GM-Ab [10]. The GM-Ab–GM-CSF complex might be incorporated into alveolar macrophages through Fc receptors, which were remarkably reduced in aPAP [11], and thus the clearance of the complex was considered to be heavily impaired.

Consistent with our phase II study [9], the serum GM-Ab levels measured by ELISA [7] were unchanged during the treatment in both high and low responders (fig. 1b). The concentration in BALF, however, decreased significantly in high responders, but not in low responders after GM-CSF treatment (fig. 1c). The concentration tended to be higher in low responders than in high responders, but this was not statistically significant. Importantly, the mean molar ratios of GM-Ab to GM-CSF in BALF before and after GM-CSF inhalation were 2.6×10^4 and 4.9×10^4, respectively, indicating that most GM-Ab was capable of binding GM-CSF in the lung.

The serum neutralising capacity against GM-CSF estimated using a GM-CSF-dependent cell line, TF-1 [10], was unchanged during the treatment in both groups (fig. 1d). However, the capacity was reduced in BALF obtained from high responders but not in low responders (fig. 1e). The decrease in BALF neutralising capacity during the treatment was probably due to the decrease in BALF GM-Ab concentration, because these two parameters significantly correlated with each other before and after the treatment (table 1). However, GM-Ab in the lung was considered dependent on circulating GM-Ab, because the concentration of GM-Ab and the neutralising capacity in BALF were closely correlated with those parameters in the serum before and after the treatment (table 1). Moreover, ratios of post- to pre-treatment GM-Ab levels in BALF were strongly correlated with those of total immunoglobulin G in BALF ($r=0.708$, $p=0.0021$), which significantly decreased ($p<0.02$) during GM-CSF inhalation treatment. Taken together with the stable serum GM-Ab level during the treatment, the decrease in GM-Ab levels in the BALF of high responders is probably due to restoration of the local clearance capacity by terminally differentiated macrophages in the lung.

Since GM-CSF inhalation differs from subcutaneous administration in dose and administration route, mechanisms for therapeutic efficacy may differ between the two therapies. As indicated in this study, the amount of GM-CSF was far less than the amount of GM-Ab in the BALF and, therefore, it is unlikely that the inhaled GM-CSF bound to GM-Ab had directly contributed to the reduced concentration of GM-Ab detected by ELISA. Because pulmonary lesions of aPAP are typically distributed in a patchy manner, as indicated by the geographical pattern of ground-glass opacity in high-resolution computed tomography, inhaled GM-CSF may first reach the mildly affected pulmonary regions in the lungs and improve the dysfunction of alveolar macrophages at these sites. The functionally improved alveolar macrophages may contribute to promoting the clearance of accumulated surfactant and reducing the diffusion barrier, shunt fraction and/or ventilation–perfusion mismatching. Conversely, GM-CSF administered subcutaneously may bind to GM-Ab, and only a small part may directly reach the lungs. Most may reach the lymph nodes or bone marrow as immune complexes with GM-Ab that might be associated with immunological modulation, including suppression of autoantibody production.

In conclusion, we confirmed that GM-CSF inhalation was associated with a decrease of GM-Ab in the BALF in improved lungs, which was probably due to the restoration of clearance, and that GM-CSF inhalation might not affect autoantibody production. We believe that the data presented in this study enhance our understanding of the mechanism for effective GM-CSF inhalation therapy and may provide us with important information for determining the regimens of the treatment.

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Deficit of osteoprotegerin release by osteoblasts from a patient with cystic fibrosis

To the Editors:

Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations of the CF transmembrane conductance regulator (CFTR), a cyclic adenosine monophosphate (cAMP)-dependent anion channel expressed mostly in epithelia. Bone deficiency is commonly seen in patients with CF and begins at a young age. Low bone mass affects children and young adults with CF and is associated with significant morbidity due to fractures and decreased lung function. Brittle bones in CF disease have been confirmed by densitometric data, the presence of fractures, and impaired quality of life of young and adult patients [1]. Whether or not this is caused by bone disease around puberty due to a poor acquisition of peak bone mass and worsens with age, lower bone mineral density (BMD) gains are already being observed in CF children with mild disease and normal nutritional status, suggesting that CF-related low BMD may, in part, be due to a primary defect in bone metabolism [2]. In human bone cells, the expression of CFTR protein has been identified by immunohistological observations [3]; we further reported the expression of CFTR mRNA and protein in primary human osteoblasts (the cells of bone formation) [4]. Although there is a report demonstrating a direct association between the F508del mutation and CF-related low BMD in young CF adults with at least one F508del allele [5], the effect of mutations in CFTR, specifically the F508del allele in bone cell metabolism is, to date, unknown.

