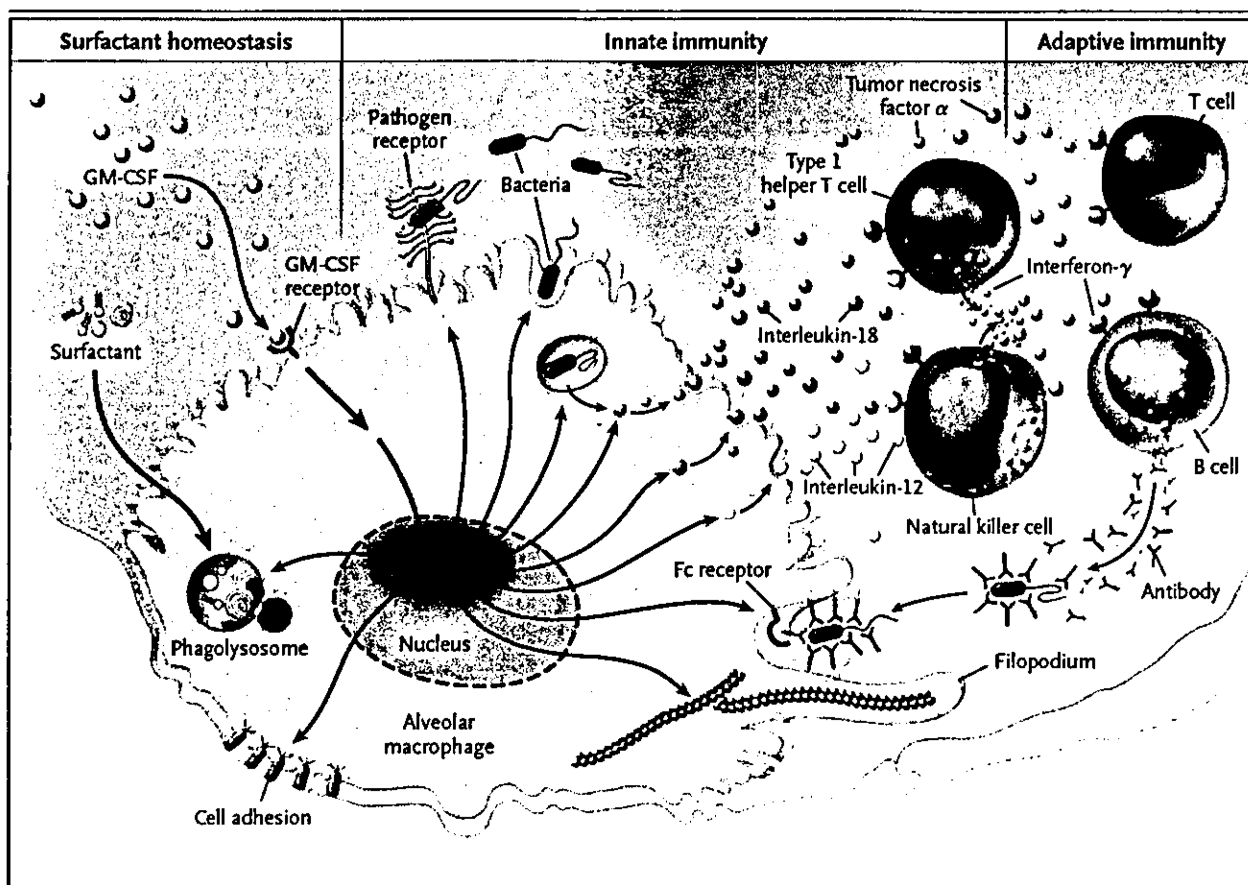

Figure 4. Role of Granulocyte–Macrophage Colony-Stimulating Factor (GM-CSF) in Modulating the Function of Alveolar Macrophages in Mice.

In vivo, pulmonary GM-CSF stimulates an increase in the level of PU.1, a transcription factor, in alveolar macrophages in the lung. In vitro, alveolar macrophages from knockout mice without the *GM-CSF* gene have a number of functional defects, including defects in cellular adhesion, catabolism of surfactant proteins and surfactant lipids, expression of pathogen-associated molecular pattern receptors (e.g., toll-like receptors and the mannose receptor), toll-like–receptor signaling, phagocytosis of pathogens (bacteria, fungi, and viruses), intracellular killing of bacteria (independent of uptake), pathogen-stimulated secretion of cytokines (tumor necrosis factor α, interleukin-12, and interleukin-18), and Fc receptor–mediated phagocytosis. Cytoskeletal organization is abnormal and may in part account for defects in phagocytosis. The inability of alveolar macrophages to release interleukin-12 and interleukin-18 severely impairs the interferon-γ response to pulmonary infection, thus impairing an important molecular connection between innate and adaptive immunity in the lung. Retroviral-vector–mediated, constitutive expression of PU.1 in alveolar macrophages from $GM^{-/-}$ mice corrects all these defects, suggesting that GM-CSF stimulates terminal differentiation of the macrophages through the global transcription factor PU.1. The blue arrows represent the functions of PU.1 that are affected by the absence of GM-CSF.

cytes, suggesting the involvement of a circulating inhibitor in the pathogenesis of the disease.[99]

Role of GM-CSF

The demonstration that GM-CSF deficiency caused pulmonary alveolar proteinosis in mice prompted a reevaluation of the pathogenesis of the acquired form of the disease in humans. A clue came from a report that the systemic administration of recombinant human GM-CSF had produced radiographic, physiological, and symptomatic improvement in one affected patient.[100] Similar treatment of additional patients (discussed below) failed to produce the expected neutrophilia[100] — a curious finding that was confirmed in subsequent studies.[30,101] Attempts to identify mutations in the genes encoding GM-CSF and its receptor in acquired pulmonary alveolar proteinosis have been unsuccessful to date,[102] in contrast to findings in the congenital form of the disease.[15] Furthermore, the levels of GM-CSF in

bronchoalveolar-lavage fluid and plasma are actually elevated in the acquired form, thus ruling out the possibility that the disease is due to the absence of GM-CSF itself.[103]

