospinal fluid) in the presence of one representative healthy plasma sample negative for anti-GM-CSF autoantibodies (Healthy/Aab−), of one healthy plasma sample positive for anti-GM-CSF autoantibodies (Healthy/Aab+), or of one representative CNS cryptococcosis patient's plasma sample positive for anti-GM-CSF autoantibodies (CNSC/Aab+), and p-STAT5 production was measured by flow cytometry by intracellular staining for p-STAT5.

not available for confirmation in this study, it is likely that these strains are *C. gattii* for two reasons. First, a majority of the clinical laboratories have commonly reported the etiologic agents for cryptococcosis as *C. neoformans* without attempting to distinguish between the two agents of the disease since the therapy for both species is the same (10). Second, the three patients reported to have been infected by *C. neoformans* were from either Southern California (two patients) or Thailand (one patient), both known to be regions of endemicity for *C. gattii* (1). Taking the results together, our report is the first to present evidence that presence of the anti-GM-CSF autoantibodies is an underlying differential risk factor for *C. gattii* infection but not necessarily for *C. neoformans*.

Anti-GM-CSF autoantibodies have long been considered the mechanistic explanation for most PAP (8, 11, 12), but their role in susceptibility to infection independent of lung disease has only recently been appreciated (7). Although the initial reports of PAP described infectious complications (9), this was prior to the identification of anti-GM-CSF autoantibodies as a causative factor (8, 12), leading to diagnostic heterogeneity in the underlying lung disease. While it is unclear if the pulmonary infections reported previously (9) were secondary to structural lung disease or to an intrinsic immunologic defect, the recent study that described CNS cryptococcosis in the absence of other lung pathology (7) suggests that the functional defect of GM-CSF that associates with immune function such as innate immunity, including phagocytic activity (13–15) and T-cell response (16, 17), could be a predisposing factor for CNS cryptococcosis. Our report showing the high prevalence of anti-GM-CSF autoantibodies in *C. gattii* infection and not in *C. neoformans* infection may offer some clues to address the differences in the host's immunological reactions to these two etiological agents.

Although *C. gattii* possesses all the virulence factors identified in *C. neoformans*, cryptococcosis is predominantly caused by *C. neoformans* and a majority of the cases are reported in immunocompromised patients (3, 4, 18, 19). Cryptococcosis due to *C. gattii* is observed in only about 20% of the disease globally and is reported more frequently in immunocompetent patients than *C. neoformans* (4, 19–21). Apart from the risk of exposure, the mechanisms by which *C. gattii* causes cryptococcosis in immunocompetent hosts more often than *C. neoformans* have yet to be elucidated. Recently, several studies have indicated differences in the pathogenesis of these two species, which include the ability to