Since its initial discovery in 1997 as a key regulator in bone density [6], osteoprotegerin (OPG), a product of osteoblasts, is now well known as an inhibitor of osteoclastogenesis. The OPG protein has been shown not only to inhibit osteoclast-mediated bone resorption, but also to exert direct osteoanabolic effects by increasing alkaline phosphatase activity and mineralisation in human osteoblasts [7]. To our knowledge, it is not known whether the F508del mutation in CFTR has a direct effect on human osteoblast activity.

Here, we report, for the first time, both defective CFTR-mediated chloride channel activity and a severe deficit of OPG protein release by osteoblasts of a 25-yr-old CF male with the F508del/G542X mutation in CFTR. The CFTR-mediated chloride channel activity and the level of OPG release were investigated in primary osteoblasts cultured from fresh ankle bone fragments from the CF patient. Normal human osteoblasts, used as controls, were obtained from fresh bone fragments of healthy young adults who underwent trauma surgery. All CF and normal primary osteoblast cell culture (used at the second to third passage and after confluence within 6–8 weeks) was performed as previously described [4]. The bone samples were obtained with informed patient consent after approval by the local research ethics committee (Faculty of Medicine, Reims, France).

First, to test the chloride channel activities in primary human osteoblasts, functional analysis was performed by measuring the cAMP-regulated and CFTR-dependent iodide efflux in the presence of a mixture composed of forskolin (an adenylate cyclase activator raising the cellular cAMP level) and genistein (a direct activator of CFTR), as previously described [8]. As shown in figure 1a, a total absence of CFTR-dependent chloride response in F508del-CFTR osteoblasts was observed compared with normal osteoblasts. Interestingly, the two calcium-dependent and volume-dependent chloride channels were found to be fully functional in both F508del-CFTR and normal osteoblasts. Secondly, we demonstrated that the level of both basal and stimulated (tumour necrosis factor-α, 20 ng·mL^{-1} for 4 h) release of OPG protein (evaluated by ELISA) in F508del-CFTR osteoblast cultures was approximately 8–10% of that observed in normal osteoblasts (fig. 1b).

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IgM-type GM-CSF autoantibody is etiologically a bystander but associated with IgG-type autoantibody production in autoimmune pulmonary alveolar proteinosis

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Nei T, Urano S, Motoi N, Takizawa J, Kaneko C, Kanazawa H, Tazawa R, Nakagaki K, Akagawa KS, Akasaka K, Ichiwata T, Azuma A, Nakata K. IgM-type GM-CSF autoantibody is etiologically a bystander but associated with IgG-type autoantibody production in autoimmune pulmonary alveolar proteinosis. *Am J Physiol Lung Cell Mol Physiol* 302: L959–L964, 2012. First published February 24, 2012; doi:10.1152/ajplung.00378.2011.—The granulocyte-macrophage colony-stimulating factor (GM-CSF) autoantibody (GMAb) is the causative agent underlying autoimmune pulmonary alveolar proteinosis (aPAP). It consists primarily of the IgG isotype. At present, information on other isotypes of the autoantibody is limited. We detected serum the IgM isotype of GMAb (IgM-GMAb) in more than 80% of patients with aPAP and 22% of healthy subjects, suggesting that a continuous antigen pressure may be present in most patients. Levels of the IgM isotype were weakly correlated with IgG-GMAb levels but not IgA-GMAb, suggesting that its production may be associated with that of IgG-GMAb. The mean binding avidity to GM-CSF of the IgM isotype was 100-fold lower than the IgG-GMAb isotype, whereas the IC_{50} value for neutralizing capacity was 20,000-fold higher than that of IgG-GMAb, indicating that IgM-GMAb is only a very weak neutralizer of GM-CSF. In bronchoalveolar lavage fluid from nine patients, IgG-GMAb was consistently detected, but IgM-GMAb was under the detection limit in most patients, confirming that IgM-GMAb is functionally a bystander in the pathogenesis of aPAP. It rather may be involved in the mechanism for development of IgG-GMAb in vivo.

