

# Disseminated Cryptococcosis Due to Anti-Granulocyte-Macrophage Colony-Stimulating Factor Autoantibodies in the Absence of Pulmonary Alveolar Proteinosis

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## Abstract

**Introduction** Autoantibodies to granulocyte-macrophage colony-stimulating factor (GM-CSF) can cause acquired pulmonary alveolar proteinosis (PAP). Cases of acquired PAP susceptible to typical respiratory pathogens and opportunistic infections have been reported. Anti-GM-CSF autoantibodies have been reported in a few patients with cryptococcal meningitis. This study evaluated the presence of neutralizing anti-GM-CSF autoantibodies in patients without known congenital or acquired immunodeficiency with severe pulmonary or extrapulmonary cryptococcal infection but without PAP.

**Methods** We took a clinical history and performed an immunologic evaluation and screening of anti-cytokine autoantibodies in patients with cryptococcal meningitis. The impact of autoantibodies to GM-CSF on immune function was assessed by intracellular staining of GM-CSF-induced

STAT5 phosphorylation and MIP-1 $\alpha$  production in normal peripheral blood mononuclear cells incubated with plasma from patients or normal control subjects.

**Results** Neutralizing anti-GM-CSF autoantibodies were identified in four patients with disseminated cryptococcosis, none of whom exhibited PAP. Plasma from patients blocked GM-CSF signaling and inhibited STAT5 phosphorylation and production of MIP-1 $\alpha$ . One patient died of disseminated cryptococcosis involving the central nervous system, which was associated with defective GM-CSF activity.

**Conclusions** Anti-GM-CSF autoantibodies increase susceptibility to cryptococcal infection in adults without PAP. Cryptococcal central nervous system infection associated with anti-GM-CSF autoantibodies could result in neurological sequelae or be life-threatening. Therefore, timely detection of neutralizing anti-GM-CSF autoantibodies and development

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of an effective therapy are necessary to prevent deterioration of cryptococcal infection in these patients.

**Keywords** Granulocyte-macrophage colony-stimulating factor · anti-cytokine autoantibodies · cryptococcal infection · opportunistic infection

## Introduction

Anti-cytokine autoantibodies are important in the pathogenesis of various diseases [1]. Such autoantibodies include anti-interferon (IFN)- $\gamma$  autoantibodies in disseminated nontuberculous mycobacterial infection (NTM) [2–5]; anti-granulocyte-macrophage colony-stimulating factor (GM-CSF) autoantibodies in pulmonary alveolar proteinosis (PAP), which is a chronic lung disease characterized by abnormalities of surfactant metabolism [6]; anti-interleukin-17 autoantibodies in chronic mucocutaneous candidiasis [7, 8]; and anti-interleukin-6 autoantibodies in *Staphylococcus* infection [9]. However, the impact of these autoantibodies in infectious diseases and their etiology are unclear.

GM-CSF is essential for the terminal differentiation and function of alveolar macrophages, which are responsible for the catabolism of surfactant lipids and proteins in the lung [10]. Dysfunction of GM-CSF, due to anti-GM-CSF autoantibodies or a genetic defect in the GM-CSF/GM-CSF receptor, can cause defects of alveolar macrophage function, which lead to deposition of lipoproteinaceous material in the alveoli and respiratory insufficiency [11]. Moreover, clearance of GM-CSF by autoantibodies impairs the antimicrobial activity of neutrophils, which might increase susceptibility to infection [12]. Several reports of the susceptibility of patients with acquired PAP to typical respiratory pathogens and opportunistic infections including *Nocardia* [13], nontuberculous mycobacteria [14], *Histoplasma* [15], and *Cryptococcus* [16] are described. However, the association between infection susceptibility due to PAP and anti-GM-CSF autoantibodies is unclear.

Cryptococcosis is a fungal disease caused by *Cryptococcus neoformans* and *Cryptococcus gattii* and occurs most frequently in immunocompromised individuals. The organism enters the body through inhalation and establishes a pulmonary infection; it can then disseminate to the meninges and brain, causing meningitis or meningoencephalitis. Disseminated cryptococcal infection is diagnosed by the recovery of *Cryptococcus* from sterile body fluids or tissues other than the lung. Cryptococcal meningitis has also been described in patients without identified immune defects [17–22]. Rosen et al. [16, 23] recently detected anti-GM-CSF autoantibodies in human immunodeficiency virus (HIV)-uninfected immunocompetent patients with cryptococcal meningitis or disseminated nocardiosis and postulated that

anti-GM-CSF autoantibodies may predispose patients to cryptococcosis or nocardiosis with or without PAP.

This study evaluated the presence of neutralizing anti-GM-CSF autoantibodies in 11 patients without a known congenital or acquired immunodeficiency who had severe pulmonary or extrapulmonary cryptococcal infection but did not have PAP, together with control subjects.