Autoantibodies against GM-CSF

An immunologic explanation for these observations was revealed by a reexamination of the inhibitory factor in pulmonary alveolar proteinosis. Bronchoalveolar-lavage fluid from subjects with the disease, but not that from control subjects, inhibited the ability of GM-CSF to stimulate the proliferation of normal monocytes and a GM-CSF–dependent cell line and competitively inhibited the binding of GM-CSF to cells bearing GM-CSF receptors.[104] This inhibitory activity was due to a neutralizing IgG antibody against GM-CSF.[105] The antibody was present in bronchoalveolar-lavage fluid and serum from all patients with acquired pulmonary alveolar proteinosis but not those with the congenital or secondary form of the disorder, those with several other lung disorders, or normal controls (Fig. 5).[105,106] The specific association between neutralizing anti–GM-CSF autoantibodies and acquired pulmonary alveolar proteinosis[38,105,106] strongly supports the view that in this disorder, a neutralizing autoantibody against GM-CSF causes defects in the functioning of alveolar macrophages, including impairment of the catabolism of surfactant lipids and proteins and disruption of surfactant homeostasis. Further strong support for this concept comes from the recent demonstration that the presence of these antibodies is correlated with the elimination of GM-CSF bioactivity in the lungs of patients with pulmonary alveolar proteinosis.[107] The finding of this autoantibody has led to the development of a latex-agglutination test with high sensitivity (100 percent) and specificity (98 percent) for diagnosing the acquired disease.[38]

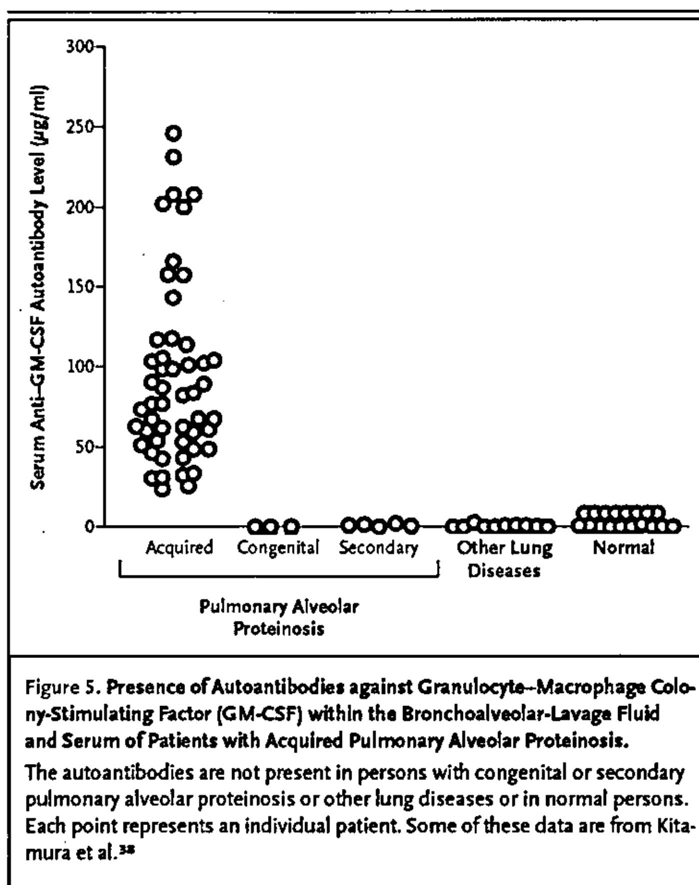

Figure 5. **Presence of Autoantibodies against Granulocyte–Macrophage Colony-Stimulating Factor (GM-CSF) within the Bronchoalveolar-Lavage Fluid and Serum of Patients with Acquired Pulmonary Alveolar Proteinosis.**
The autoantibodies are not present in persons with congenital or secondary pulmonary alveolar proteinosis or other lung diseases or in normal persons. Each point represents an individual patient. Some of these data are from Kitamura et al.[38]

PULMONARY CYTOKINES

Similarities between pulmonary alveolar proteinosis in mice and the acquired form of the disease in humans also include abnormalities of pulmonary cytokines. For example, the level of macrophage colony-stimulating factor, which is elevated in the lungs of $GM^{-/-}$ mice,[84] is also elevated in the lungs of humans with acquired pulmonary alveolar proteinosis.[106] Similarly, the level of monocyte chemotactic protein 1 is elevated in the lungs of both $GM^{-/-}$ mice and humans with the acquired disease.[35,83] The mechanism of these cytokine changes is not known, but the latter may explain the increased numbers of lymphocytes in the lungs of mice[60] and patients with pulmonary alveolar proteinosis.[35]

THERAPEUTIC APPROACHES

CURRENT APPROACHES

The treatment of pulmonary alveolar proteinosis depends on the underlying cause. Current therapy for the congenital form of the disorder is supportive,[7] although successful lung transplantation has been reported.[108] Therapy for secondary pulmonary alveolar proteinosis generally involves treatment of the underlying condition; for example, when the disorder is associated with a hematologic cancer, successful chemotherapy or bone marrow transplantation corrects the associated pulmonary disorder.[12]

Acquired pulmonary alveolar proteinosis has been treated successfully since the early 1960s by whole-lung lavage, and this procedure remains the standard of care today.[109-113] Although it has not been tested in prospective, randomized trials,

whole-lung lavage improves clinical, physiological, and radiographic findings. A retrospective analysis of 231 cases found clinically significant improvement in arterial oxygen tension and in measures of pulmonary function (forced expiratory volume in one second, vital capacity, and diffusing capacity for carbon monoxide).[19] Such therapy also improves survival: in a group of 146 patients, the mean (±SD) rate of survival at five years was 94±2 percent with lavage, as compared with 85±5 percent without lavage (P=0.04).[19] The median duration of clinical benefit from lavage has been reported to be 15 months.[19] Interestingly, therapeutic whole-lung lavage improves defects in the migration[114] and phagocytosis[115] of alveolar macrophages. Successful treatment of pulmonary alveolar proteinosis by lobar lavage through fiberoptic bronchoscopy has also been reported, although the practical clinical utility of this approach is unclear.[116]

GM-CSF THERAPY

Several prospective phase 2 trials of GM-CSF therapy for acquired pulmonary alveolar proteinosis have been undertaken. The first, conducted from 1995 through 1998, evaluated the effectiveness of subcutaneous GM-CSF (at a dose of 5 μg per kilogram of body weight per day) for 6 to 12 weeks in 14 patients.[30] Five patients had a response to this dose, with a mean improvement in the alveolar–arteriolar diffusion gradient of 23.2 mm Hg; four of the patients who did not have a response then received 20 μg per kilogram per day and had a response to that dose. The remaining five patients did not have a response at the higher dose. An ongoing study, initiated in 1998, reported a response in three of four initial patients who received daily subcutaneous injections of GM-CSF in escalating doses over a period of 12 weeks.[101,117] These three patients had symptomatic, physiological, and radiographic improvement as well as a reduction in the mean alveolar–arteriolar diffusion gradient from 48.3 mm Hg at base line to 18.3 mm Hg after 16 weeks of treatment. These initial results are encouraging, but the mechanism of the effect of GM-CSF treatment is unclear. The observation of a reduction in pulmonary levels of anti–GM-CSF antibody in association with clinical improvement suggests that desensitization to GM-CSF may be involved.[36]

CONCLUSIONS

Clinical investigations, research in transgenic mice, and translation of findings from the bench to the bedside have considerably changed our concepts of the pathogenesis and treatment of pulmonary alveolar proteinosis. In addition to illuminating the mechanism of this disorder, research has revealed critical roles for GM-CSF in the regulation of mature alveolar macrophages in the lung, the regulation of surfactant homeostasis, and the stimulation of multiple mechanisms that protect the lung against microbial invasion.

