Anti–GM-CSF Autoantibodies in Patients with Cryptococcal Meningitis

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Anti–GM-CSF Autoantibodies in Patients with Cryptococcal Meningitis

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Cryptococcal meningitis has been described in immunocompromised patients, as well as in those for whom no immune defect has been identified. GM-CSF regulates the function of phagocytes and pulmonary alveolar macrophages, critical elements in cryptococcal control. We performed clinical histories, immunological evaluation, and anticytokine autoantibody screening in four current patients with cryptococcal meningitis and identified and tested 103 archived plasma/cerebrospinal fluid samples from patients with cryptococcal meningitis. We assessed the ability of anti–GM-CSF autoantibody–containing plasmas to inhibit GM-CSF signaling. We recognized anti–GM-CSF autoantibodies in an otherwise healthy female with cryptococcal meningitis who later developed pulmonary alveolar proteinosis (PAP). Her diagnosis prompted screening of patients with cryptococcal meningitis for anticytokine autoantibodies. We identified seven HIV-negative patients with cryptococcal meningitis who tested positive for higher anti–GM-CSF autoantibodies. Two of the seven later developed evidence of PAP. Plasma from all patients prevented GM-CSF–induced STAT5 phosphorylation and MIP-1α production in normal PBMCs. This effect was limited to their IgG fraction. Anti–GM-CSF autoantibodies are associated with some cases of cryptococcal meningitis in otherwise immunocompetent patients. These cases need not have associated PAP. The Journal of Immunology, 2013, 190: 000–000.

Anti–GM-CSF autoantibodies were shown to cause acquired pulmonary alveolar proteinosis (PAP) (1, 2), a chronic lung disease characterized by abnormalities of surfactant metabolism. PAP generally occurs in adulthood and is characterized by accumulation of dense proteinaceous infiltrates and associated respiratory insufficiency. Both respiratory and extrapulmonary infections, often with unusual pathogens, have also been reported (3–11). The role of serum anti–GM-CSF autoantibodies in infection susceptibility outside the lung has not been clearly defined.

Anti–GM-CSF autoantibodies in patients with PAP contribute to a range of defects in alveolar macrophage function, including impaired chemotaxis, adhesion, phagocytosis, microbicidal activity, and phagolysosome fusion (12, 13). GM-CSFR–deficient mice show that GM-CSF induces PU.1, which is critical to both surfactant homeostasis and TLR signaling (14), potentially explaining surfactant accumulation, as well as the infection susceptibility seen in PAP. GM-CSF−/− mice and PAP patients have defective neutrophil phagocytosis, adhesion, oxidative burst, and bacterial killing (15). Furthermore, many of the infecting organisms in PAP, such as Nocardia spp. (3, 5, 6, 8–10), Histoplasma (7), and Cryptococcus (4, 9, 16, 17), are ones against which phagocytes are known to be important. However, the majority of these reports originated prior to the identification of anti–GM-CSF autoantibodies as a major cause of acquired PAP.

Anti-cytokine autoantibodies are now known to be a cause of adult-onset infection susceptibility (18), with examples including anti–IFN-γ autoantibodies with disseminated nontuberculous mycobacterial infections (19–22) and anti–IL-17 autoantibodies with chronic mucocutaneous candidiasis (23, 24). Cryptococcal meningitis is a recognized opportunistic infection in those with immunocompromise, such as lymphoma, steroid use, or HIV infection, but it was also described in those without identified immune defects (25–31).

Identification of a patient with cryptococcal meningitis and PAP prompted us to recognize anti–GM-CSF autoantibodies. To evaluate whether anti–GM-CSF autoantibodies were specifically associated with cryptococcal meningitis, we screened banked archived sera or plasma and, in some cases, cerebrospinal fluid of previously healthy, HIV-negative adults with cryptococcal meningitis.