granulocyte-macrophage colony-stimulating factor; isotype; autoantibody

IN 1999, WE DISCOVERED a high concentration of granulocyte-macrophage colony-stimulating factor (GM-CSF) autoantibody (GMAb) in sera and bronchoalveolar lavage fluid (BALF) of patients with idiopathic pulmonary alveolar proteinosis (PAP) (4). Following this, 90% of patients with acquired PAP were found to be positive for the antibody and were reclassified as having autoimmune PAP (aPAP) (4).

Studies on mice defective for GM-CSF signaling (1, 2, 14) revealed that neither production of surfactant by type II cells nor its uptake by alveolar macrophages was altered. Rather, the defect is due to impaired catabolism of surfactant in alveolar macrophages (20). Subsequently, GM-CSF in the lung was found to be the critical factor for regulation of surfactant catabolism in alveolar macrophages (13). As GMAb in aPAP shows high avidity and strong neutralizing capacity against GM-CSF, it is rational to consider that the antibody blocks GM-CSF bioactivity in the lung, resulting in maturation arrest of alveolar macrophages and the development of PAP (4). This hypothesis was recently proven by establishing a PAP model in primates by injecting patient-derived GMAb (10, 11).

In our earlier studies, we reported that GMAb in aPAP only consisted of the IgG isotype (6) predominantly featuring IgG1 and IgG2. GMAb in pharmaceutical IgG preparations was shown by Svenson et al. (15), suggesting the presence of GMAb in the normal human IgG fraction, which was later confirmed to be ubiquitous in the sera of normal subjects (18). In contrast, GMAbs in cord blood are exclusively IgM, and those from maternal peripheral blood are both IgM and IgG (8). Recently, Sergeeva et al. (12) reported that patients with myeloid leukemia or myelodysplastic syndrome had not only IgG (52% of patients) but also IgA- (33%) and IgM-GMAb (20%). Thus the isotypic distribution of GMAb is heterogeneous among both healthy and diseased states.

In the present study, we reevaluated the occurrence of immunoglobulin heavy-chain isotypes of GMAb other than IgG in aPAP. We found that many patients had low concentrations of serum IgM- and IgA-GMAb, but only the concentration of IgM-GMAb was specifically elevated in the patients. Characterization of IgM-GMAb was performed for binding avidity and neutralizing capacity. We assessed the role of IgM-GMAb in the pathogenesis of aPAP.

MATERIALS AND METHODS

Study subjects. Serum samples from 71 patients with aPAP and 23 healthy subjects as controls were collected from nine hospitals in Japan participating in a study of aPAP and stored in Niigata University Medical and Dental Hospital at −80°C. We also collected and stored BALF supernatants at the diagnosis of PAP similarly from nine of the above patients. Written informed consent was obtained under protocols approved by the institutional review boards of the hospitals. A diagnosis of aPAP was confirmed by cytological analysis of BALF, pulmonary histopathological findings, or both (17).

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Analysis of peripheral mononuclear cells population by flow cytometry. Peripheral blood mononuclear cells (PBMNCs) were separated from whole blood by density gradient centrifugation using Ficoll (GE Healthcare, Uppsala, Sweden). PBMNCs were washed twice with PBS supplemented with 2% BSA and 0.2% sodium azide and taken in different flow cytometry tubes and stained with mAbs for 60 min at 4°C in the dark. After incubation, the cells were washed and analyzed by flow cytometry (Epics XL; Beckman Coulter, Brea, CA). The following mAbs were used in this study: FITC-conjugated anti-CD3 was purchased from BD Bioscience (San Diego, CA); phycoerythrin (PE)-conjugated anti-CD4 were purchased from Dako (Carpinteria, CA); PE-cyanine 5 (PE-Cy5)-conjugated anti-CD8 and CD56 and PE-Texas Red-conjugated CD19 were purchased from Beckman Coulter; PE-conjugated anti-CD14 was purchased from Nichirei Bioscience (Tokyo, Japan).