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Granulocyte-Macrophage Colony–Stimulating Factor and Lung Immunity in Pulmonary Alveolar Proteinosis

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The anti–granulocyte-macrophage colony–stimulating factor (GM-CSF) autoantibody is inferred to cause idiopathic pulmonary alveolar proteinosis (iPAP): the antibody neutralizes GM-CSF and thereby impairs differentiation of alveolar macrophages. Administration of GM-CSF improves respiratory function of patients with iPAP, as confirmed in this study using aerosolized GM-CSF. To elucidate its mechanism, we characterized bronchoalveolar lavage fluid and alveolar macrophages obtained from three patients with iPAP who were treated successfully with aerosolized GM-CSF. Cell number, expressions of surface mannose receptor and the transcription factor PU.1, and phagocytic ability of alveolar macrophages were all restored to control levels. With treatment, the neutralizing capacity of GM-CSF activity was reduced markedly, concomitant with the decreasing autoantibody levels. Interestingly, the amount of GM-CSF autoantibody complex also decreased. In one case in which the complex was analyzed, the majority of GM-CSF binding the complex was endogenous protein, suggesting that the complex is removed immediately from the lung after treatment. Our study shows that GM-CSF administration engenders a decrease in the neutralizing capacity against the protein in the lungs. Thereby, it facilitates restoration of the normal function of alveolar macrophages.

Keywords: anti-GM-CSF antibody; bronchoalveolar fluid; GM-CSF; pulmonary alveolar proteinosis

Pulmonary alveolar proteinosis (PAP) is an uncommon lung disease characterized by an accumulation of surfactant that fills terminal airways and alveoli, thereby impairing gas exchange and engendering respiratory insufficiency (1–3). Three clinically and etiologically distinct forms of PAP are acknowledged (congenital, secondary, and idiopathic), but more than 90% of cases are idiopathic (iPAP). In iPAP, respiratory symptoms initiate insidiously, with no precipitating event or illness. Alveolar macrophages from patients with iPAP show impaired chemotactic activity, reduced adhesion to glass, and poor phagocytosis (4). Dysfunction that impairs surfactant clearance of alveolar macrophages is considered responsible for iPAP (2–4).

A serendipitous observation first suggested that abnormalities of GM-CSF signaling may be involved pathogenically in iPAP: mice lacking the hematopoietic growth factor granulocyte-macrophage colony–stimulating factor (GM-CSF) or its receptor develop histologic changes similar to those seen in PAP (5–7). In mice, GM-CSF regulates the terminal differentiation of alveolar macrophages. It is necessary for normal catabolism of surfactant lipids and proteins (8). Genetic abnormalities of GM-CSF or its receptor were reported in a small number of patients with congenital PAP (9), but were not found in iPAP (2). Instead, all patients with iPAP evaluated so far have high titers of neutralizing anti–GM-CSF autoantibody. No more than trace amounts of the antibody were detected in patients with congenital or secondary PAP, other lung diseases, or healthy volunteers (10, 11). Taken together, loss of GM-CSF activity caused by the autoantibody cripples normal functions of alveolar macrophages, thereby reducing surfactant clearance.

Recombinant human GM-CSF is used clinically to stimulate bone marrow recovery in neutropenic patients and after bone marrow transplantation. Several investigators have administered GM-CSF subcutaneously to patients with PAP and have observed varied responses (12–15). A single case report describes a patient who was treated successfully using aerosolized GM-CSF (16). A recent study demonstrated that extrinsic GM-CSF administration restored expression of a transcriptional factor, PU.1, in alveolar macrophages, and thereby improved the maturation of alveolar macrophages in patients with PAP (17, 18). Considering the preexisting autoantibody, which binds GM-CSF with high avidity and specificity (19), it is unlikely that administered GM-CSF can directly stimulate immature alveolar macrophages by binding their GM-CSF receptors.

To investigate mechanisms of action of administered GM-CSF, we observed changes in the function of alveolar macrophages, together with changes in the neutralizing activity against GM-CSF and the amount of autoantibody in bronchoalveolar lavage fluid (BALF) of three patients treated with aerosolized GM-CSF. Results suggested that inhaled GM-CSF reduced the neutralizing capacity of BALF against GM-CSF with decreased concentration of both free autoantibody and the immune complex. Consequently, inhaled extrinsic GM-CSF might condition the alveolar microenvironment in the lung, allowing alveolar macrophages' functional recovery and clearance of proteinaceous materials. Some of the results of this study have been reported previously in the form of an abstract (20).

METHODS

See the online supplement for further details on the methods.

Patients and GM-CSF Administration

The institutional review board approved this study. It was conducted after obtaining written, informed consent from each participant between

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December 2000 and October 2002. We treated a series of three individuals with iPAP (one man and two women; age range, 51–57 years) with aerosolized GM-CSF (Table 1). BAL, transbronchial lung biopsy, and anti–GM-CSF antibody in the serum confirmed the iPAP diagnosis. The patients were administered recombinant GM-CSF (125 μg in 2 ml normal saline; Leucomax; Novartis AG, Switzerland) by aerosol (LC Plus jet nebulizer; PARI Respiratory Equipment, Inc.) twice daily, during alternate weeks for 24 weeks. This schedule of treatment was based on a report by Anderson and coworkers (21). They administered aerosol GM-CSF to seven patients with metastatic lung tumors and found low toxicity. Improvement was defined as 10 mm Hg or greater decrease in the alveolar–arterial oxygen gradient (A-aDO_2).