## Methods

### Subjects

Eleven patients with cryptococcal infection were screened for anti-GM-CSF autoantibodies. The diagnosis of cryptococcosis was based on culture of blood, cerebrospinal fluid, or tissue. Laboratory tests to differentiate *C. neoformans* and *C. gattii* were not performed. This study was performed with the approval of the Ethics Committees of Chang Gung University and China Medical University. Plasma was obtained from heparinized venous whole blood by centrifugation and stored in aliquots at  $-80^{\circ}\text{C}$ . All subjects provided written informed consent under the Institutional Review Board-approved protocols DMR99-IRB-075, 103-7395B, and 104-1357A3.

### Identification of Anti-GM-CSF Autoantibodies and Analysis of IgG Subclass in Plasma

A clear polystyrene 96-well, flat-bottomed plates (Nunc) was coated with 100  $\mu\text{l}$  of human GM-CSF recombinant protein (2  $\mu\text{g}/\text{ml}$ ) in bicarbonate buffer (pH 9.6) per well and incubated at  $4^{\circ}\text{C}$  overnight. The plate was washed five times with phosphate-buffered saline (PBS)-Tween 0.05% and then blocked with PBS - 5% human normal serum albumin (Aventis) for 2 hr. The plate was washed again, serially diluted plasma ( $10^{-2}$  to  $10^{-4}$ ) from patients and healthy donors was added, and the plate was incubated at  $4^{\circ}\text{C}$  overnight. The plate was thoroughly washed, and Fc-specific peroxidase-conjugated AffiniPure Goat anti-human IgG (Cappel) was added at a ratio of 1:2500. The plate was incubated for 90 min at  $37^{\circ}\text{C}$  and then washed five times with PBS-Tween 0.05%. *p*-Nitrophenyl phosphate (pNPP) solution (100  $\mu\text{l}/\text{well}$ ) was added, and the plate was incubated for 20 min at  $37^{\circ}\text{C}$ . It was determined absorbance at OD 405 nm with a VICTOR X3 Multilabel Plate Reader (PerkinElmer). Data from a representative experiment are shown. Similar results were obtained in independent experiments. Subsequently, for IgG subclass analysis, the plate was thoroughly washed and peroxidase-conjugated mouse anti-human IgG1, 2, 3, and 4 (Abcam) antibodies were added at a ratio of 1:2000 after the diluted plasma incubation (1:100). The plate was incubated for 60 min at room temperature and then washed five times

with PBS-Tween 0.05%. 3,3',5,5'-Tetramethylbenzidine (TMB) solution (100  $\mu$ l/well) was then added, and the plate was incubated for 20 min at room temperature. The absorbance was detected at OD 450 nm with a VICTOR X3 Multilabel Plate Reader (PerkinElmer).

### Intracellular Staining of Phospho-STAT5

To demonstrate that plasma containing anti-GM-CSF autoantibodies blocked GM-CSF signaling,  $2 \times 10^5$  PBMCs from a healthy donor were cultured in Roswell Park Memorial Institute (RPMI)-1640 with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin containing control or patient plasma (10 or 50%) and then incubated with or without GM-CSF (10 ng/ml) and IL-3 (10 ng/ml) for 30 min at 37 °C. Monocytes were identified by anti-human CD14-FITC (BD Pharmingen) surface staining, followed by fixation and permeabilization for intracellular staining with an anti-pSTAT5 (Y694)-PE antibody (BD Phosflow™), as described previously [16]. Data were collected using FACSVerse (BD Bioscience) and analyzed using FlowJo software (TreeStar).

### Titration of Neutralizing Anti-GM-CSF Activity in Plasma

U937 cells were cultured in RPMI-1640 with 10% FBS and 1% penicillin/streptomycin. Recombinant human GM-CSF was serially diluted from  $5 \times 10^3$  to  $10^{-5}$  ng/ml in 5% human plasma and pre-incubated at 37 °C for 15 min. Then U937 cells were added in a final concentration of  $1 \times 10^6$  cells/ml. After the incubation with GM-CSF for 30 min at 37 °C, U937 cells were fixed and permeabilized for intracellular staining with an anti-pSTAT5 (Y694)-PE antibody (BD Phosflow™). Data were collected and analyzed using FACSVerse (BD Bioscience) and graphed with Prim6 (GraphPad).

### Plasma Inhibition of GM-CSF-Induced MIP-1 $\alpha$

To evaluate inhibition of GM-CSF activity, PBMCs from a healthy donor were incubated with control or patient plasma (10%) overnight with or without GM-CSF (10 ng/ml). Supernatants were collected and MIP-1 $\alpha$  levels were determined using a Human MIP-1 $\alpha$  ELISA Kit (eBioscience) according to the manufacturer's instructions.

## Results

The clinical manifestations of the following four patients are summarized in Tables 1 and 2.