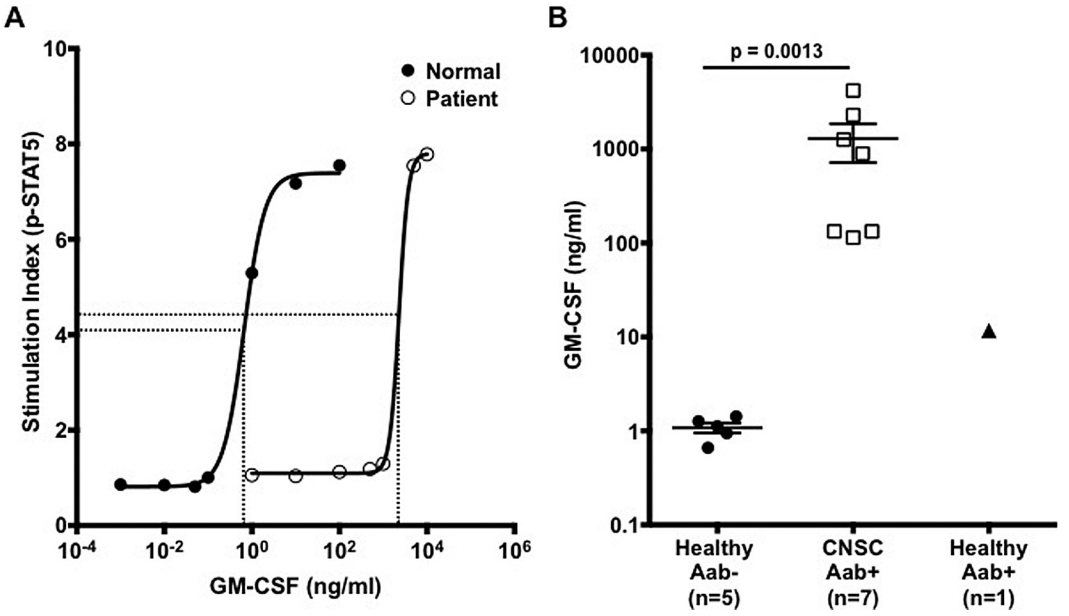

FIG 3 Anti-GM-CSF autoantibodies containing plasma blunts the response of PBMCs to GM-CSF stimulation. (A) A representative dose-response curve was depicted by measuring p-STAT5 production in normal PBMCs with 10% plasma from a patient or from a healthy individual under conditions of stimulation at increasing concentrations (between 0.001 ng/ml and 10 μg/ml) of GM-CSF. The concentration of GM-CSF required to phosphorylate 50% of STAT5 (EC_{50}) was 2,298 ng/ml ($R^2 = 0.9998$) and 0.6609 ng/ml ($R^2 = 0.9990$) for the patient's plasma and for the normal plasma, respectively. (B) The concentration of GM-CSF required to phosphorylate 50% of STAT5 (EC_{50}) was determined from the dose-response curves generated for each of the anti-GM-CSF autoantibody-positive CNS cryptococcosis (CNSC) patients' plasma samples ($n = 7$), plasma from anti-GM-CSF autoantibody-negative healthy volunteers ($n = 5$), and plasma from an anti-GM-CSF autoantibody-positive healthy volunteer ($n = 1$). Aab, anti-GM-CSF autoantibodies.

provoke an immune reaction in the host (22–24), the sites of infection (25), and the proliferation rate in host macrophages (26, 27). With respect to the host's defense against *Cryptococcus*, both innate immunity and adaptive immunity, especially the Th1 type, are required for the elimination of this pathogen (28).

GM-CSF regulates innate immune cells, including macrophages, neutrophils, and dendritic cells (DCs) (13–15, 29), and is important for Th1-type cytokine production (30, 31). Huston et al. indicated that *C. gattii* strains can evade DCs and T-cell-mediated adaptive immunity because they fail to induce DC maturation and the release of cytokines such as tumor necrosis factor alpha (TNF-α) that are important for DC maturation (32). Low production of proinflammatory cytokines such as TNF-α and gamma interferon (IFN-γ) in *C. gatiii* infection (22) and suppression of these proinflammatory cytokines under GM-CSF-neutralizing conditions (31) may impair establishment of T-cell-mediated protective immunity to *C. gattii*. The interaction between DCs and T cells during infection by *C. neoformans* has not been evaluated thoroughly, but $CD11b^+$ DCs necessary for Th1 immunity accumulate in response to *C. neoformans* infection (33) and cytokines such as TNF-α and IFN-γ are produced more abundantly in response to *C. neoformans* than *C. gattii* (22). Furthermore, histopathological observations of infected lungs in mice showed that *C. neoformans* elicited more inflammatory cells than *C. gattii* (22, 25). These indicate that *C. neoformans* probably provokes redundant immune reactions to compensate for the immune defect resulting from GM-CSF neutralization compared to *C. gattii*, thereby leading to the prevalence of *C. gattii* infection in GM-CSF-neutralized individuals. However, Schoffelen et al. recently reported that the production of proinflammatory cytokines, including TNF-α and interleukin-6 (IL-6), in human PBMCs infected by heat-killed *C. gattii* was more prominent than that seen with *C. neoformans* (23), which is opposite the pattern observed in the murine infection model. There are several factors to be considered regarding this discrepancy such as differences between humans and mice in the immunological reaction, the status of the *Cryptococcus* cells used in each study (viable or heat killed), and the time point of cytokine measurement. Hence, we hypothesize that the presence of anti-GM-CSF autoantibodies represents a higher risk for cryptococcosis due to *C. gattii* than for that due to *C. neoformans*.

Deepe et al. reported that endogenous GM-CSF is essential for host survival in primary but not in secondary infection by *Histoplasma capsulatum* (31). They found that mice with primary *H. capsulatum* infection given GM-CSF-neutralizing antibodies had a significant increase in fungal burden with drastic decreases in survival. However, mice with secondary infection cleared fungal cells and all survived during the observation period despite GM-CSF neutralization. This study suggests that adaptive immunity is less affected by GM-CSF deficiency and can protect the host even in the absence of functional GM-CSF. Interestingly, histoplasmosis and cryptococcosis have been reported in PAP (34).