[2] [3]

BAL Procedures

Three 50-ml aliquots of normal saline were instilled and suctioned sequentially from the right middle lobe under bronchoscopy and processed immediately. Cells were stained by modified Giemsa; 400 nucleated cells were counted differentially in cytocentrifuge preparations. Then 200 alveolar macrophages were measured for lengthwise diameter and classified into the following two morphologic groups based on Iyonaga and colleagues (22): (*1*) nonfoamy, monocyte-like cells and (*2*) foamy cells.

Electron Micrograph of Alveolar Macrophages

BALF was incubated in a plastic culture dish at 37°C for 1 hour. After removing nonadherent cells by gentle washing, adherent cells (alveolar macrophages) were fixed and processed for Epon-embedding sections to be observed with a transmission electron microscope.

[4]

Phagocytic Activity of Alveolar Macrophages

Alveolar macrophages, isolated as previously described, were suspended in RPMI/10% fetal calf serum and plated in a four-well chamber slide (LabTek Chamber). After placing at 37°C for 2 hours, cells were incubated with 0.5% PE-labeled latex beads (Sigma-Aldrich Corp., MO) for 30 minutes and fixed in 4% paraformaldehyde at 4°C for 15 minutes. Cells were then stained with a 1:3,000 dilution of Syber green (Dojindo Laboratories, Japan). The alveolar macrophages that had phagocytosed beads were counted using a confocal laser microscope.

[5] [6] [7] [8]

Immunohistochemical Staining

Alveolar macrophages were fixed with 4% paraformaldehyde and stained with antimannose receptor antibody (Beckman Coulter, Inc.) and horseradish peroxidase-labeled antimouse IgG antibody (Nichirei Corp.) to examine expression of the mannose binding protein, a maturation marker for macrophages. We examined PU.1 expression by double immunostaining using a rabbit polyclonal anti-PU.1 antibody (Santa Cruz Biotechnology, Inc., CA), which was detected using an alkaline phosphatase-labeled antirabbit IgG (Promega Corp., WI) and a mouse anti-CD68 monoclonal antibody labeled with horseradish peroxidase. Two pathologists independently determined quantification of a ratio of macrophages expressing PU.1. They counted the number of cells stained through a binary decision. Mean values are presented.

[9] [10]

To observe localization of PU.1, alveolar macrophages were immunostained with a rabbit anti-PU.1 polyclonal antibody and PE-labeled antirabbit polyclonal antibody (DakoCytomation), counterstained with 1:3,000 dilution of Syber green; they were then examined using confocal laser microscopy.

[11]

Quantification of Anti–GM-CSF Autoantibody

Autoantibody concentrations in BALF or in serum were measured using purified autoantibody as a standard (15, 23).

Neutralizing Capacities against GM-CSF in BALF

The GM-CSF bioactivity was quantified using TF-1, a GM-CSF–dependent cell line, as described elsewhere (19).

Detection of GM-CSF in GM-CSF–Autoantibody Immune Complexes

Protein samples obtained from BALF of patients with iPAP and normal control subjects using protein-A sepharose were subjected to ELISA and Western blotting to detect GM-CSF, as described previously (19).

Statistical Analyses

Statistical analyses were performed using StatView version 4 software (SAS Institute, Inc., CA), using the Mann-Whitney's U test or Kruskal-Wallis rank sum procedures for nonparametric data. Correlation of variables was assessed using the Spearman rank correlation coefficient. We considered $p < 0.05$ to be significant.

[12]

RESULTS

Population, Morphology, and Function of Alveolar Macrophages during GM-CSF Treatment

The 24-week course of inhaled GM-CSF therapy showed improved oxygenation of arterial blood with no side effects. All three patients showed a 10 mm Hg decrease or more in A-aDO_2 after treatment (Table 1). Serum levels of surfactant protein-D, lactate dehydrogenase, and carcinoembryonic antigen were also improved (Figure 1; Figures E1 and E2 in the online supplement) (24). Case 1 recurred 20 months after the GM-CSF therapy (*see* Figure 1 and the online supplement for further details). Table 2 summarizes general characteristics of the cells in BALF. Alveolar macrophages increased after a 24-week GM-CSF inhalation ($p < 0.05$), whereas extracellular proteinaceous material and cell debris markedly decreased (Figures 2A–2C and 3A). Although the percentage of macrophages decreased in Case 3 after treatment, the absolute number of macrophages in 1 ml of BALF increased, for the substantial increase of total BAL cells (Table 1 and Figure 3A). Foamy macrophages decreased after treatment (Figure 3B). Nonfoamy alveolar macrophages, smaller than normal control ($p < 0.01$) before the treatment, were of normal

TABLE 1. PATIENT PROFILE

	Case 1	Case 2	Case 3[†]
Age and sex	51-yr female	56-yr male	57-yr female
Smoking	None	Ex-smoker	Smoker[‡]
Diagnostic procedure	BALF, TBLB, Ab	BALF, TBLB, Ab	BALF, TBLB, Ab
Prior treatment	Left lung lavage	Oxygen treatment immunosuppressants*	Oxygen treatment
A-aDO_2 decrease after GM-CSF inhalation (torr)	17	20	27

Definition of abbreviations: A-aDO_2 = alveolar–arterial oxygen gradient; Ab = serum titer of anti–GM-CSF antibody; BALF = bronchoalveolar lavage fluid; GM-CSF = granulocyte-macrophage colony–stimulating factor; TBLB = transbronchial lung biopsy.

* Case 2 had received prednisone and cyclosporine as the treatment for Wegener's granulomatosis, which was diagnosed 17 months earlier to the onset of idiopathic pulmonary alveolar proteinosis (*see* the online supplement for further details).

[†] Brief profile and the effects of GM-CSF inhalation on a mucinlike glycoprotein, KL-6, and serum anti–GM-CSF antibody of Case 3 was reported previously (24).

[‡] Case 3 had just stopped smoking when pulmonary aveolar proteinosis was diagnosed.