## Case Reports

**Patient 1** An otherwise healthy 37-year-old man presented in October 2013 after experiencing at least 1 month of headache, dizziness, nausea, vomiting, and blurred vision. The patient had experienced a 17-kg weight loss in 3 months and had progressive skin lesions on his face for 2 months (Fig. 1a). His initial laboratory studies revealed a leukocyte count of 17,230/ $\mu$ l with 90% neutrophils, hemoglobin of 10.7 g/dl (normocytic anemia), platelets of 364,000 per  $\mu$ l, and a C-reactive protein (CRP) level of 66 mg/l. His chest X-ray and CT showed an upper mediastinal mass with tracheal compression and a consolidation in the right middle lung (Fig. 1b, c). Brain magnetic resonance imaging (MRI) showed an approximately 1.4-cm mass on the right nasopharyngeal roof (Fig. 1d). Lumbar puncture revealed an opening pressure of >60 cm H<sub>2</sub>O, 40 WBCs/mm<sup>3</sup>, 20 RBCs/mm<sup>3</sup>, monocyte predominance (61%), glucose of <10 mg/dl, and protein of 144.7 mg/dl. India ink preparation of cerebrospinal fluid revealed characteristic round budding yeasts with distinct halos. Cerebrospinal fluid and serum cryptococcal antigens were positive with titers of >1:1024, and cerebrospinal fluid culture grew cryptococci. Skin biopsy revealed numerous encapsulated yeasts by hematoxylin and eosin staining (Fig. 1e) and Periodic acid-Schiff staining (Fig. 1f). Chest CT-guided aspiration and bronchoscopic biopsy also demonstrated numerous mucoid encapsulated yeasts consistent with *Cryptococcus* (Fig. 1g). The immune status of the patient was investigated. He was nonreactive to HIV, and his serum immunoglobulins, complement levels, and lymphocyte subsets were within the normal range except for a low CD4<sup>+</sup> T cell percentage. Therefore, the diagnosis of disseminated cryptococcal infection with meningitis and pulmonary and cutaneous involvement in an otherwise immunocompetent individual was made. However, there was no clinical or microbiological improvement after 3 months of amphotericin B plus flucytosine treatment and therapeutic lumbar punctures. The patient died of sequelae of cryptococcal CNS infection.

**Patient 2** A 40-year-old man with a history of gout presented in May 2014 after experiencing 1 day of headache and weakness in the left limbs. His initial laboratory studies revealed a leukocyte count of 10,700/ $\mu$ l with 70% neutrophils, hemoglobin of 14.6 g/dl, platelets of 343,000/ $\mu$ l, and CRP level of 67.5 mg/l. His chest X-ray showed a consolidation in the left lower lung (Fig. 2a). Brain MRI showed a large mass on the right frontal lobe and a small nodule in the right caudate head (Fig. 2b, c). Under the impression of glioblastoma multiforme, the patient underwent craniotomy to remove the tumor. However, the pathologic examination revealed cryptococcal spores. Serum cryptococcal antigen was positive with a titer of 1:512. Therefore, the diagnosis was disseminated cryptococcal infection with pulmonary involvement and brain abscess.

**Table 1** Demographics, laboratory data, treatment, and outcome of patients with cryptococcal infection and anti-GM-CSF autoantibodies

Clinical parameter	Patient 1	Patient 2	Patient 3	Patient 4
Age at diagnosis (years)	37	40	59	37
Sex	Male	Male	Female	Male
Underlying disease	No	Allergic rhinitis, asthma, gout	Hypertension	Chronic hepatitis C
Presenting symptoms	Headache, vomiting, blurred vision, seizure	Headache, left limb weakness, confusion	Blurred vision	Fever, cough Headache, vomiting
Infection site	CNS, skin, lung	CNS, lung	Eye	CNS, lung
Peripheral total WBC count (cells/ $\mu$ l)	17,230	10,700	8100	14,900
(3900–10,600)				
Granulocytes (cells/ $\mu$ l)	15,558	7522	5848	12,248
(1638–7844)				
Lymphocytes (cells/ $\mu$ l)	999	2215	1547	1579
(780–5936)				
Monocytes (cells/ $\mu$ l)	637	717	510	939
(0–1272)				
Serum cryptococcal antigen	>1:1024	1:512	1:64	1:256
Cerebrospinal fluid data				
Opening pressure (mm H <sub>2</sub> O)	>60	NA	NA	130
WBC (cells/mm <sup>3</sup> )	40	NA	NA	534
Granulocyte (cells/mm <sup>3</sup> )	5	NA	NA	48
Lymphocytes (cells/mm <sup>3</sup> )	10	NA	NA	459
Monocytes (cells/mm <sup>3</sup> )	24	NA	NA	27
Microbiology	India ink (+) Cryptococcal antigen (>1:1024) and culture (+)	Cryptococcal fungal spores and culture (+)	Cryptococcal antigen (1:64)	Cryptococcal antigen (1:256)
Protein (mg/dl)	144.7	NA	NA	92
Glucose (mg/dl)	<10	NA	NA	34
PAP	No	No	No	No
HIV Ab	Negative	Negative	Negative	Negative
Antifungal agents	AmB + 5-FC for 3 months	AmB + 5-FC for 3 months; then fluconazole	AmB (intraocular); then voriconazole	AmB + 5-FC; then fluconazole
Outcome and sequelae	Hydrocephalus, status epilepticus, coma, death	Hydrocephalus, focal seizure, left hemiplegia	Impaired vision	No sequelae

CNS central nervous system, WBC white blood cell, PAP pulmonary alveolar proteinosis, HIV Ab human immunodeficiency virus antibody, AmB amphotericin B, 5-FC 5-fluorocytosine, NA not available

