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

Tomomi Saijo,a Jianghan Chen,b Sharon C.-A. Chen,c,d Lindsey B. Rosen,e Jin Yi,b Tania C. Sorrell,c,d John E. Bennett,f Steven M. Holland,a Sarah K. Browne,a Kyung J. Kwon-Chunga

Molecular Microbiology Section, Laboratory of Clinical Infectious Diseases, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA; Department of Dermatology and Key Laboratory of Medical Mycology, Changzheng Hospital, Second Military Medical University, Shanghai, China; Center for Infectious Diseases and Microbiology, Westmead Hospital, Westmead, New South Wales, Australia; Marie Bashir Institute, University of Sydney, New South Wales, Australia; Immunopathogenesis Section, Laboratory of Clinical Infectious Diseases, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA; Laboratory of Clinical Infectious Diseases, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA

T.S. and J.C. contributed equally to this article.

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 Southern 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 immunocompromised 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.
C. 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 these patients to this mycosis (7). Interestingly, anti-GM-CSF autoantibodies 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.

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 VGII 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 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

| Patient Race | Gender Age (yr) | Aab | 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 | VGI |
| A9 Asian | Male NA         | –   | C. gattii | VGI |

Aab, anti-GM-CSF autoantibody; C, patients in China; A, patients in Australia; NA, not available for this study.
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 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 2 | Each healthy individual’s gender and age and the presence of anti-GM-CSF autoantibodies in plasma* |
|-----------------|---------------------|---------|---------|
| **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.

**TABLE 3** | Each patient’s origin and the cryptococcal strains isolated from the patients described in a previous study* |
|-----------------|-----------------|-----------------|-----------------|
| **Patient** | **Race** | **Gender** | **Age (yr)** | **Aab** | **Cryptococcal species** | **Identification in previous study** | **Identification in this study** |
| 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 | + | *Cryptococcus* | *C. gattii* | VGIII | NA |
| P6  | Caucasian | Male | 34 | + | *Cryptococcus* | *C. gattii* | VGIII | New Jersey |
| P7  | Caucasian | Male | 32 | + | *Cryptococcus* | *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).
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
Anti-GM-CSF Autoantibodies for *C. gattii* Meningitis

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 CN5 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. gattii* 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 \textit{C. neoformans} is more prevalent than that to \textit{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 \textit{C. gattii} than to \textit{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 \textit{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 \textit{C. neoformans} (38) but also in \textit{C. gattii} (39) as shown by molecular typing techniques. It is possible that concomitant immune suppression with GM-CSF neutralization might reactivate only dormant \textit{C. gattii}, and not \textit{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 \textit{C. gattii}. (ii) Dormant infection with \textit{C. gattii}, but not with \textit{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 \textit{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 \textit{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 \textit{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 \textit{C. gattii}. There are two representative areas where \textit{C. gattii} is endemic, Oceania and Northwest America/Vancouver (19). While in Oceania, especially in Australia, the VGI molecular type strain of \textit{C. gattii} is prevalent in the environment and CNS involvement by \textit{C. gattii} is very high (85%) (20), in Northwest America/Vancouver, the VGI strains are most prevalent in the environment but CNS involvement in cryptococcosis patients infected by VGI 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 \textit{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 \textit{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.** \textit{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 \textit{C. neoformans} or \textit{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 \textit{C. neoformans} and one as \textit{C. gattii} whereas nine strains from Australia included eight \textit{C. gattii} strains (seven VGI type and one VGI type) and one \textit{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 \textit{C. neoformans} and one \textit{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 latex 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-peptide-dextrose (YPD) agar media \((2%\text{ glucose, }1%\text{ yeast extract, }2%\text{ peptone, }2%\text{ Bacto agar})\) 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-phycocerythrin (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, 10% FBS, and 100 U/ml penicillin-streptomycin with 10^6 cells/ml. Monocytes were treated with anti-human CD14 (BD Pharmingen) antibodies for surface staining and then fixed and permeabilized for intracellular staining with anti-STAT5 (Santa Cruz Biotechnology, Santa Cruz, CA) 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).