Materials and Methods

Subjects

Three patients were seen at the National Institutes of Health (NIH) and consented to evaluation and treatment of cryptococcal meningitis under Institutional Review Board (IRB)–approved protocol 93-I-0119 or 93-I-0106. One patient was seen and gave consent in Thailand under NIH IRB–approved protocol 09-I-N060. Normal PBMCs were obtained though the NIH Blood Bank under appropriate IRB-approved protocols. Plasma from healthy controls (n = 64) and diseased controls (n = 43) who had not been previously tested for autoantibodies included healthy adult volunteers and adult patients with an undiagnosed immunodeficiency, respectively.

Abbreviations used in this article: CT, computerized tomography; IRB, Institutional Review Board; MRI, magnetic resonance imaging; NIH, National Institutes of Health; PAP, pulmonary alveolar proteinosis; PAS, periodic acid-Schiff.
<table>
<thead>
<tr>
<th>Clinical Parameter</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5</th>
<th>Patient 6</th>
<th>Patient 7</th>
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</thead>
<tbody>
<tr>
<td>Age at diagnosis (y)</td>
<td>20</td>
<td>31</td>
<td>48</td>
<td>47</td>
<td>26</td>
<td>34</td>
<td>32</td>
</tr>
<tr>
<td>Gender</td>
<td>Female</td>
<td>Female</td>
<td>Male</td>
<td>Male</td>
<td>Male</td>
<td>Male</td>
<td>Male</td>
</tr>
<tr>
<td>Presenting symptoms</td>
<td>Fever, headache, neck pain, diplopia, confusion</td>
<td>Headache</td>
<td>Back pain, cough, fever</td>
<td>Cough, weakness, tremors</td>
<td>Back/neck pain, nausea, vomiting, ptosis</td>
<td>Blurred vision, nausea, vomiting, fever, chills</td>
<td></td>
</tr>
<tr>
<td>Peripheral total WBC count (cells/μl)</td>
<td>8,600</td>
<td>9,900</td>
<td>6,800</td>
<td>8,200</td>
<td>6,300</td>
<td>10,800</td>
<td>5,000</td>
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<td>Granulocytes (cells/μl)</td>
<td>7,482</td>
<td>8,400</td>
<td>3,046</td>
<td>5,035</td>
<td>3,150</td>
<td>9,288</td>
<td>3,500</td>
</tr>
<tr>
<td>Lymphocytes (cells/μl)</td>
<td>795</td>
<td>800</td>
<td>1,714</td>
<td>1,870</td>
<td>1,071</td>
<td>972</td>
<td>1,250</td>
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<tr>
<td>Monocytes (cells/μl)</td>
<td>353</td>
<td>700</td>
<td>802</td>
<td>713</td>
<td>378</td>
<td>540</td>
<td>250</td>
</tr>
<tr>
<td>Serum cryptococcal Ag</td>
<td>1:128</td>
<td>1:256</td>
<td>1:1024</td>
<td>1:512</td>
<td>Not available</td>
<td>Not available</td>
<td>Not available</td>
</tr>
<tr>
<td>Lumbar puncture</td>
<td>Opening pressure (mm H2O)</td>
<td>250</td>
<td>400</td>
<td>250</td>
<td>Not done</td>
<td>200</td>
<td>360</td>
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<tr>
<td>WBC (cells/mm³)</td>
<td>17</td>
<td>219</td>
<td>98</td>
<td>241</td>
<td>158</td>
<td>60</td>
<td>53</td>
</tr>
<tr>
<td>Granulocytes (cells/mm³)</td>
<td>1</td>
<td>6</td>
<td>2</td>
<td>22</td>
<td>48</td>
<td>14</td>
<td>Not available</td>
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<tr>
<td>Lymphocytes (cells/mm³)</td>
<td>16</td>
<td>82</td>
<td>94</td>
<td>219</td>
<td>0</td>
<td>32</td>
<td>Not available</td>
</tr>
<tr>
<td>Monocytes (cells/mm³)</td>
<td>0</td>
<td>12</td>
<td>0</td>
<td>110</td>
<td>4</td>
<td>Not available</td>
<td></td>
</tr>
<tr>
<td>Microbiology</td>
<td>Cryptococcal Ag⁺ (1.256)</td>
<td>Cryptococcal Ag⁺ (1.8) and culture⁺</td>
<td>Cryptococcal Ag⁺ and culture⁺</td>
<td>Cryptococcal Ag⁺ (1:16)</td>
<td>Budding yeast/India ink⁺</td>
<td>India ink and culture⁺</td>
<td>Direct stain and culture⁺</td>
</tr>
<tr>
<td>Protein (mg/dl)</td>
<td>33</td>
<td>91</td>
<td>85</td>
<td>75</td>
<td>68</td>
<td>36</td>
<td>Not available</td>
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<tr>
<td>Glucose (mg/dl)</td>
<td>51</td>
<td>47</td>
<td>36</td>
<td>36</td>
<td>43</td>
<td>140</td>
<td>Not available</td>
</tr>
<tr>
<td>PAP</td>
<td>Yes, 2 y later</td>
<td>No</td>
<td>No</td>
<td>Asymptomatic ground-glass opacities on chest CT</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Anti-infectives</td>
<td>AmB + 5FC; then fluconazole</td>
<td>AmB + 5FC; then fluconazole + 5FC</td>
<td>AmB; then fluconazole; antituberculous therapy</td>
<td>AmB + fluconazole</td>
<td>AmB + 5FC</td>
<td>AmB IT and i.v. + 5FC</td>
<td>AmB IT and i.v. + 5FC</td>
</tr>
<tr>
<td>Outcome and sequelae</td>
<td>Required whole-lung lavage for PAP with subsequent improvement</td>
<td>Full recovery</td>
<td>Full recovery</td>
<td>Recovered; remains on maintenance fluconazole</td>
<td>Full recovery</td>
<td>Seizures, homonymous hemianopsia, normal pressure hydrocephalus, chronic brain syndrome</td>
<td>Seizures, neurogenic bladder</td>
</tr>
</tbody>
</table>