**Table 2** Immunophenotypes of the patients

Parameter	Patient 1	Patient 3	Patient 4
CD3 (%) (49.9–84.7)	63.2	69.0	66.2
CD4 (%) (41–53)	37.5	33.2	37.7
CD8 (%) (24–33)	35	35.5	27.2
CD19 (%) (7.8–22.8)	14.3	9.9	30.8
CD3 <sup>-</sup> /CD56 <sup>+</sup> (%) (1–35)	12.8	NA	NA
IgG (mg/dl) (680–1620)	968.1	1500	NA
IgM (mg/dl) (57–288)	35.6	NA	NA
IgA (mg/dl) (84–438)	325.7	NA	NA

The data of patient 2 were not available

He received amphotericin B and flucytosine treatment for 3 months, followed until June 2016 by fluconazole. He had severe sequelae of hydrocephalus, seizure, and left hemiplegia.

**Patient 3** A 59-year-old woman with a history of hypertension presented to our ophthalmologic department due to a red left eye and blurred vision for 2 months and 2 weeks, respectively. Fundoscopic examination disclosed a choroidal tumor in the left eye. Brain computed tomography showed a pseudotumor in the left sclera layer. One week later, this patient underwent microincision vitreoretinal surgery, and the extracted tissue was submitted for pathological examination. The pathologist reported cryptococci within the tissue. A blood cryptococcal antigen test was positive with a titer of 1:64. Under the diagnosis of cryptococcosis of the left eye, intraocular injection of amphotericin B was administered, followed by oral voriconazole. The CD4<sup>+</sup> T cell count was within the normal range, and a HIV test was negative. This patient received 14 weeks full-dose (200 mg twice daily) voriconazole therapy, followed by secondary prophylaxis (150 mg daily). No recurrence of disease was reported during the follow-up period.

**Patient 4** A 37-year-old man with a history of chronic hepatitis C infection presented to our emergency room with cough and fever for >10 days. In the emergency room, physical examination disclosed bilateral rhonchi in the lungs, and the laboratory data showed an elevated CRP (43.8 mg/l) and leukocytosis (14,900/ $\mu$ l). Numerous consolidated lesions in both lung fields were noted on the chest X-ray. The patient was admitted with a presumptive diagnosis of community-acquired pneumonia. During the course of hospitalization, however, persistent fever, new-onset headache, and vomiting

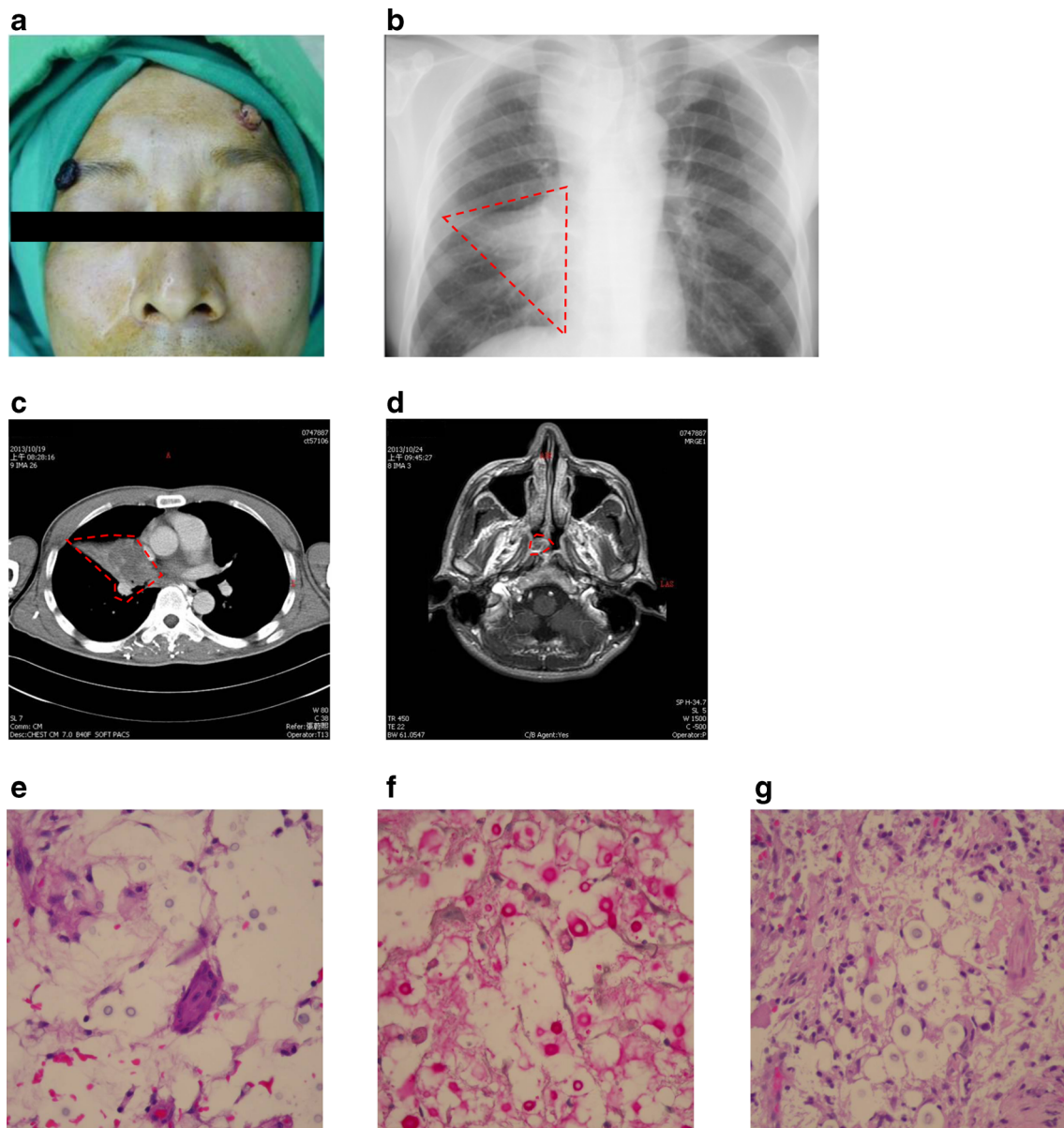
were reported by the patient. Lumbar puncture was performed to exclude meningitis. The lumbar puncture opening pressure was 130 mm H<sub>2</sub>O. The cerebrospinal fluid showed lymphocyte-predominant pleocytosis (534/ $\mu$ l, 86%) with an elevated protein (92 mg/dl) but reduced glucose (34 mg/dl) level. No pathogen was isolated from the cerebrospinal fluid, but cryptococcal antigen was detected in the cerebrospinal fluid and serum with a titer of 1:256. A sputum culture grew *Cryptococcus* species. The patient was HIV-negative and had a CD4<sup>+</sup> T cell count within the normal range. This patient was treated with amphotericin B and flucytosine. The antifungal therapy resulted in improvement in the clinical symptoms and reduced the size of the pulmonary lesions. Two weeks after systemic antifungal therapy, the patient was discharged with maintenance fluconazole therapy.