Reagents. Recombinant human GM-CSF (rhGM-CSF; Leukine, Immunex, WA) was dialyzed against PBS (pH 7.4) and biotinylated using the NHS-PEO-biotin kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. The purity of biotinylated GM-CSF (bGM-CSF) was almost 100%. IgG was purified from a pooled serum or plasma sample of aPAP using protein A/G (mixed 7:3, GE Healthcare, affinity chromatography) according the to manufacturer's instructions. The bound IgG was eluted with glycine-HCl (10 mM, pH 2.8; bound fraction). For isolating IgG-GMAb, the eluate containing IgG or the flow through (containing both IgM and IgA) were independently loaded onto GM-CSF-coupled NHS HiTrap columns and eluted with glycine-HCl (10 mM, pH 2.8). The IgM/A-GMAb fraction was further purified by loading onto HiTrap IgM Purification HP (GE Healthcare) columns, according to the manufacturer's instructions. The purities of isolated GMAbs were close to 100% for both IgG and IgM, as determined by ELISA assay (Bethyl Laboratories, Montgomery, TX). Pharmaceutically prepared immunoglobulin was kindly provided by Benesis, Osaka, Japan (Venoglobulin IH) and Kaketsuken (The Chemo-sero Therapeutic Research Institute, Kumamoto, Japan) (Kenketsu Venilon-I). Monoclonal IgG-GMAb was kindly provided from Dr. Kenzo Takada (Evec, Sapporo, Japan) and was used as the standard in ELISA. Monoclonal IgM-GMAb was obtained from immortalized Epstein-Barr virus-transformed peripheral mononuclear cells from patients with aPAP as described previously (8), and the stable cell culture supernatant with the highest IgM-GMAb production was used as the standard antibody for IgM-GMAb-ELISA as described below.

GMAb-ELISA. The GMAb concentration in the serum, culture medium, and BALF was measured using direct ELISA as described previously (18). Each isotype-GMAb was detected using peroxidase-labeled anti-human Fcγ, Fcμ, Fcα, or Fcδ antibody (Dako), and Fcε antibody (GeneTex, Irvine, CA). The sera were diluted to 1 in 3,000 to measure the concentration of IgG-GMAb. For measurement of IgM- or IgA-GMAb, serum samples were diluted 1 in 300. The lower detection limit of IgG- and IgM-GMAb, which was defined as the concentration corresponding to mean blank optical density (OD) + 2 SD, were 0.26 and 0.12 μg/ml, respectively. For IgA-GMAb, the OD value at lower detection limit was 0.062. For measurement of GMAb in BALF, BALF was diluted consistently to 1 in 10 with the lower detection limit of 0.009 and 0.004 μg/ml for IgG- and IgM-GMAb, respectively.

Characterization of GMAb. The GM-CSF binding avidity of purified GMAb was determined using bGM-CSF binding assay. After being coated with anti-Fcγ or anti-Fcμ antibody (Dako) overnight, 96-well microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were washed five times with PBS/0.1% Tween 20 (PBST) and blocked with Stabilcoat (SurModics, Eden Prairie, MN). The purified IgG- or IgM-GMAb was applied to each well, incubated for 1 h at room temperature, washed three times with PBST, and reacted with bGM-CSF (75 μg/ml) for 1 h at room temperature. After being washed five times with PBST, bGM-CSF-bound GMAb was reacted with alkaline phosphatase-streptavidin (Invitrogen, Carlsbad, CA). The activity of alkaline phosphatase was detected by CDP-Star (Applied Biosystems, Carlsbad, CA) using a chemiluminescence plate reader (Berthold Technology, Bad Wildbad, Germany). On the basis of the Lineweaver-Burk plot, the dissociation constant (Kd) was determined from the concentration of bGM-CSF at 50% of maximal binding.