Serum antibodies specific to the organism are indicative of adaptive immunity, and several studies on serum antibodies against *Cryptococcus* have been published. Seaton et al. demonstrated that serum antibodies to *C. gattii* were positive in 22% to 53% of healthy individuals in Papua New Guinea, a region of endemicity for *C. gattii* (35). Abadi and Pirofski showed that 100% of healthy as well as HIV-infected schoolchildren tested in New York were positive for antibodies to *C. neoformans* (36), and Goldman et al. indicated that 70% of serum samples from children older than 5 years had many reactive antibodies to *C. neoformans*

proteins (37). Although the sensitivities and specificities of the antibodies used in the two studies are not comparable due to the differences in the methods by which each antibodies were made, it is possible that acquired immunity to *C. neoformans* is more prevalent than that to *C. gattii*. Furthermore, the previous report indicated that cryptococcal meningitis patients with anti-GM-CSF autoantibodies remained well after successful therapy (7). It is plausible that the lack of acquired immunity may lead to higher susceptibility to *C. gattii* than to *C. neoformans* as a primary encounter in the context of GM-CSF neutralization. GM-CSF deficiency impairs innate immunity, but once adaptive immunity is established after primary infection, the host may be protected from *C. gattii* infection, even in the presence of anti-GM-CSF autoantibodies.

Reactivation of the fungus after long-term dormancy has been suggested not only in *C. neoformans* (38) but also in *C. gattii* (39) as shown by molecular typing techniques. It is possible that concomitant immune suppression with GM-CSF neutralization might reactivate only dormant *C. gattii*, and not *C. neoformans*, but the natural history of functional anti-GM-CSF autoantibodies in the host remains unknown. Based on the median age (39 years) at the time of diagnosis in PAP (11) and the range of the ages (20 to 49 years, except for the Australian patients) of the patients with CNS cryptococcosis who had anti-GM-CSF autoantibodies as shown in this study as well as in a previous study (7), the functional defect of GM-CSF appears to occur in adults. Anti-GM-CSF autoantibodies are detectable not only during disease but also while subjects are healthy (40–42). Although anti-GM-CSF autoantibodies in cord blood were exclusively IgM and did not neutralize GM-CSF (42), those in healthy adults and PAP patients were only IgG and strongly neutralized GM-CSF (41, 43). Our study and a previous study (7) indicated that functional anti-GM-CSF autoantibodies in CNS cryptococcosis patients were all IgG. It is postulated that immature B-cell clones that produce IgM anti-cytokine autoantibodies may enter the germinal center and that subsequent class switching and hypermutation can cause the production of neutralizing IgG autoantibodies (44). Also, in the induction of several anti-cytokine autoantibodies, repeated intrinsic cytokine exposure is considered to be a possible mechanism (44). Taking the data together, two different hypotheses can be postulated. (i) The patient who initially had anti-GM-CSF IgM autoantibodies can produce IgG autoantibodies by class switching and hypermutation, which leads to higher susceptibility to *C. gattii*. (ii) Dormant infection with *C. gattii*, but not with *C. neoformans*, stimulates the production of GM-CSF for a long period of time and causes the induction of functional anti-GM-CSF autoantibodies, which leads to reactivation of *C. gattii* infection. Monitoring of anti-GM-CSF autoantibodies, including both IgM and IgG, GM-CSF titer, and anti-cryptococcal antibodies in cryptococcosis patients as well as in healthy individuals who reside in the region of *C. gattii* endemicity might address this hypothesis. One healthy control in this study was found to be positive for anti-GM-CSF autoantibodies (Fig. 1). It is advisable to monitor this person's health status over time for future evidence of invasive opportunistic infections.

This study as well as a previous study (7) focused on CNS cryptococcosis patients to evaluate the prevalence of anti-GM-CSF autoantibodies. Although the function of GM-CSF in brain immunity has not been extensively studied, this cytokine has been reported to induce functionally competent DCs in the mouse brain (45). Further, GM-CSF gene expression has been observed to be upregulated in *Toxoplasma* encephalitis (46), suggesting that GM-CSF has some role in brain immunity. It is also important to investigate whether the anti-GM-CSF antibodies are equally prevalent in pulmonary cryptococcosis patients without CNS involvement and in the patients with CNS involvement caused by *C. gattii*. There are two representative areas where *C. gattii* is endemic, Oceania and Northwest America/Vancouver (19). While in Oceania, especially in Australia, the VGI molecular type strain of *C. gattii* is prevalent in the environment and CNS involvement by *C. gattii* is very high (85%) (20), in Northwest America/Vancouver, the VGII strains are most prevalent in the environment but CNS involvement in cryptococcosis patients infected by VGII strains is low to moderate (18% to 50%) (21, 47). It is necessary to investigate the prevalence of anti-GM-CSF autoantibodies in healthy individuals as well as in cryptococcosis patients from these two regions.