### Detection of Anti-GM-CSF Autoantibodies

Plasma samples from controls ( $n = 5$ ) and patients with severe cryptococcal infection ( $n = 11$ ) at a serial dilution ( $10^{-2}$  to  $10^{-4}$ ) were tested for anti-GM-CSF autoantibodies. Plasma samples from five PAP patients served as positive controls. Four patients were positive for anti-GM-CSF autoantibodies (Fig. 3a). The major IgG subclass of anti-GM-CSF autoantibodies in the four patients was IgG<sub>1</sub> (Fig. 3b).

### Inhibition by Anti-GM-CSF Autoantibodies of Phospho-STAT5 Signaling

Non-neutralizing anti-GM-CSF autoantibodies and a low titer thereof have been described in healthy individuals and those with various diseases [24]. To determine the inhibitory capacity of plasma containing anti-GM-CSF autoantibodies, control PBMCs were incubated with or without GM-CSF or IL-3 in the presence of normal or patient plasma (10%) to detect the phosphorylation level of STAT5. The plasma of six patients containing anti-GM-CSF autoantibodies blocked STAT5 phosphorylation to a greater degree than plasma from healthy controls, whereas IL-3 which inducing STAT5 phosphorylation through sharing the  $\beta$  unit of the GM-CSF receptor served as control (Fig. 4a). Moreover, the inhibitory level of anti-GM-CSF autoantibodies in patient plasma was evaluated through stimulating U937 cells in patient or control plasma (5%) with increasing amounts of GM-CSF and calculated the phosphorylation level of STAT5 protein. The amount of GM-CSF required to achieve 50% of maximum STAT5 phosphorylation (half-maximal effective concentration = EC<sub>50</sub>) was determined from each dose-response curve (Fig. 4b). Plasma samples from health donors had the similar response to GM-CSF. However, patient plasma containing anti-GM-CSF autoantibodies required a 2–5 log higher concentration of GM-CSF to obtain 50% of maximum pSTAT5 level compared to control plasma.



**Fig. 1** Skin lesions, radiographic and cytopathic manifestations of patient 1. **a** Umbilicated papules of various sizes located mainly on the face. **b** Chest X-ray showing consolidation of the right middle lobe and mediastinal mass with airway compression (*red dashed line*). **c** Chest CT demonstrating a pulmonary cryptococcal lesion (*red dashed line*). **d** MRI