GM-CSF neutralizing capacity. The GM-CSF neutralizing capacity of GMAb was estimated by a conventional bioassay using a GM-CSF-dependent cell line, TF-1 cells, as described previously (5, 19). Briefly, TF-1 cells (20,000 cells/well) were cultured (37°C, 5% CO_2) in microtiter plates for 3 days in macrophage serum-free medium (Invitrogen) containing rhGM-CSF in concentrations of 0.5 ng/ml with GMAb ranging 0–12 μg/ml. TF-1 cell survival was evaluated using the MTT assay as described previously (4). The percentage of growth was calculated using the equation, % growth inhibition = $100 - 100 \times (A - B)/(C - B)$, where A is the absorbance of TF-1 cells grown in the presence of rhGM-CSF and GMAb, B is the absorbance of TF-1 cells grown in medium only, and C is the absorbance of TF-1 cells grown without GMAb but containing GM-CSF (20 ng/ml).

Immunoblotting of GMAbs. The highly purified IgG-GMAb, non-IgG-GMAb, and GMAb including whole isotypes described above was analyzed using SDS-PAGE with 2–15% gradient gels under reducing conditions using 2-mercaptoethanol (30 mA, 50 min). Fractionated proteins were transferred onto PVDF membranes by electroblotting (12 V, 75 min). Membranes were incubated with blocking solution [PBS containing 1% (wt/vol) BSA and 0.1% (vol/vol) Tween 20] at 4°C overnight, washed, incubated with horseradish-peroxidase-conjugated anti-human IgG or IgM antibody (room temperature, 60 min), and visualized by ECL Plus (GE Healthcare).

Statistical analysis. Statistical analyses were performed on a microcomputer using JMP software (SAS Institute, Cary, NC). Numerical data were evaluated for a normal distribution using the Shapiro-Wilk test and for equal variance using the Levine median test. Statistical comparisons of nonparametric data were made with the Mann-Whitney *U*-test for two-group comparisons. Paired samples were evaluated with the Wilcoxon signed-rank test. Spearman's correlation coefficients were calculated to assess a correlation between two parameters. *P* values of <0.05 were considered significant.

RESULTS

Profile of PBMNC population in aPAP. To characterize the immunological modulation by aPAP, we compared the PBMNC population between 24 subjects with aPAP and 23 healthy subjects. As shown in Table 1, there was no difference in cell counts of $CD3^+$, $CD4^+CD3^+$, $CD19^+$, and $CD14^+$ cells, corresponding to total T cells, $CD4^+$T cells, total B cells, and monocytes, respectively. Only $CD8^+CD3^+$cells significantly decreased in the patients compared with controls ($P < 0.01$). Thus aPAP seemed to alter no remarkable changes in major populations of PBMNCs.

Occurrence of Ig isotypes of GMAb. In previous studies, we demonstrated that GMAb in patients with aPAP were mainly of the IgG isotype but did not rule out the occurrence of low concentrations of other isotypes. To elucidate this, free GMAb (able to bind with GM-CSF) was isolated from pooled patient sera by GM-CSF-coupled affinity chromatography, concentrated, and applied to immunoblotting. As shown in Fig. 1*A*, bands specific for IgG-, IgM-, and IgA-GMAb were detected at the predicted molecular weights, but neither IgD- nor IgE-GMAb were detected. The binding of IgG-, IgM-, or IgA-GMAb to biotinylated GM-CSF was completely blocked by an excess amount of nonlabeled GM-CSF, indicating the specificity of the binding (Fig. 1*B*).

Concentrations of IgG-, IgM-, and IgA-GMAb. All sera from patients (n = 71, 51 males, 20 females, median 44.0 yr, range

Table 1. *Comparison of lymphocyte and monocyte population between patients with aPAP and healthy subjects*