In summary, the results of this study strongly suggest that anti-GM-CSF autoantibodies are a risk factor for *C. gattii* CNS cryptococcosis in otherwise immunocompetent individuals and emphasize the importance of determining the presence of GM-CSF-neutralizing autoantibodies in patients with CNS cryptococcosis. Further work is required to investigate the relationship between the prevalence of anti-GM-CSF autoantibodies and *C. gattii* infection in areas where different molecular types are endemic.

MATERIALS AND METHODS

Subjects. Plasma samples were collected from healthy individuals (n = 20) and otherwise healthy CNS cryptococcosis patients in China (n = 21) and in Australia (n = 9) under Institutional Review Board (IRB)-approved protocols at each institution and stored in aliquots at −80°C until use. CNS cryptococcosis was diagnosed by a combination of clinical symptomatology, computed tomography (CT) or magnetic resonance imaging (MRI) of the brain, detection of encapsulated yeasts in cerebrospinal fluid by the use of India ink (1), and determination of cryptococcal antigen titers of cerebrospinal fluid samples. Blood samples from healthy donors for isolation of PBMCs were collected through the National Institutes of Health blood bank under conditions of appropriate IRB-approved protocols.

Cryptococcal strains. *Cryptococcus* strains were recovered from the cerebrospinal fluid of 21 and 9 otherwise healthy patients in China and Australia, respectively. The strains had been identified as either *C. neoformans* or *C. gattii* by conventional laboratory tests in each country of origin. Molecular typing of the Australian strains was carried out at the time of isolation in Australia. Of the 21 cryptococcal strains from China, 20 were identified as *C. neoformans* and one as *C. gattii* whereas nine strains from Australia included eight *C. gattii* strains (seven VGI type and one VGII type) and one *C. neoformans* strain (molecular type unknown). To reconfirm the species status and to determine the molecular types of the Chinese strains, we studied seven strains (six *C. neoformans* and one *C. gattii*) from China. In addition, we obtained three of seven strains isolated from the patients in the United States whose plasma had been reported as positive for anti-GM-CSF autoantibodies (7); those were available for the identification of the species and molecular types. L-Canavanine-glycine bromthymol blue (CGB) agar media, which differentiates between the two species (1), was used for identification of the species, and their serotypes were determined by the use of a Iatron Crypto kit (Mitsubishi Kagaku, Tokyo, Japan) (no longer available). The molecular types were determined using *URA5* gene restriction patterns (48). Glycerol stocks were made for all strains and stored at −80°C until use. Yeast extract-peptone-dextrose (YPD) agar media (2% glucose, 1% yeast extract, 2% peptone, 2% Bacto agar) was used for growth of the strains.

Detection of anti-GM-CSF autoantibody. Plasma samples were screened for the presence of anti-GM-CSF autoantibodies using previously described particle-based technology (7, 49) with slight modifications. Briefly, one set of fluorescent beads (Bio-Rad) was conjugated with 2.5 μg of human GM-CSF (R&D Systems). Beads were combined and incubated for 30 min with plasma diluted at 1:100, washed, and incubated with biotinylated mouse anti-human total IgG (eBioscience). Beads were washed again, incubated with streptavidin-phycoerythrin (PE) (Bio-Rad), and then run on a Bio-Plex (Bio-Rad) instrument. Fluorescence intensity was plotted as a function of antibody concentration (GraphPad Prism, version 6.0c).

Detection of p-STAT5 in PBMCs by flow cytometry. GM-CSF-induced phosphorylation of STAT5 (p-STAT5) was evaluated by flow cytometry as described previously (7) with slight modifications. Briefly, normal PBMCs (1×10^6 cells) were isolated by density-gradient centrifugation as described previously (50) and resuspended in complete medium composed of RPMI 1640 (Life Technologies), 2 mM glutamine, 20 mM HEPES, and 0.01 mg/ml penicillin-streptomycin with 10% plasma from each subject. The PBMCs were either left unstimulated or stimulated with GM-CSF (R&D Systems) at the desired concentration (between 0.001 ng/ml to 10 μg/ml) for 30 min at 37° C. Monocytes were treated with anti-human CD14 (BD Pharmingen) antibodies for surface staining and then fixed and permeabilized for intracellular staining with anti-pSTAT5 (Y694) antibodies (BD Pharmigen) as described previously (7, 51). Data were collected using FACSCalibur (BD Biosciences) and analyzed using FlowJo software (TreeStar). A stimulation index (ratio of stimulated/unstimulated geometric mean channels) was calculated for each sample.

Statistics. For comparisons between healthy controls and patients, a two-tailed unpaired *t* test was applied, using Prism 6 software (GraphPad Prism, version 6.0c).

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