AmB, Amphotericin B; 5FC, flucytosine; IT, intrathecal; MTB, M. tuberculosis.
All subjects were seen and consented under appropriate National Institutes of Allergy and Infectious Diseases IRB-approved protocols.

One hundred and three patients were identified as having been treated at the NIH for cryptococcal meningitis between 1955 and 1984. Archived plasma samples that had been stored at −80°C were screened under IRB-approved protocol 09-I-N162. Cerebrospinal fluid collected for diagnosis and management of cryptococcal meningitis was stored at −80°C for some patients and, when available, it was screened. Anonymous cerebrospinal fluid collected at NIH for other purposes and designated as medical waste by the clinical laboratory was used as controls.

All current patients underwent a history and physical examination, as well as routine laboratories, including HIV testing and complete blood count with differential, serum electrolytes, renal and hepatic function chemistries, quantitative Ig levels, and lymphocyte markers (i.e., total T cells [CD3+; BD Pharmingen], CD4+ T cells [Immunotech], CD8+ T cells [BD Pharmingen], CD80+ T cells [Immunotech], CD20+ B cells [BD Pharmingen], CD20+/CD27+ memory B cells [BD Pharmingen], CD16+ or CD56+/CD3− NK cells [BD Pharmingen], and CD56+/CD3+ NKT cells). Medical charts were reviewed for patients for whom archived serum had been collected to document microbiological evidence of cryptococcal meningitis and to identify possible comorbidities that could contribute to cryptococcal susceptibility.

**Determination of anti-cytokine autoantibody titers**

Plasma from patients and healthy controls was screened for anti-cytokine autoantibodies using a particle-based technology that was described previously (32). Briefly, six sets of differentially fluorescing magnetic beads (Bio-Rad) were conjugated to 2.5 μg recombinant human GM-CSF, IFN-α, IFN-γ, IL-12p70, IL-17A, or IL-22 (R&D Systems). Beads were combined and incubated for 1 h with subject or control plasma at 1:100 dilution, washed and incubated with biotinylated mouse anti-human total IgG, as well as IgG subclasses, and IgA, IgM, and IgE (Sigma). Beads were washed again and incubated with Streptavidin-PE (Bio-Rad) before being run in a multiplex assay on the Bio-Plex (Bio-Rad) instrument. Fluorescence intensity for each bead type was plotted as a function of Ab titer (GraphPad Prism, version 5.0c). Stored cerebrospinal fluid from patients identified as having anti-GM-CSF autoantibodies was examined in the same manner using neat fluid.