Populations In Peripheral Blood	aPAP (n = 24, counts/μl)	Healthy Subjects (n = 23, counts/μl)	P value*
Total lymphocytes	1183.7 ± 701.6 (212.0–2908.6)	1407.3 ± 383.6 (706.5–2383.9)	NS
T cells			
Total $CD3^+$ cells	736.5 ± 583.4 (77.4–2188.3)	906.6 ± 259.3 (467.2–1463.7)	NS
Total $CD3^+CD4^+$ cells	397.8 ± 340.8 (27.9–1164.2)	419.4 ± 155.5 (180.1–917.1)	NS
Total $CD3^+CD8^+$ cells	266.4 ± 182.2 (21.4–667.4)	388.6 ± 155.5 (67.7–701.6)	$P < 0.01$
B cell			
Total $CD19^+$ cells	235.4 ± 195.1 (28.5–815.1)	215.3 ± 93.1 (62.1–406.6)	NS
Monocyte			
Total $CD14^+$ cells	226.3 ± 160.4 (60.9–623.8)	199.2 ± 140.6 (66.2–710.2)	NS

All data are expressed as means ± SD (range). *Statistical difference between patients with autoimmune pulmonary alveolar proteinosis (aPAP) and healthy subjects. Data are analyzed by Mann-Whitney U-test.

9–74) and 12 sera from controls (n = 23, 12 males, 11 females, median 36.0 yr, range 22–59) were over the detection limit. The median serum IgG-GMAb was 17.4 μg/ml (1.4~138.6) and 0.3 μg/ml (0.3~0.5), for patients and healthy controls, respectively ($P < 0.001$) (Fig. 2*A*, *left*). The concentrations of IgG-GMAb in patients were similar to levels reported previously (3). When the cut-off value was set at 0.5 μg/ml, both the sensitivity and specificity were 100%.

In our previous studies, sera diluted 1 in 1,500 were applied to the GMAb-ELISA, but neither IgM- nor IgA-GMAb was detected (6). In this study, the sera were diluted 1 in 300 for measuring IgM- or IgA-GMAb.

Using monoclonal IgM-GMAb, the concentrations of those IgM-GMAb were estimated to be 1.1 μg/ml (0.2~10.7) and 0.4 μg/ml (0.1~1.0) for patients and controls, respectively (median, $P < 0.001$). When the cut-off value was set at 0.13 μg/ml, the sensitivity and specificity were 78.3% and 80.3%, respectively (Fig. 2*A*, *middle*). Because an IgA-GMAb standard was not available, the concentration was not determined, but the frequency of detection and the OD values were comparable between aPAP and controls (Fig. 2*A*, *right*). The lower detection limit was 0.12 μg/ml for IgM-GMAb and was 0.062 for IgA-GMAb (as absorption at 450 nm). We could detect both IgM- and IgA-type GMAb in 57 (81%) and 45 (63%) of