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

ACKNOWLEDGMENTS

This study was funded by the Intramural Program of the National Institutes of Allergy and Infectious Diseases, National Institutes of Health, and the Shanghai Committee of Science and Technology, China, 2012 (grant number 1241070000). We thank David Judd in Australia for his assistance in retrieving specimens and Ashok Varma for critical readings of the manuscript.

REFERENCES

1. Kwon-Chung KJ, Bennett JE. 1992. Medical mycology, p 44–78. Lea & Febiger, Pennsylvania, PA.
2. Sorrell TC. 2001. Cryptococcus neoformans variety gattii. Med. Mycol. 39:155–168. http://dx.doi.org/10.1080/714031012
3. Hajjeh RA, Conn LA, Stephens DS, Baughman W, Hamill R, Graviss E, Pappas PG, Thomas C, Reingold A, Rothrock G, Huttlinger LC, Schuchat A, Brandt ME, Pinner RW. 1999. Cryptococcus: population-based multistate active surveillance and risk factors in human immunodeficiency virus-infected persons. Cryptococcal Active Surveillance Group. J. Infect. Dis. 179:449–454. http://dx.doi.org/10.1086/314606.
4. Chen S, Sorrell T, Nimmo G, Speed B, Currie B, Marriott D, Pfeiffer T, Parr D, Byth K. 2000. Epidemiology and host- and variety-dependent characteristics of infection due to Cryptococcus neoformans in Australia and New Zealand. Australasian Cryptococcus Study Group. Clin. Infect. Dis. 31:499–508. http://dx.doi.org/10.1086/313992.
5. Choi YH, Ngamskulrungroj P, Varma A, Sionov E, Yamada Y, Kanegasaki S, Nakata K. 2009. Idiopathic pulmonary alveolar proteinosis as an autoimmune disease with neutralizing antibody against granulocyte/macrophage colony-stimulating factor. J. Exp. Med. 190:875–880. http://dx.doi.org/10.1084/jem.190.6.875.
6. Rosen LB, Freeman AF, Yang LM, Jutivorakool K, Olivier KN, Ang-kaewskini N, Suputtathamgkol Y, Bennett JE, Pyrgos V, Williamson PR, Ding L, Holland SM, Browne SK. 2013. Anti-GM-CSF autoantibodies in patients with cryptococcal meningitis. J. Infect. 190:3959–3966. http://dx.doi.org/10.1007/s11576-013-0252-0.
7. Nakata K, Yamamoto Y, Yanagihara K, Miyazaki Y, Kohno S. 2008. Detection of anti-GM-CSF autoantibody. J. Clin. Microbiol. 46:3206–3208. http://dx.doi.org/10.1128/JCM.02014-08.
8. Uchida K, Beck DC, Yamamoto T, Berclaz PY, Abe S, Staudt MK, Carey BC, Filippi MD, Wert SE, Bak N, Currie B, Hajkowicz K, Korman TM, McBride WJ, Meyer NEJ. 2003. Pulmonary alveolar proteinosis. N. Engl. J. Med. 349:2527–2539. http://dx.doi.org/10.1056/NEJMoa032226.
9. Tranpell BC, Whitsett JA, Nakata K. 2003. Pulmonary alveolar proteinosis. N. Engl. J. Med. 349:2527–2539. http://dx.doi.org/10.1056/NEJMoa032226.
10. Sorrell TC, Chen SC-A, Phillips P, Marr KA. 2010. Clinical perspectives on Cryptococcus neoformans and Cryptococcus gattii: implications for diagnosis and management, p 595–606. In Heitman J, Kozel TR, Kwon-Chung KJ, Perfect JR, Casadevall A (ed), Medical mycology, p 44–78. Lea & Febiger, Pennsylvania, PA.
11. Nishino S, Shimoji H, Yamamoto Y, Yanagihara K, Miyazaki Y, Kohno S. 2008. Detection of anti-GM-CSF autoantibody and neutralizing dysfunction in pulmonary alveolar proteinosis. N. Engl. J. Med. 356:567–579. http://dx.doi.org/10.1056/NEJMoa0802505.
12. Subramanian Vignesh K, Landero Figueroa JA, Porollo A, Caruso JA, Deepes GS, Jr. 2013. Granulocyte macrophage-colony stimulating factor induced Zn sequestration enhances macrophage superoxide and limits intracellular pathogen survival. Immunity 39:947–950. http://dx.doi.org/10.1016/j.immuni.2013.09.006.
13. Shi Y, Liu CH, Roberts AI, Das J, Xu G, Ren G, Zhang Y, Zhang L, Yuan ZR, Tan HS, Das G, Devadas S. 2006. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and T-cell responses: what we do and don’t know. Cell Res. 16:126–133. http://dx.doi.org/10.1038/cr.7310017.
14. Wada H, Noguchi Y, Marino MW, Dunn AR, Old LJ. 1997. T cell responses in granulocyte-macrophage colony-stimulating factor deficient mice. Proc. Natl. Acad. Sci. U. S. A. 94:12557–12561. http://dx.doi.org/10.1073/pnas.94.23.12557.
15. Sorrell TC, Chen SC-A, Phillips P, Marr KA. 2010. Clinical perspectives on Cryptococcus neoformans and Cryptococcus gattii: implications for diagnosis and management, p 595–606. In Heitman J, Kozel TR, Kwon-Chung KJ, Perfect JR, Casadevall A (ed), Cryptococcus: from human pathogen to model yeast. ASM Press, Washington, DC.
16. Meyer W, Trilles L. 2010. Genotyping of the Cryptococcus neoformans/ C. gattii species complex. Aust. Biochem. 41:12–15. http://www.asbmb.org.au/magazine/2010-April_Issue41-1-Showcase/62%20-%20Meyer.pdf.
17. Chen SC, Slavin MA, Heath CH, Playford EG, Byth K, Marriott D, Kidd SE, Bak N, Currie B, Hajkowicz K, Korman TM, McBride WJ, Meyer W, Murray R, Sorrell TC; Australia and New Zealand Mycoses Interest Group (ANZMIG)—Cryptococcus Study. 2012. Clinical manifestations of Cryptococcus gattii infection: determinants of neurological sequelae and death. Clin. Infect. Dis. 55:789–798. http://dx.doi.org/10.1093/cid/cis529.
18. Galanis E, Macdougall L, Kidd S, Morshed M, British Columbia Cryptococcus gattii Working Group. 2009. Epidemiology of Cryptococcus gattii, British Columbia, Canada, 1999-2007. Emerg. Infect. Dis. 16: 351–357. http://dx.doi.org/10.3201/eid1602.090900.
19. Cheng PY, Sham A, Kronstad JW. 2009. Cryptococcus gattii isolates from the British Columbia cryptococcosis outbreak induce less protective inflammation in a murine model of infection than Cryptococcus neoformans. Infect. Immun. 77:4284–4294. http://dx.doi.org/10.1128/IAI.00628-09.
20. Schoffenil T, Illnait-Zaragozi MT, Joosten LA, Netea MG, Boekhout T, Meis JF, Spong T. 2013. Cryptococcus gattii induces a cytokine pattern
that is distinct from other cryptococcal species. PLoS One 8:e55579. http://dx.doi.org/10.1371/journal.pone.0055579.