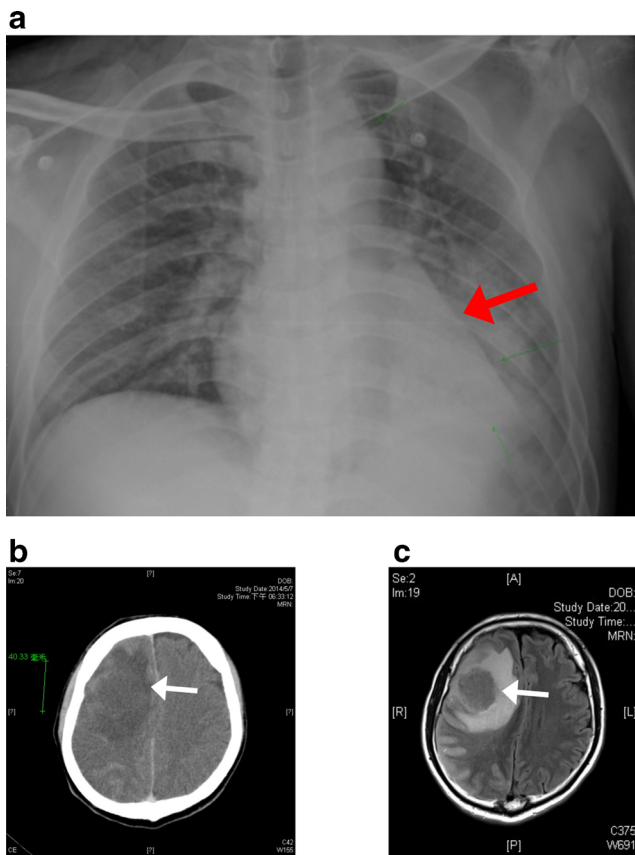
of the brain showing the right nasopharyngeal roof mass (*red dashed line*). **e** Hematoxylin and eosin stain of the cutaneous lesion showing numerous encapsulated yeasts. **f** Periodic acid-Schiff stain of the cutaneous lesion showing numerous yeast cells. **g** Sample obtained by CT-guided biopsy revealed numerous encapsulated yeast forms

### Plasma Containing Anti-GM-CSF Autoantibodies Inhibits MIP-1 $\alpha$ Production

The effect of plasma containing anti-GM-CSF autoantibodies on GM-CSF-induced MIP-1 $\alpha$  production from PBMCs of controls was determined. Control PBMCs were incubated with or without GM-CSF in the presence of control or patient plasma (10%) (Fig. 4c). Plasma containing anti-GM-CSF autoantibodies decreased GM-CSF-induced MIP-1 $\alpha$  production in comparison with control plasma ( $p < 0.05$ ).

### Discussion

We have herein reported an association between biologically inhibitory anti-GM-CSF autoantibodies and four cases of severe cryptococcal infection without concurrent PAP. Fourteen cases of cryptococcal meningitis [16, 24] and three cases of CNS nocardiosis [23] associated with anti-GM-CSF autoantibodies have been reported in previous studies; none had concurrent PAP, although it developed later in two patients. Therefore, our data support the hypothesis that anti-GM-CSF autoantibodies increase susceptibility to fungal infection



**Fig. 2** Radiographic manifestations of patient 2. **a** Chest X-ray showing consolidation of the left lower lobe (red arrow). **b** Brain CT showing a large mass in the right frontal lobe with perifocal edema (white arrow). **c** MRI of brain showing an approximately 5-cm mass in the right frontal lobe with central necrosis (white arrow)

in the absence of PAP. The presence of anti-GM-CSF autoantibodies should be considered in patients with CNS cryptococcal infection, and earlier diagnosis and antifungal treatment,

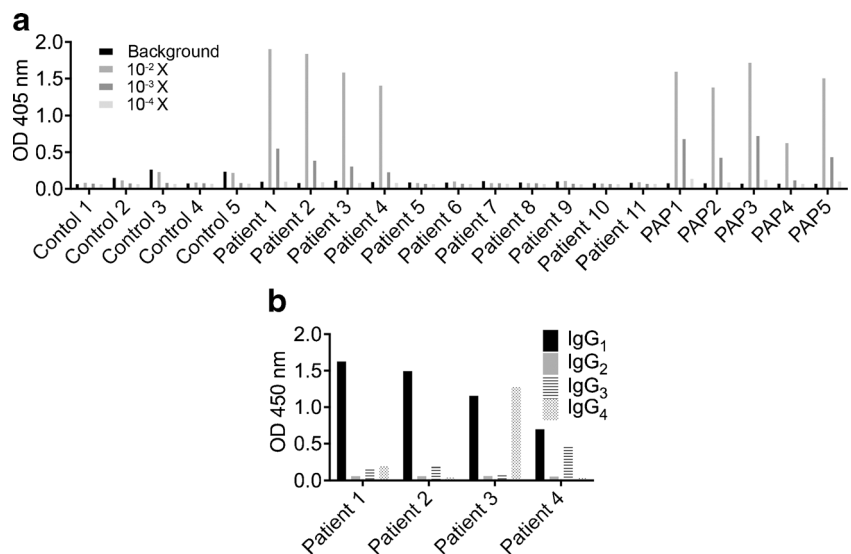
including depletion of autoantibodies, might be critical to prevent mortality.

The reason for the diverse clinical manifestations of anti-GM-CSF autoantibodies is unclear. Non-neutralizing anti-GM-CSF autoantibodies and a low concentration thereof have been reported in patients with various diseases and in healthy individuals [24]. Immune complex formation in the presence of a high concentration of anti-GM-CSF autoantibodies was recently reported to block the function of GM-CSF in patients with PAP [25]. Consistent with this observation, anti-GM-CSF antibody therapy did not cause PAP in a clinical trial, suggesting that immune complex formation or neutralizing epitopes are linked to PAP.