**Plasma isolation, cell culture, and stimulation**

Plasma from each subject was isolated from whole blood and stored at −80°C until testing. For current patients, PBMCs were obtained from the remaining cell pellets by density-gradient centrifugation, as described previously (33), and cultured at 10^5 cells/ml in complete medium consisting of RPMI 1640 (Life Technologies BRL), 2 mM glutamine, 20 mM HEPEs, and 0.01 mg/ml penicillin/streptomycin with 10% plasma from patients or from healthy blood bank donors.

**Detection of p-STAT5 by flow cytometry**

To demonstrate that patient cells had intact GM-CSF signaling when washed free of autologous plasma, PBMCs (5 × 10^5 cells) from patients were cultured in complete RPMI media containing control or patient plasma (10%) and were left unstimulated or stimulated with GM-CSF (10 ng/ml) or IL-3 (10 ng/ml) (both from R&D Systems) for 30 min at 37°C. To demonstrate that patient plasma blocked GM-CSF signaling, PBMCs (5 × 10^5 cells) from healthy controls were cultured in complete RPMI 1640 media containing control or patient plasma (10%) and left unstimulated or stimulated with GM-CSF (10 ng/ml) or IL-3 (10 ng/ml) (both from R&D Systems) for 30 min at 37°C. Monocytes were identified by CD14 (BD Pharmingen) surface staining before being fixed and permeabilized for intracellular staining with p-STAT5 (Y694) Ab (BD Pharmingen), as described previously (22). Data were collected using a FACSCalibur (BD Biosciences), analyzed using FlowJo software (TreeStar), and graphed with Prism5 (GraphPad).

**Plasma inhibition of GM-CSF–induced MIP-1α**

To evaluate cellular immune function and plasma inhibition of GM-CSF activity, normal PBMCs in normal plasma or subject plasma (10%) were left unstimulated or stimulated overnight with GM-CSF (10 ng/ml; R&D Systems) for 30 min at 37°C. Supernatants were collected and stored at −20°C until measurement of MIP-1α levels using the Bio-Plex cytokine assay (Bio-Rad), per the manufacturer’s instructions.

**Statistics**

To evaluate differences between healthy controls and patients, a two-tailed unpaired t test was applied using Prism 5 (GraphPad).

**Results**

**Case reports**

**Patient 1.** An otherwise healthy HIV-negative 20-y-old woman from California presented in June 2008 after experiencing a few days of fever, debilitating headache, neck pain, double vision, vomiting, and confusion (Table I). Lumbar puncture found an opening pressure of 250 mm H2O, 17 WBCs/mm3, lymphocyte predominant, glucose of 51 mg/dl, and protein of 33 mg/dl. The cerebrospinal fluid cryptococcal Ag was positive at 1:256, and culture grew Cryptococcus neoformans. Brain magnetic resonance imaging (MRI) showed three hyperintense gadolinium-enhancing lesions in the bilateral occipital lobes and the right parietal temporal lobe (Fig. 1A). A left lower lobe mass was confirmed to be a cryptococcoma by needle aspiration. She received liposomal amphotericin B, flucytosine, and later fluconazole, with resolution of her meningitis. Lymphocyte phenotyping was normal, with the exception of a low NK cell number (Table II).

Chest computerized tomography (CT) 2 y later showed resolution of the cryptococcoma but new scattered, patchy, ground-glass opacities in both lungs (Fig. 1B). She denied pulmonary symptoms, despite regularly dancing and singing. Bronchoalveolar lavage with transbronchial biopsy was consistent with PAP (Fig. 1C). Because she was asymptomatic, no intervention was performed. About 1 y later, exertional dyspnea and inspiratory chest pain prompted repeat chest CT, which showed worsened bilateral ground-glass opacities associated with a decrease in total lung capacity from 4.13 l (85% predicted) to 3.77 l (77%) but no significant change in diffusing capacity (14.4 ml/mm Hg/min; 46%) or exertional oxyhemoglobin desaturation. Bilateral whole-lung lavage led to improvement in symptoms.