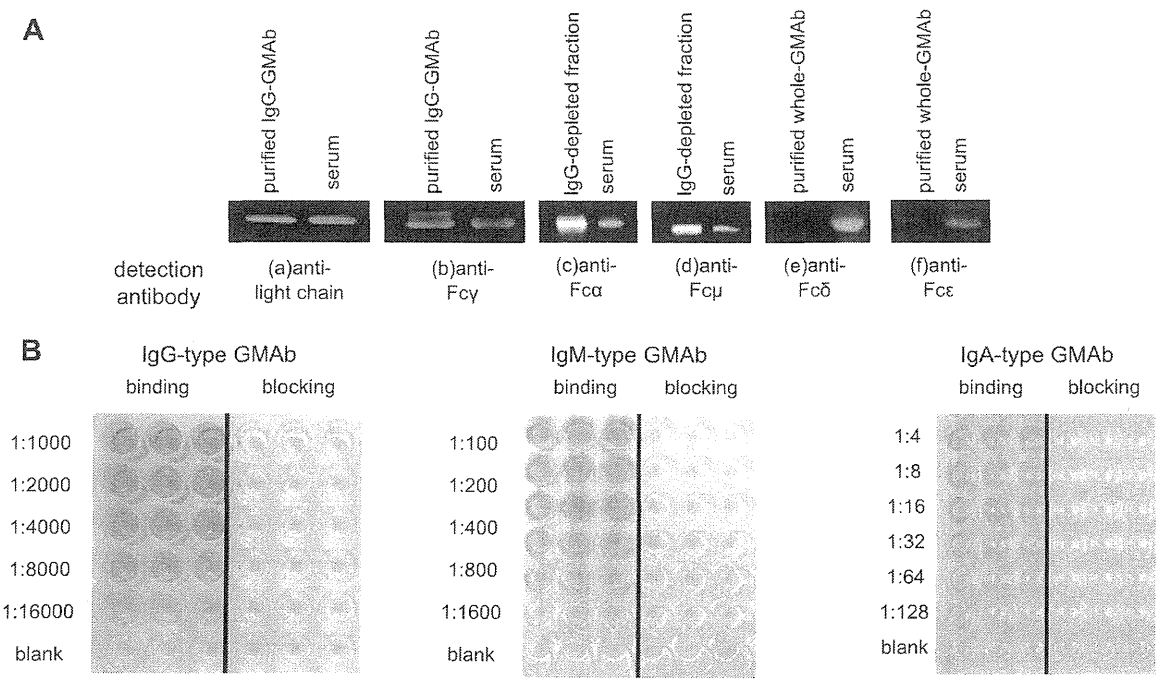

Fig. 1. Occurrence of granulocyte-macrophage colony-stimulating factor (GM-CSF) autoantibody isotypes in autoimmune pulmonary alveolar proteinosis (aPAP). *A*: highly purified IgG-GMAb was loaded in columns *a* and *b*, and highly purified non-IgG-GMAb was loaded in columns *c* and *d*. Highly purified GM-CSF antibody (GMAb) including whole isotypes was loaded in columns *e* and *f*. Each isotype was detected by immunoblotting with antibody specific to light chain (*a*), Fcγ chain (*b*), Fcα chain (*c*), Fcμ chain (*d*), Fcδ chain (*e*), and Fcε chain (*f*), corresponding to whole Ig light chain, IgG, IgA, IgM, IgD, and IgE, respectively. For each isotype control, normal human serum (right lane) was also tested. *B*: elucidation of the binding specificity of IgG- (*left*), IgM- (*middle*), or IgA-GMAbs (*right*) with human GM-CSF. In each panel, the pooled patient's serum was incubated with biotinylated GM-CSF (ng/ml) with (right 3 lanes; blocking) or without (left 3 lanes; binding) excess amounts of nonlabeled GM-CSF (20 μg/ml), serially diluted (0 to 1,000-fold, 0 to 100-fold, and 0 to 4-fold dilution for IgG-, IgM-, and IgA- type GM-CSF autoantibodies, respectively), and was applied to a streptavidin-coated plate in triplicate manner, followed by detection with peroxidase-labeled anti-isotype-specific antibody.