24. Leongson K, Cousineau-Côté V, Goupil M, Aumont F, Sénéchal S, Gaboury I, Jolicoeur P, Kroonstad JW, de Repentigny L. 2013. Altered immune response differentially enhances susceptibility to Cryptococcus neoformans and Cryptococcus gattii infection in mice expressing the HIV-1 transgene. Infect. Immun. 81:1100–1113. http://dx.doi.org/10.1128/IAI.01339–12.

25. Ngamskulrungroj P, Chang Y, Sionov E, Kwon-Chung KJ. 2012. The primary target organ of Cryptococcus gattii is different from that of Cryptococcus neoformans in a murine model. mBio 3:e00103–12. http://dx.doi.org/10.1128/mBio.00103–12.

26. Ma H, Hagen F, Stelk DJ, Johnston SA, Sionov E, Fark R, Polacheck I, Bockhout T, May RC. 2009. The fatal fungal outbreak on Vancouver Island is characterized by enhanced intracranial parasitism driven by mitochondrial regulation. Proc. Natl. Acad. Sci. U. S. A. 106:12980–12985. http://dx.doi.org/10.1073/pnas.0902963106.

27. Voelz K, Lammans DA, May RC. 2009. Cytokine signaling regulates the outcome of intracranial macrophage parasitism by Cryptococcus neoformans. J. Immunol. 183:3450–3457. http://dx.doi.org/10.1128/JI.00297–09.