**Patient 2.** An otherwise healthy 31-y-old HIV-negative woman from southern California presented in November 2010 with a severe headache. Lumbar puncture showed opening pressure of 400 mm H2O, 219 WBCs/mm3, with lymphocytic predominance, glucose of 47 mg/dl, and protein of 91 mg/dl (Table I). Cerebrospinal fluid...
cryptococcal Ag was positive at 1:8, and culture grew *Cryptococcus gattii*. Brain MRI was normal. Chest CT scan showed a sharply circumscribed solid mass in the left lung apex that, on biopsy, contained yeast consistent with *Cryptococcus*. She received i.v. liposomal amphotericin B and flucytosine for 8 d and then fluconazole plus flucytosine. Diarrhea led to discontinuation of flucytosine. Five months later she remained on fluconazole and felt well; her lung mass improved, and she resumed working full-time.

**Patient 3.** A previously healthy HIV-negative 48-y-old Thai man experienced 2 mo of severe back pain, chronic cough, and low-grade fever. Chest CT showed a broad-based mass in the posterior right thorax invading the right seventh rib, with fibronodular infiltration of the left upper lung (Fig. 1D). CT-guided aspiration demonstrated numerous mucoid encapsulated yeasts consistent with *Cryptococcus*; serum cryptococcal Ag was 1:1024. Lumbar puncture had a leukocyte count of 98 WBCs/mm³, with 96% lymphocytes, a glucose level of 36 mg/dl, a protein level of 85 mg/dl, and a positive cryptococcal Ag and *C. neoformans* culture. The patient received 2 wk of amphotericin B deoxycholate, which was changed to fluconazole because of nephrotoxicity. Eleven months later, the patient complained of a low-grade fever, productive cough, and 3-kg weight loss. New consolidations were seen in the right upper lung and medial segment of the right middle lung, as well as with cavitation in the left lower lung. Bronchoscopy identified *Mycobacterium tuberculosis*. Standard antituberculosis treatment was promptly initiated and continued for 9 mo. The patient was well off all antimicrobial therapy after 1 y.

**Patient 4.** A previously healthy HIV-negative 47-y-old Mexican man living in California since age 12 presented in 2008 with persistent cough prompting chest x-ray. A large perihilar mass led to bronchoscopic biopsy that showed *C. neoformans*. His serum cryptococcal Ag was 1:512, and fluconazole was initiated. Approximately 1 wk later, he complained of weakness and tremors. Lumbar puncture showed cryptococcal Ag positive at 1:16, with

### Table II. Baseline immunophenotyping

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Mean ± SEM</th>
<th>Normal Range</th>
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<tr>
<td><strong>CD4/CD3</strong> (cells/μl)</td>
<td>1399</td>
<td>950</td>
<td>817</td>
<td>773</td>
<td>985 ± 143</td>
<td>362–1275</td>
</tr>
<tr>
<td><strong>CD8/CD3</strong> (cells/μl)</td>
<td>490</td>
<td>305</td>
<td>340</td>
<td>1076</td>
<td>553 ± 179</td>
<td>344–911</td>
</tr>
<tr>
<td><strong>CD20</strong> (cells/μl)</td>
<td>246</td>
<td>163</td>
<td>365</td>
<td>251</td>
<td>256 ± 41</td>
<td>49–424</td>
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<tr>
<td><strong>CD16+ or CD56+CD3-</strong> (cells/μl)</td>
<td>56</td>
<td>105</td>
<td>157</td>
<td>86</td>
<td>101 ± 21</td>
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<tr>
<td><strong>CD16+ or CD56+CD3+</strong> (cells/μl)</td>
<td>86</td>
<td>42</td>
<td>19</td>
<td>145</td>
<td>73 ± 28</td>
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<tr>
<td><strong>IgG (mg/dl)</strong></td>
<td>843</td>
<td>901</td>
<td>1490</td>
<td>1350</td>
<td>1146 ± 161</td>
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<td><strong>IgM (mg/dl)</strong></td>
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<td>64</td>
<td>120</td>
<td>106</td>
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<tr>
<td><strong>IgA (mg/dl)</strong></td>
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<td>154</td>
<td>175</td>
<td>286</td>
<td>196 ± 30</td>
<td>34–342</td>
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<tr>
<td><strong>IgE (IU/ml)</strong></td>
<td>86</td>
<td>16</td>
<td>ND</td>
<td>49</td>
<td>50 ± 20</td>
<td>0–90</td>
</tr>
</tbody>
</table>

ND. Not determined.