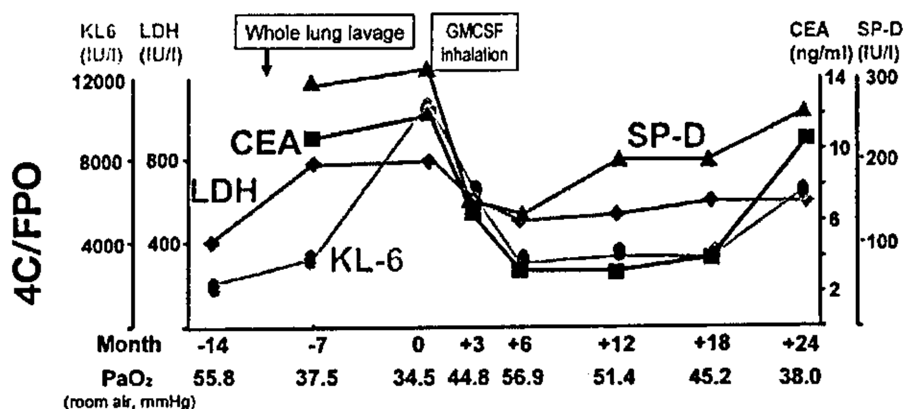


Figure 1. Clinical course of Case 1. Laboratory data for Pa_{O_2} and serum markers for idiopathic pulmonary alveolar proteinosis, including lactate dehydrogenase (LDH), mucinlike glycoprotein KL-6, surfactant protein-D (SP-D), and carcinoembryonic antigen (CEA), are presented with clinical information. GM-CSF = granulocyte-macrophage colony-stimulating factor.

size after GM-CSF treatment (Figure 3C). Alveolar macrophages after the treatment showed mature ultrastructural features with the development of microvilli and clear organelles, compared with those before treatment (Figure 2D).

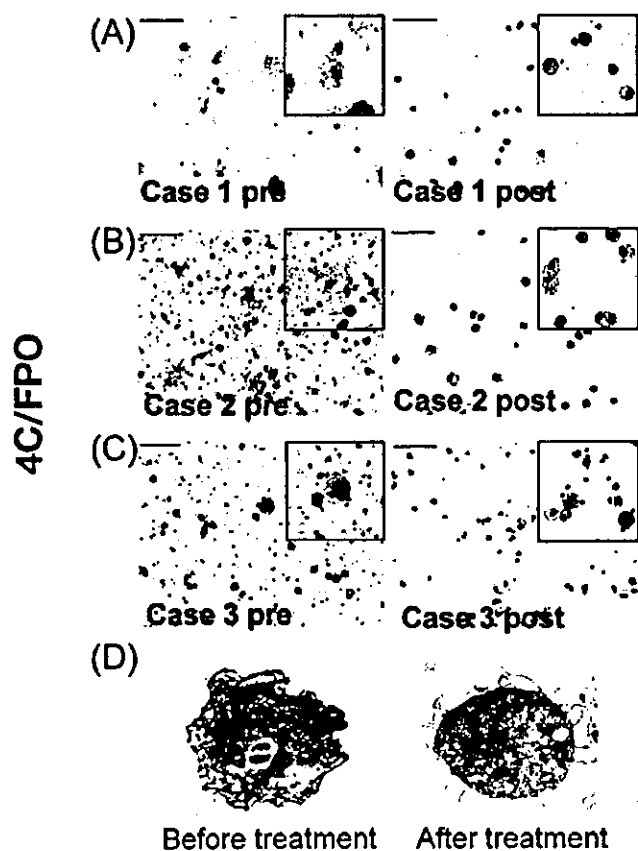


Figure 2. (*A–C*) Wright-Giemsa staining of the cells in bronchoalveolar lavage fluid before (*left*) and after (*right*) the GM-CSF treatment (×200; scale, 40 μm). Insets show higher magnifications of the cells (×400). (*D*) Electron micrographs of the alveolar macrophages of Case 1 before (*left*) and after (*right*) the GM-CSF treatment (×3,000).

We examined alveolar macrophages before and after treatment for changes in phagocytic activity and in the expression of two molecules. Phagocytic activity, as measured using the number of the cells harboring beads, was increased after the treatment (Figure 4). Expression of the mannose receptor, a crucial molecule for macrophages to phagocytose microorganisms, and expression of PU.1, a critical transcription factor regulating differentiation and maturation, were both increased after treatment (Figures 5 and 6). These results suggest that GM-CSF treatment promoted differentiation and restored the normal functions of alveolar macrophages.

Neutralizing Capacity against GM-CSF in BALF Was Reduced after Treatment with a Decreased Level of the Autoantibody

Anti–GM-CSF antibodies in patients with iPAP have a wide range of target epitopes. In addition, the crude amount of the autoantibody may not be correlated with the biological effect or a state of the disease (20). Consequently, to investigate the effect of GM-CSF inhalation on autoantibody levels in the lung, we examined BALF after treatment for the following: (*1*) neutralization capacity, which suppresses biological activities of GM-CSF using a GM-CSF–dependent cell line, and (*2*) the amount of IgG binding to GM-CSF by EIA (Table 3). In the three cases of that study, the neutralizing capacity against GM-CSF declined remarkably to normal levels after GM-CSF treatment ($p < 0.05$). Consistently, the amount of the anti–GM-CSF antibody was also markedly decreased after GM-CSF inhalation ($p < 0.05$). The serum titer of the antibody after treatment was approximately 60 to 70% of the titer before the treatment. The neutralizing capacity of GM-CSF in BALF exhibited significant correlation with serum carcinoembryonic antigen ($r = 0.886$, n = 6, p = 0.0476), serum surfactant protein-D ($r = 0.943$, n = 6, p = 0.035), and serum KL-6 ($r = 0.943$, n = 6, p = 0.035). It also showed marked correlation with the titer of anti–GM-CSF antibody in BALF ($r = 0.829$, n = 6, p = 0.0639) and P_{O_2} ($r = -0.829$, n = 6, p = 0.0639), but not with the serum titer of anti–GM-CSF antibody ($r = 0.143$, n = 6, p = 0.7494). Our results suggest that inhalation of GM-CSF restored bioactivity in the lung of patients with iPAP by reduction of neutralizing capacity against GM-CSF with a proportionate reduction in the amount of the autoantibody in BALF.