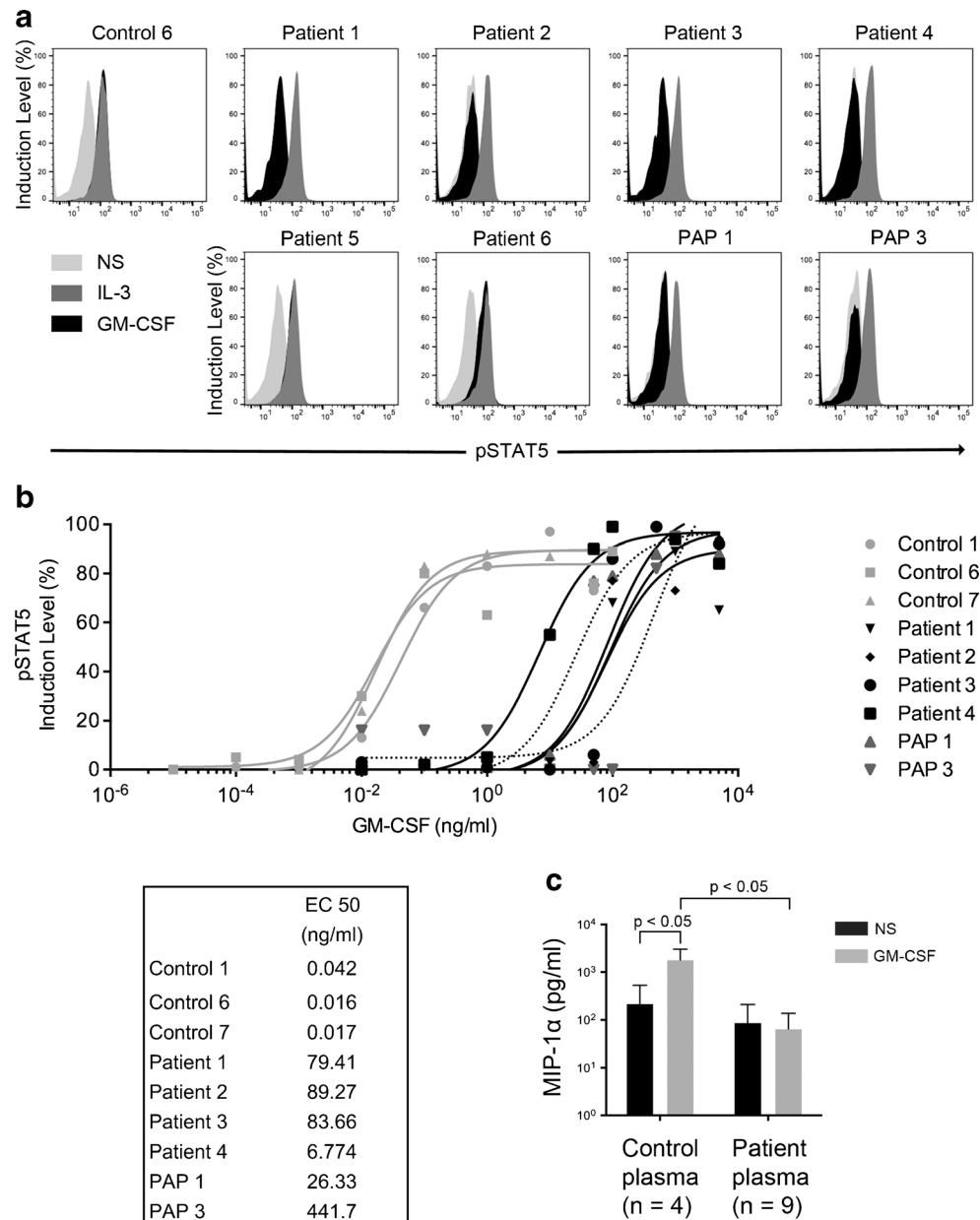
The brain is protected by the blood–brain barrier, and only 0.1% of antibodies are found in the cerebrospinal fluid. The anti-GM-CSF autoantibody concentration in cerebrospinal fluid was not measured in the present study due to lack of availability. Anti-GM-CSF autoantibodies in cerebrospinal fluid were reported in a previous study; however, their concentration and inhibitory activity were not measured [16]. The antibody concentration in the cerebrospinal fluid and the presence of neutralizing epitopes or immune complex formation may explain the diverse clinical manifestations in patients with anti-GM-CSF autoantibodies. Investigation of the concentration or even the neutralizing titer of anti-GM-CSF autoantibodies in patients’ cerebrospinal fluid, cloning and characterization of anti-GM-CSF autoantibodies from patients with CNS infection are thus necessary to improve our understanding of the two clinical manifestations of anti-GM-CSF autoantibody disease.