**FIGURE 2.** Anti-cytokine autoantibody evaluation. (A) Anti–GM-CSF autoantibodies in controls, both healthy (*n = 64*) and diseased (*n = 43*); patients with known acquired PAP; and patients with cryptococcal meningitis (CM), with and without known immunocompromise (IC). (B) Multiplex screen for anti-cytokine autoantibodies against GM-CSF, IFN-α, IFN-γ, IL-12p70, IL-17A, and IL-22 in anti–GM-CSF autoantibody positive (aab+) cryptococcal meningitis (CM) patients and 10 normal controls. Evaluation of anti–GM-CSF autoantibody–positive cryptococcal meningitis patients for other anti–GM-CSF Ig isotypes (C) and anti–GM-CSF IgG subclasses (D). (E) Anti–GM-CSF autoantibodies in cerebrospinal fluid.
WBCs/mm³, lymphocyte predominant, glucose of 36 mg/dl, and protein of 75 mg/dl. Amphotericin B was given for 14 d but was changed to fluconazole because of renal toxicity. The patient was maintained on fluconazole and had no evidence of recurrence of infection; however, a chest CT scan performed in April 2012 demonstrated a new small region with ground-glass opacification. A repeat chest CT in July 2012 demonstrated expansion of this infiltrate, with multiple new similar regions identified bilaterally; bronchoalveolar lavage revealed periodic acid-Schiff (PAS)–positive material on cytology, consistent with a diagnosis of PAP. Because the patient remains asymptomatic with normal pulmonary function testing, clinical monitoring will be continued without therapeutic intervention at this time.

**Archived samples**

Plasma samples, and cerebrospinal fluid when available, were obtained for 103 patients with microbiologically proven cryptococcal meningitis collected between 1958 and 1982. Clinical histories were reviewed, and patients were categorized as those without evidence of immunodeficiency (n = 67) and those with either a history of iatrogenic immunosuppression or an underlying medical condition, including diabetes mellitus or hematological malignancy, prior to their diagnosis of cryptococcal meningitis (n = 36).

**Anti-cytokine autoantibody detection**

Plasma from controls (n = 107), patients with known PAP (n = 3), the four patients described above with cryptococcal meningitis, and the archived samples from patients with cryptococcal meningitis (n = 103) were tested for anti–GM-CSF autoantibodies at 1:100 plasma dilution. Patients with PAP and the four current patients with cryptococcal meningitis were positive for anti–GM-CSF autoantibodies (Fig. 2A). Three of 67 archived patient samples (Patients 5–7, Table I) without recognized immunodeficiency were positive for anti–GM-CSF autoantibodies compared with none of the healthy controls (n = 64), diseased controls (n = 43), or patients with recognized immunocompromise (n = 36). Anti-cytokine autoantibodies against IFN-α, IFN-γ, IL-12p70, IL-17A, and IL-22 were absent in those patients with GM-CSF autoantibodies (Fig. 2B). The anti–GM-CSF autoantibodies were all IgG isotype (Fig. 2C) and were predominantly IgG1 subclass (Fig. 2D). Anti–GM-CSF autoantibodies were also identified at lower titers in cerebrospinal fluid in four patients (Patients 1, 4, 6, and 7) for whom cerebrospinal fluid was available (Fig. 2E).

**Anti–GM-CSF IgG inhibits GM-CSF–induced p-STAT5**

To demonstrate biological activity of plasma containing anti–GM-CSF autoantibodies, normal PBMCs were left unstimulated or stimulated with GM-CSF or IL-3 in the presence of patient or normal plasma (10%) to evaluate p-STAT5 production. Only plasma containing anti–GM-CSF autoantibodies prevented GM-CSF–induced p-STAT5, whereas signaling induced by IL-3, which shares the β unit of the GM-CSFR and also causes STAT5 phosphorylation, was not affected (Fig. 3A) (p < 0.0001). However, in the absence of autologous plasma, PBMCs from Patients 1 and 2 were normally responsive to both GM-CSF and IL-3 (Fig. 3B), indicating that their defect was humoral and not cell intrinsic.