GM-CSF–Autoantibody Immune Complex Was Reduced after the Treatment

The effects of inhaled exogenous GM-CSF on reduction of both the neutralizing capacity and titer of the autoantibody suggested

TABLE 2. BRONCHOALVEOLAR LAVAGE FLUID ANALYSES BEFORE AND AFTER AEROSOLIZED GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR THERAPY

	Case 1		Case 2		Case 3	
	Before	After	Before	After	Before	After
Recovery, % of 150 ml	69	60	53	48	40	70
Cell counts, $\times 10^4$/ml	2.9	15.9	4.0	16.2	7.5	27.1
Macrophages, %	62	76	66	95	47	24
Lymphocytes, %	29	21	32	4	40	76
Neutrophils, %	9	3	2	1	12	0
Eosinophils, %	0	0	0	0	0	0
CD4/8 ratio	2.2	2.2	3.2	1.9	1.7	5.0

that exogenous GM-CSF bound to the free autoantibody and thereby reduced the free autoantibody detected by both ELISA and bioassay. If that occurs, GM-CSF bound to the autoantibody in BALF may increase after treatment. To elucidate this, the concentration of GM-CSF bound or unbound to the autoantibody in BALF was measured and compared before and after treatment. Unexpectedly, concentrations of GM-CSF bound to the autoantibody were reduced consistently to a level below the range of detection after treatment (Table 4). On the other hand, concentrations of GM-CSF that was unbound to the autoantibody in BALF were at levels below the range of detection in any cases before or after treatment, suggesting that GM-CSF in BALF was trapped completely by the autoantibody in the lung of patients with iPAP. To investigate GM-CSF bound to the autoantibody, we performed immunoblotting assay of GM-CSF stripped from the immune complexes in the BALF of Case 1. The assay demonstrated a band of 23 kD corresponding to intrinsic GM-CSF, which was larger than extrinsic GM-CSF of 14.5 kD. Furthermore, the band of extrinsic GM-CSF was not detected in BALF.

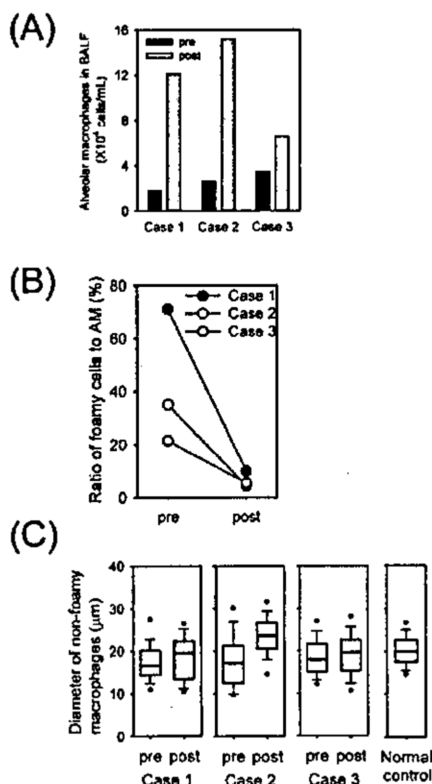


Figure 3. (*A*) Cell counts of alveolar macrophages in bronchoalveolar lavage fluide (BALF) of the patients with pulmonary alveolar proteinosis (PAP) before (*black bars*) and after (*gray bars*) the 24-week GM-CSF inhalation. (*B*) The ratio of foamy macrophages to the total number of macrophages in BALF of the patients with PAP before (pre) and after (post) the 24-week GM-CSF inhalation. (C) Diameters of nonfoamy macrophages in BALF of the patients with PAP before and after the 24-week GM-CSF inhalation. *Central bars* show median, *box plots* show 25th and 75th percentiles, *error bars* show 10th and 90th percentiles; *dots* show the minima and the maxima.

DISCUSSION

Alveolar macrophages in the BALF of patients with iPAP in severe cases show defective mature alveolar macrophage functions (25, 26). Surfactant catabolism and host defense immunity regulated by transcription factor PU.1 are typical of such functions (27). Our previous studies suggested that maturation arrest of alveolarmacrophages is caused by abundant autoantibody against GM-CSF in the lung (19). Because the therapeutic efficacy of extrinsic GM-CSF on iPAP has been established in clinical trials over the last decade (13–16), it is plausible to hypothesize that administered GM-CSF alters the unclear balance between GM-CSF and the autoantibody in the pulmonary microenvironment.

Several investigators have addressed the mechanism of extrinsic GM-CSF action on the pathologic status of iPAP. Seymour and colleagues (28) reported that patients with iPAP who were treated with 5 µg/kg/day of GM-CSF showed an impaired hematopoietic response to GM-CSF. Schoch and coworkers (15) demonstrated that GM-CSF treatment restored morphology and adhesive function of alveolar macrophages in patients with iPAP. The serum anti–GM-CSF titer has been reported to decrease with improvement of iPAP in patients treated with GM-CSF or plasmapheresis (24, 29). Bonfield and colleagues (18) showed that suppressed expression of PU.1 and macrophage colony–stimulating factor receptor in alveolar macrophages of patients with PAP was changed to upregulation by GM-CSF treatment in both *in vitro* experiments and *in vivo* after subcutaneous injection.

These studies demonstrated that treatment accelerated maturation of alveolar macrophages, but they did not explore alterations of the pulmonary microenvironment in which alveolar macrophages reside. Consequently, we have conducted analyses that specifically address the following two points: (*1*) estimation of the neutralizing capacity of the BALF against GM-CSF during the treatment and

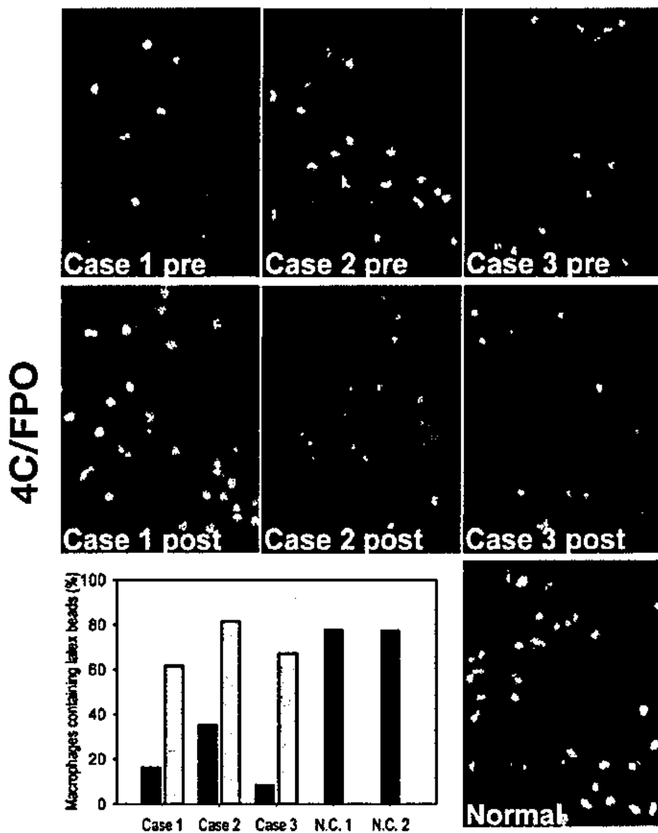


Figure 4. Phagocytosis assay using latex beads. The *upper* and *middle panels* show confocal microscopy images of alveolar macrophages from the patients (*upper panel*: before GM-CSF treatment; *middle panel*: after GM-CSF treatment) incubated with PE-labeled latex beads. The *lower panel* shows confocal microscopy images of alveolar macrophages of a normal control (N.C.) incubated with PE-labeled latex beads, and the ratio of macrophages containing latex beads to total macrophages before (*black bars*) and after (*gray bars*) the GM-CSF inhalation.