28. Olszewski MA, Zhang Y, Huffnagle GB. 2010. Mechanisms of cryptococcal virulence and persistence. Future Microbiol. 5:1269–1288. http://dx.doi.org/10.2217/fmb.10.93.

29. van de Laar I, Coffer PJ, Wolman AM. 2012. Regulation of dendritic cell development by GM-CSF: molecular control and implications for immune homeostasis and therapy. Blood 119:3383–3393. http://dx.doi.org/10.1182/blood-2011-11-370130.

30. Lacey DC, Achuthan A, Fleetwood AJ, Dinh H, Roiniotis J, Scholz GM, Chang MW, Beckman SK, Cook AD, Hamilton JA. 2012. Defining intravital macrophage colony-stimulating factor produced by cord blood-derived B cell lines immortalized by Epstein-Barr virus in vitro. Cell. Immunol. 204:114–127. http://dx.doi.org/10.1016/cimm.2000.1704.

31. Kitamura T, Uchida K, Tanaka N, Tsuichiya T, Watanabe J, Yamada Y, Hanaoka K, Seymour JF, Schoch OD, Doyle I, Inoue Y, Sakatani M, Kudoh S, Azuma A, Nukiwa T, Tanihata Y, Katagiri M, Fujita A, Kurashima A, Kanagasaki S, Nakata K. 2000. Serological diagnosis of idiopathic pulmonary alveolar proteinosis. Am. J. Respir. Crit. Care Med. 162:658–662. http://dx.doi.org/10.1164/rccm.200005-055579.

32. Watanabe M, Uchida K, Nakagaki Y, Trapnell BC, Nakata K. 2010. High avidity cytokine autoantibodies in health and disease: pathogenesis and mechanisms. Cytokine Growth Factor Rev. 21:263–273. http://dx.doi.org/10.1016/j.cytogfr.2010.03.003.

33. Mausberg AK, Jander S, Reichmann G. 2009. Intracerebral granulocyte-macrophage colony-stimulating factor induces functionally competent dendritic cells in the mouse brain. Glia 57:1341–1350. http://dx.doi.org/10.1002/glia.20853.

34. Hunter CA, Roberts CW, Alexander J, 1992. Kinetics of cytokine mRNA production in the brains of mice with progressive toxoplasmic encephalitis. Eur. J. Immunol. 22:2317–2322. http://dx.doi.org/10.1002/eji.180220921.

35. Centers for Disease Control and Prevention. 2010. Emergence of Cryptococcus gattii—Pacific Northwest, 2004-2010. MMWR Morb. Mortal. Wkly. Rep. 59:865–868.

36. Meyer W, Castañeda A, Jackson S, Huynh M, Castañeda E; IberoAmerican Cryptococcal Study. 2003. Molecular typing of IberoAmerican Cryptococcus neoformans isolates. Emerg. Infect. Dis. 9:189–195. http://dx.doi.org/10.3201/eid0902.020246.

37. Ding L, Mo A, Juutivaraa K, Pancholi M, Holland SM, Browne SK. 2012. Determination of human anticytokine autoantibody profiles using a particle-based approach. J. Clin. Immunol. 32:238–245. http://dx.doi.org/10.1007/s10875-011-9621-8.

38. García-Hernoso D, Janbon G, Dromer F. 1999. Epidemiological evidence for dormant Cryptococcus neoformans infection. J. Clin. Microbiol. 37:3204–3209.

39. Hagen F, Colom MF, Swinne D, Tintelnot K, Iatta R, Montagna MT, Torres-Rodriguez JM, Cogliati M, Velegazra A, Burgaflra A, Kamermans A, Sweere JM, Meis JF, Klaassen CH, Bockhout T. 2012. Autochthonous and dormant Cryptococcus gattii infection in Europe. Emerg. Infect. Dis. 18:1618–1624. http://dx.doi.org/10.3201/eid1810.120608.

40. Svenson M, Hansen MB, Ross C, Diamant M, Kienek K, Nielsen H, Bendtz K. 1998. Antibody to granulocyte-macrophage colony-stimulating factor is a dominant anti-cytokine activity in human IgG preparations. Blood 91:2054–2061.