To confirm that the IgG fraction contained the inhibitory factor, normal PBMCs were stimulated with GM-CSF in the presence of the purified IgG or the other plasma fractions. Only the IgG fraction...
in all seven patients, but not the remaining plasma fractions, prevented GM-CSF–induced STAT5 phosphorylation, as shown for Patients 1, 2, and 6 (Fig. 3C). To further demonstrate the specificity of GM-CSF autoantibodies in patient plasma, we generated dose-response curves by stimulating normal PBMCs in patient or normal plasmas (10%) with increasing amounts of GM-CSF and evaluating p-STAT5 production (Fig. 4A). Anti–GM-CSF autoantibody–containing plasma required a 2–3 log higher concentration of GM-CSF to obtain 50% of maximum p-STAT5 production compared with normal plasma (Fig. 4B) ($p = 0.0015$).

Anti–GM-CSF autoantibody–containing plasma inhibits GM-CSF–induced MIP-1α

To further evaluate the downstream effects of anti–GM-CSF autoantibodies, plasma was tested for its ability to prevent GM-CSF–induced MIP-1α production from normal PBMCs. Normal and patient PBMCs washed of autologous plasma demonstrated that GM-CSF induced a 34- and 22-fold increase in MIP-1α production, respectively ($p = \text{NS}$), whereas normal PBMCs in the presence of anti–GM-CSF autoantibody–containing plasma demonstrated no increase in MIP-1α from baseline ($p < 0.0001$) (Fig. 5).

Discussion

Cryptococcal meningitis is typically seen in immunocompromised hosts, but it also occurs in those for whom no underlying immune defect has been found (25–31). In this article, we describe an association between biologically inhibitory anti–GM-CSF autoantibodies and cryptococcal meningitis in seven otherwise healthy HIV-negative individuals. None of these patients carried the diagnosis of PAP at presentation of meningitis, and only one patient developed symptomatic PAP in the following years; a second patient had radiographic and cytopathologic changes without symptoms. Review of the medical records related to archived patient samples found no reports of pulmonary disease consistent with PAP. To our knowledge, Cryptococcus has been described in four patients with active PAP, including two with meningitis (4, 9, 17, 34), but all of these reports antedated the recognition of anti–GM-CSF autoantibodies as a cause of PAP.

Anti-cytokine autoantibodies, including those against GM-CSF, have been reported in normal hosts (18, 35, 36). Therefore, it is possible that there are asymptomatic people in the general population who have anti–GM-CSF autoantibodies that could lead to cryptococcal infection following sufficient exposure. Alternatively, GM-CSF is induced by cryptococcal infection, and anti–GM-CSF autoantibodies might develop as a reactive phenomenon. The absence of high-titer anti–GM-CSF autoantibodies in both healthy and diseased controls ($n = 107$, Fig. 2A), as well as in >180 previously reported controls (healthy volunteers and patients with tuberculosis) (1, 37, 38), is consistent with a direct relationship between cryptococcal disease and anti–GM-CSF autoantibodies in a subset of patients.
Anti–GM-CSF autoantibodies in the context of PAP have broad immunological effects on monocytes, macrophages, and neutrophils (12–15), which appear to be important for host control of Cryptococcus. Studies in mice and in humans with HIV showed that GM-CSF therapy may augment the effects of triazole therapy and increase cryptococcal killing by monocytes (39, 40), suggesting a physiologic role for GM-CSF in host control of Cryptococcus. Additionally, Cryptococcus may diminish GM-CSF production by NK cells and T lymphocytes, thereby decreasing phagocyte and macrophage activity (41, 42). The IgG fraction of plasma from all seven patients with anti–GM-CSF autoantibodies inhibited GM-CSF–induced STAT5 phosphorylation (Fig. 3) and eliminated GM-CSF–induced MIP-1α protein expression (Fig. 5), proving that these autoantibodies are biologically active. GM-CSF plays a critical role in alveolar macrophage function and activation and pulmonary host defense, as clearly manifest by PAP and its complications. We hypothesize that GM-CSF–mediated macrophage function against Cryptococcus is important and that autoantibodies that block its function are etiologic and preceding the infection, causing cryptococcal susceptibility. As is the case with many autoantibody-mediated diseases, such as anti–IFN-γ autoantibodies and adult-onset immunodeficiency, the trigger for the culprit autoantibody is unknown.