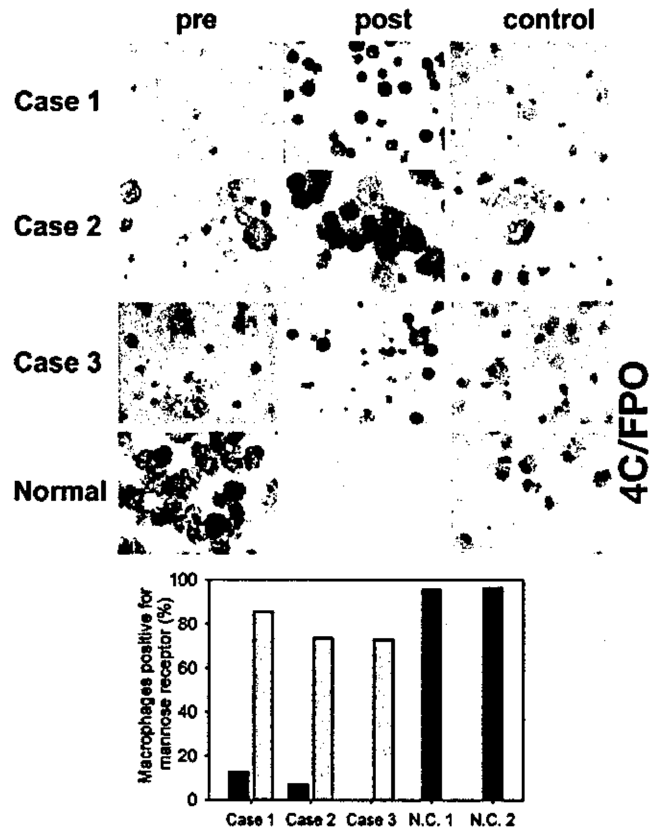
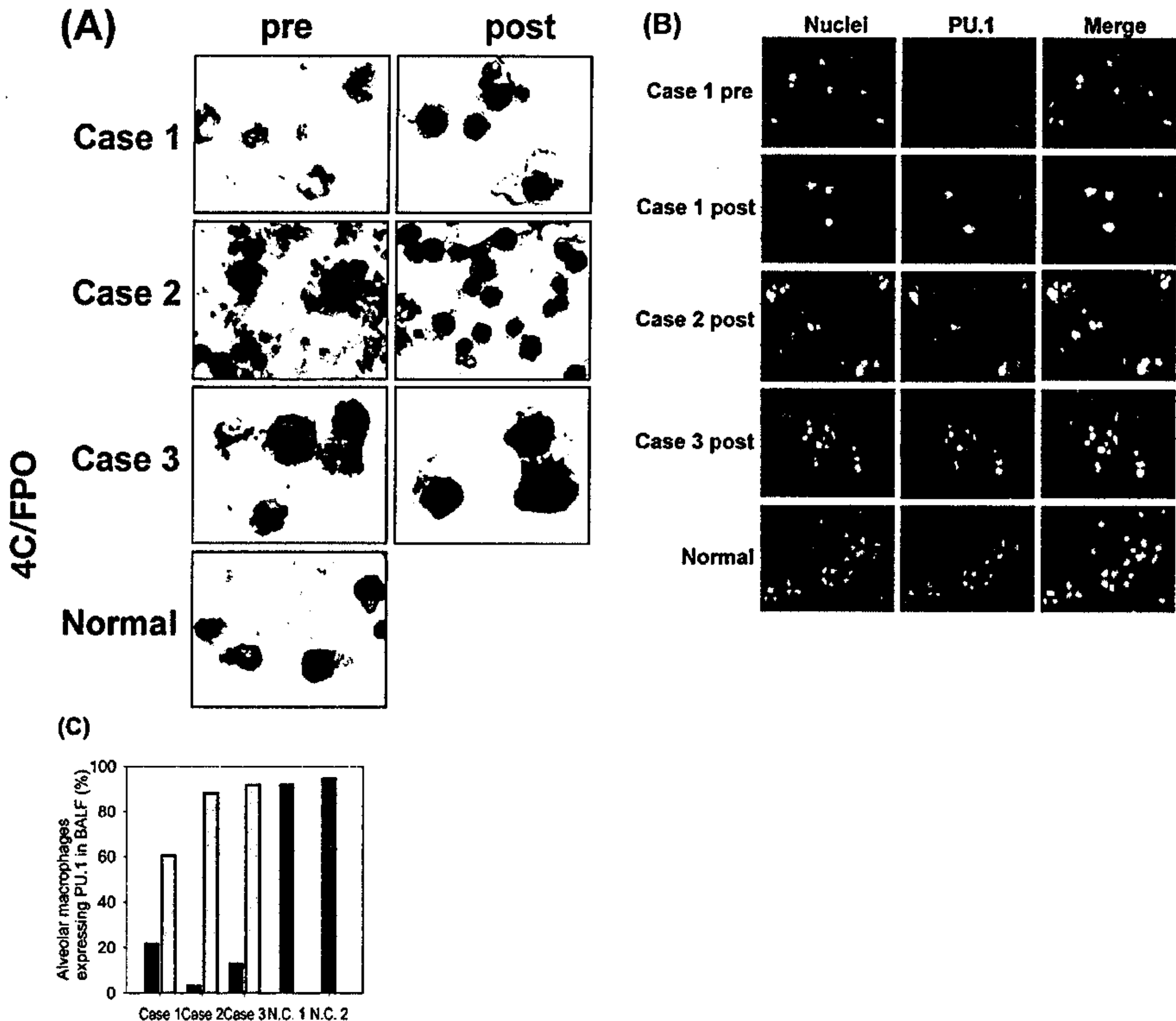


Figure 5. Immunohistochemical staining of alveolar macrophages obtained from patients with PAP and normal control subjects with mannose receptor. The *upper panel* shows alveolar macrophages expressing mannose receptor (*red*) from the patients before treatment (*left*) and after treatment (*middle*), and control staining using murine IgG (*right*). The *lower panel* shows the percentage of macrophages expressing mannose receptor to total macrophages before (*black bars*) and after (*gray bars*) the GM-CSF inhalation.