Rosen et al. [16] reported that five of seven patients with anti-GM-CSF autoantibodies responded well to standard antifungal treatment. In contrast, two of our patients with anti-GM-CSF autoantibodies showed a poor response to treatment,

**Fig. 3** Characterization of anti-GM-CSF autoantibodies in patients with *Cryptococcus* infection. **a** Detection of anti-GM-CSF autoantibodies in controls ( $n = 5$ ), patients with cryptococcal infection (patients 1–11), and patient with PAP (PAP 1–5). **b** Identification of IgG subclass in plasma containing anti-GM-CSF autoantibodies from patient with cryptococcal infection



**Fig. 4** The inhibitory capacity of anti-GM-CSF autoantibodies in patient plasma. **a** Inhibition by anti-GM-CSF autoantibodies of phospho-STAT5 signaling. Control PBMCs were incubated with control or patient plasma and stimulated with or without GM-CSF. Intracellular staining for phospho-STAT5 was analyzed by flow cytometry. Only patient 4 was under 50% plasma, the remaining subjects were under 10% plasma. **b** Representative dose-response curves for phospho-STAT5 level in U937 cells incubated with plasma from healthy donors, patient with cryptococcal infection or PAP and stimulated with increasing amounts of GM-CSF. The concentration required for 50% pSTAT5 level (half-maximal effective concentration =  $EC_{50}$ ) was shown and presented as nanogram per milliliter in the column, respectively. **c** Anti-GM-CSF autoantibodies in plasma inhibited GM-CSF-induced MIP-1 $\alpha$  protein expression. Control PBMCs were incubated with control or patient plasma and stimulated with or without GM-CSF (10 ng/ml). The MIP-1 $\alpha$  protein level was measured by ELISA and is presented as picogram per milliliter



resulting in mortality and severe sequelae, respectively. The reason for this difference is unclear. However, the increased severity might reflect lower residual GM-CSF activity in the presence of anti-GM-CSF autoantibodies, particularly in the CNS. Patients with complete loss-of-function mutations of IFNGR1 or IFNGR2 can suffer from lethal NTM infections. Despite suffering from NTM infections, patients with hypomorphic mutations have a better clinical outcome, likely due to residual IFN- $\gamma$  activity. It seems that anti-IFN- $\gamma$  autoantibody disease is a phenocopy of hypomorphic IFNG receptor deficiency because patients with anti-IFN- $\gamma$  autoantibodies retain a degree of IFN- $\gamma$  activity. This might also be the case for anti-GM-CSF autoantibodies and cryptococcal CNS infection: patients with the most severe *Cryptococcus*

infection might have minimal GM-CSF activity in the CNS. In this situation, removing autoantibodies by plasmapheresis and B cell depletion therapy, which have been reported for treating anti-IFN- $\gamma$  autoantibody disease [26], should be considered in addition to standard antifungal therapy.

*C. neoformans* and *C. gattii* are the two major species of the genus *Cryptococcus* considered to be pathogenic in humans, and both can cause pulmonary and CNS disease. *C. gattii* is mainly associated with immunocompetent patients, but can also infect immunosuppressed patients, while *C. neoformans* infection is reported predominantly in immunocompromised patients [27]. *C. gattii*, but not *C. neoformans*, has been linked to meningitis associated with anti-GM-CSF autoantibodies [24]. However, molecular techniques that can distinguish



these two species are not widely available, including in Taiwan. Therefore, we were unable to specify the *Cryptococcus* isolates from our patients. Identification of *C. gattii* should be performed in all patients with CNS cryptococcal infection, and if *C. gattii* is identified, the presence of anti-GM-CSF autoantibodies should be evaluated due to their influence on clinical management.

Anti-IFN- $\gamma$  autoantibodies are a common etiology of disseminated NTM infection, particularly in patients in Southeast Asia [5, 28–31]. Recently, we demonstrated that production of these autoantibodies was associated with specific genetic factors, the human leukocyte antigen (HLA) class II molecules DRB1\*15:02/16:02 and DQB1\*05:01/05:02. Moreover, these HLA molecules mediated the geographic/ethnic specificity of this disease [5, 30]. Due to the small number of patients, it is unclear whether or not anti-GM-CSF autoantibodies are linked to a particular geographic location or ethnicity. Also, whether production of anti-GM-CSF autoantibodies is associated with specific HLA molecules should be the subject of future research. Further studies and evaluation of a larger number of patients with fungal infections that have anti-GM-CSF autoantibodies are needed to address this issue.

We did not observe the sign of PAP in our patients; however, it is still possible that the patients might develop the PAP later. Reported by Rosen et al., two patients of cryptococcal meningitis with anti-GM-CSF autoantibodies later developed evidence of PAP [16]. Long-term follow-up in cryptococcal infection patients with anti-GM-CSF autoantibodies is necessary to dissect the relationship between PAP and cryptococcal meningitis. Further prospective work must be conducted to dissect the molecular mechanism of these two clinical manifestations induced by anti-GM-CSF autoantibodies.

## Conclusions

*Cryptococcus* infection of the CNS in otherwise healthy patients suggests acquired immunodeficiency due to anti-GM-CSF autoantibodies, even in the absence of signs of PAP. The manifestations of CNS cryptococcal infections in these patients range from moderate to severe and life-threatening illness. The presence of anti-GM-CSF autoantibodies should be considered in patients with cryptococcal CNS infection because earlier diagnosis and treatment might prevent mortality.

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## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

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