Complex factors, such as titer, epitope, IgG subclass, and Ab avidity, may contribute to disease severity and phenotype (i.e., PAP, cryptococcal meningitis, or both). Indeed, these factors were shown to contribute to disease activity in other autoantibody-mediated syndromes (43–45). Furthermore, susceptibility to Cryptococcus may only be identified in the case of sufficient exposure to this environmentally restricted organism.

In those for whom cerebrospinal fluid was available, our patients with anti–GM-CSF autoantibodies in serum also had detectable levels in cerebrospinal fluid, albeit at lower levels (Fig. 2E). It is unclear whether these Abs play a pathogenic role in permitting cryptococcal entry to the CNS. GM-CSF does not seem to influence CNS microglial killing of Cryptococcus, implicating other host defense mechanisms (46). For example, the lung may be an important portal of entry where defective alveolar macrophage (47, 48) activity allows for dissemination and subsequent CNS penetration.

Two patients from our archived samples had low positive anti–GM-CSF autoantibody titers (Fig. 2A) that were not inhibitory in vitro (data not shown). Given that these samples had been stored for >40 y, it is possible that their binding was nonspecific or, alternatively, that they had lost activity over the time they were in storage. Additionally, these patients had alternative explanations for their infection susceptibilities: one had end-stage cirrhosis, and the other had diabetes mellitus and systemic lupus erythematosus for which he was receiving chronic steroid therapy. Conversely, Patient 4 had low anti–GM-CSF autoantibody titers, but they were fully inhibitory. The plasma sample for this patient was collected 4 y after his initial meningitis diagnosis and in the setting of inactive disease, raising the possibility that his titers may have declined over time. Decreasing titers in concordance with clinical improvement have been demonstrated in other anticytokine autoantibody syndromes (44, 49).

Consistent with previous reports (50), all seven patients with anti–GM-CSF autoantibodies and the three with PAP had IgG1 subclass Abs. Conversely, in other autoantibody syndromes, such as anti–IFN-γ autoantibodies and pemphigus vulgaris (anti-desmoglein autoantibodies), the predominating subclasses were either IgG3 or IgG4 (37, 51). The influence of infection, cytokine, age, and gender on pathologic IgG autoantibody subclass and disease pathogenesis remains to be dissected.

All four contemporary patients with cryptococcal meningitis and anti–GM-CSF autoantibodies responded well to standard antifungal therapy. One patient had symptomatic PAP, which was treated with whole-lung lavage alone, and one patient has radiographic and cytopathologic evidence of PAP without clinical manifestations. Somewhat surprisingly, and in contrast to the experience with anti–IFN-γ autoantibody-associated disseminated mycobacterial disease (32), the patients with cryptococcal meningitis have remained well after successful therapy. Subcutaneous and inhaled GM-CSF showed activity in other PAP patients, and anti–CD20 therapy (rituximab) demonstrated encouraging results for refractory acquired PAP (49, 52), as well as against anti–IFN-γ autoantibodies (44).

Further prospective work must be done to definitively establish the relationship between anti–GM-CSF autoantibodies in otherwise immunocompetent patients and the development of cryptococcal meningitis. Finding anti–GM-CSF autoantibodies should prompt consideration of PAP, which may develop after meningitis. Together, these data implicate GM-CSF as a critical actor in the host defense against Cryptococcus and support a direct role for anti–GM-CSF autoantibodies in some cases of cryptococcal infection.

Disclosures
The authors have no financial conflicts of interest.

References
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