(*2*) determination of the GM-CSF–autoantibody immune complex. We found the following: (*1*) the neutralizing capacities and the levels of autoantibody against GM-CSF were decreased in BALF of patients with iPAP after aerosolized GM-CSF treatment (Table 3), (*2*) the amounts of GM-CSF–autoantibody immune complexes were also decreased in BALF after the treatment (Table 4), and (*3*) GM-CSF bound to the immune complex in BALF was not extrinsic recombinant protein but rather to natural glycosylated human protein in Case 1.

Aerosolized recombinant human GM-CSF given to cynomolgus monkeys increased the total number of BAL cells more effectively than intravenous infusion of GM-CSF (30). Aerosolized GM-CSF also improved lung histology, alveolar macrophage differentiation, and SP-B immunostaining to normal levels in GM-CSF–deficient mice (8). These results suggest that inhalation of GM-CSF might be an effective approach to affect alveolar macrophages' proliferation and functional maturation. It is notable that Case 3 demonstrated the increase of lymphocytes in BALF after GM-CSF treatment. The increase of lymphocytes was greater than in other two cases, and it might be associated with smoking cessation (Table 1). BAL lymphocytosis was also observed in a patient with PAP throughout the treatment course of subcutaneous GM-CSF injection, despite clinical improvement (15). The pulmonary infiltrates of lymphocytes in GM-CSF–deficient mice decreased but remained under successful treatment with aerosolized GM-CSF (8). Aerosolized GM-CSF itself increased lymphocytes in BALF of healthy cynomolgus macaques (30). The mechanism of the persistent BAL lymphocytosis during PAP treatment with GM-CSF remains to be elucidated.

The lungs of patients with iPAP contain abundant anti–GM-CSF antibody, and they produce GM-CSF to the comparable extent of normal lung (19). Decreased levels of the anti–GM-CSF antibody and the immune complex in BALF of the post-treatment patients suggested that aerosolized GM-CSF might affect the regulatory mechanism of production/disposition of anti–GM-CSF antibody locally or systemically. We infer that the reduced antibody restores bioactivity of intrinsic GM-CSF, engendering an increase of alveolar macrophages. To test the assumption, we attempted to demonstrate the presence of biologically active endogenous GM-CSF in BALF using TF-1, a GM-CSF–dependent cell line. However, neither BALF from normal control subjects nor BALF from the post-treatment



21 22 *Figure 6.* (*A*) Alveolar macrophages expressing PU.1 (*dark blue*) from the patients before (*left*) and after (*right*) treatment and from a normal control subject. (*B*) Confocal microscopy of alveolar macrophages obtained from the patients and stained with cyber green (*left panel*: "Nuclei") and PE-labeled anti-PU.1 antibody (*middle panel*: "PU.1"). Merge images are shown in the *right panel*. (*C*) The percentage ratio of macrophages that were positive for PU.1 to the total macrophages before and after GM-CSF inhalation.

patients sustained cell survival; their GM-CSF activities were below the detectable range (data not shown).

It remains unclear why treatment with extrinsic GM-CSF can decrease both the amount and the neutralizing capacity of autoantibody against GM-CSF in BALF of patients with iPAP (14, 15). It is a remarkable finding that the aerosolized GM-CSF therapy decreased the titer and neutralizing capacity of the anti-GM-CSF antibody in BALF during administration of immunosuppressants in Case 2. Further study should address the following: (*1*) the immune complex might modify a profile of T-cell population that regulates the autoantibody production and (*2*) apoptosis of the B cells that produce anti–GM-CSF antibody might be triggered by the immune complex of extrinsic GM-CSF and the autoantibody through Fc receptors, such as inhibitory FcγRIIB, as in the process of negative selection of B cells (31).

The clinical implication of the present study is that quantification of anti–GM-CSF antibody in BALF is useful to predict the response to GM-CSF treatment in each patient. The neutralizing capacity of GM-CSF in BALF is correlated significantly with serum markers including carcinoembryonic antigen, KL-6, and surfactant protein-D. It is also strongly correlated with the titer of anti–GM-CSF antibody in BALF and P_{O_2}. Clinical trials of GM-CSF treatment revealed the existence of patients who showed no improvement in clinical parameters such as P_{O_2}, computed tomographic, and pulmonary function tests (13, 14). Furthermore, these clinical markers often showed delayed response to GM-CSF therapy in some cases. Techniques to evaluate the 17

(23) TABLE 3. EFFECTS OF GM-CSF INHALATION ON THE ANTIBODY AGAINST GM-CSF AND ITS NEUTRALIZING CAPACITY

	Anti–GM-CSF Ab (μg/ml)		Neutralizing Capacity
	BALF	Serum	(IC50 GM-CSF ng/BALF ml)
Case 1			
Pre	1.38	30.54	4.13
Post	0.10	21.85	0.47
Case 2			
Pre	0.58	57.40	1.33
Post	0.03	33.70	0.32
Case 3			
Pre	5.40	NA	10.27
Post	0.19	NA	0.21

Definition of abbreviations: Ab = serum titer of anti–GM-CSF antibody; BALF = bronchoalveolar lavage fluid; GM-CSF = granulocyte-macrophage colony-stimulating factor; NA = not available.

amount and the neutralizing capacity of anti–GM-CSF antibody in BALF during GM-CSF treatment would be useful tools to enable prediction of the response to GM-CSF treatment.

In conclusion, the present study demonstrated the importance of evaluating microenvironments surrounding macrophages in lungs as well as functions of alveolar macrophages in patients with iPAP. Techniques for detecting the neutralizing capacity and amount of anti–GM-CSF autoantibody in BALF could contribute to optimization of treatment for patients with iPAP.

Conflict of Interest Statement: R.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; E.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; T.A. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; H.O. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; O.I. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; K.U. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; M.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; J.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; M.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; Y.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; I.I. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; Y.E. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; K.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; M.E. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; Y.I. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; K.N. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; T.N. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

TABLE 4. CONCENTRATION (pg/ml) OF GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR BOUND OR UNBOUND TO THE AUTOANTIBODY IN BRONCHOALVEOLAR LAVAGE FLUID

	Before Treatment		After Treatment	
	Bound	Unbound	Bound	Unbound
Case 1	71.3	ND	54.1	ND
Case 2	24.0	ND	ND	ND
Case 3	10.7	ND	ND	ND

Definition of abbreviation: ND = not detected.

(24) The lower detection range of the granulocyte-macrophage colony-stimulating factor EIA kit we used is 2.8 pg/ml.

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