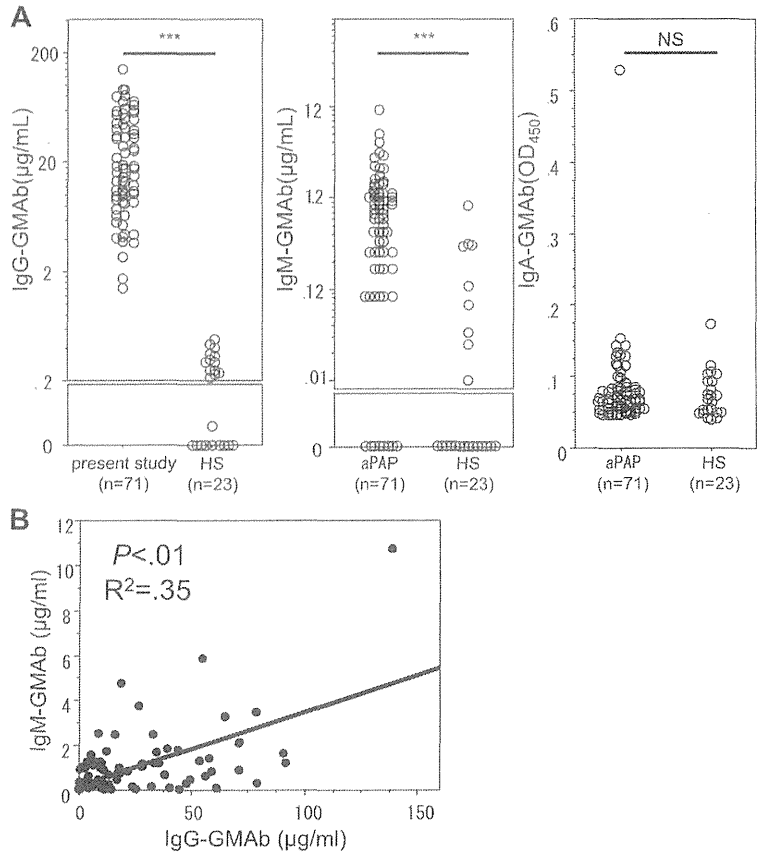

Fig. 2. The concentration of each isotype of GM-CSF autoantibody. *A*: concentrations of IgG- (*left*), IgM- (*middle*), or IgA-GMAbs (*right*) in sera from patients or healthy subjects were determined as described in MATERIALS AND METHODS. The vertical axes show the concentration (μg/ml) for IgG-GMAb or IgM-GMAb and optical density at 450 nm for IgA-GM-CSF autoantibody. Data are expressed as a logarithmic scale except for the range of 0–0.2 μg/ml and 0–0.8 μg/ml for IgG and IgM, respectively. Statistic difference was shown as $***P < 0.001$. *B*: correlation of the concentrations of IgG- and IgM-GMAbs in the sera from patients with aPAP.

71 patients, respectively. Five (22%) of 23 control subjects were positive for IgM-GMAb, and 13n (57%) were positive for IgA-GMAb. Although IgG, IgM, and IgA GMAb were detected in the sera of both patients and healthy controls, our data indicated that only IgG and IgM GMAb specifically increased in aPAP.

When the concentration of IgG, IgM, and IgA-GMAb were compared, a weak correlation was recognized between the concentrations of IgG- and IgM-GMAb (Fig. 2*B*, $R^2 = 0.35$ $P < 0.01$). Other clinical parameters including age, sex, smoking status, occupational dust inhalation, arterial oxygen pressure, and serum KL-6 and SP-D were not correlated with either IgG- or IgM-GMAb.

Characterization of IgM-GMAb properties. To assess the pathophysiological roles of IgM-GMAb, we investigated the properties of IgM-GMAb compared with those of IgG-GMAb. The mean binding avidity of IgM-GMAb to GM-CSF was 10.9 ± 9.0 nM ($n = 7$), which was 136-fold lower than that of IgG-GMAb ($n = 5$, 80 ± 60 pM, Fig. 3*A* and Table 2). Previous studies have demonstrated that IgM-GMAb in neonatal or maternal subjects is nonneutralizing. The neutralizing capacity of purified IgM-GMAb from pooled patient sera was

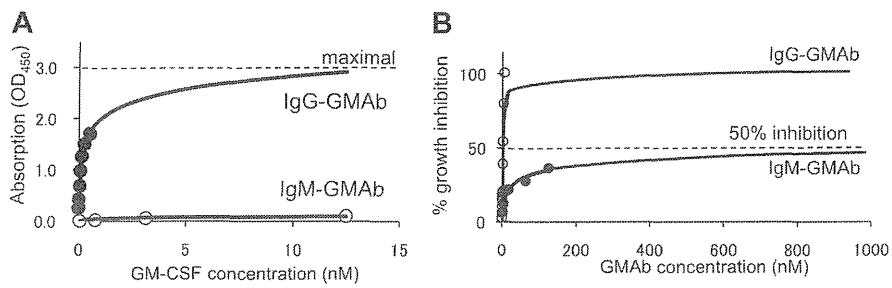

Fig. 3. Characterization of IgM-GMAb properties. *A*: saturation binding plots of biotinylated GM-CSF binding to highly purified IgG- (●) and IgM-GMAbs (○) prepared as described in MATERIALS AND METHODS. Vertical axis represents the optical density corresponding to the binding of biotinylated GM-CSF to the autoantibody. Binding avidity (Kav) was determined from the concentration of GM-CSF at 50% of maximum binding. *B*: serum GM-CSF-neutralizing capacity of purified IgG-(○) or IgM-GMAbs (●). The neutralizing capacity is expressed as percent growth inhibition of TF-1 cells (vertical axis) corresponding to increased concentrations of GMAb (horizontal axis) as described in MATERIALS AND METHODS.

more than 1 μM, whereas that of IgG-GMAb was 50 pM, indicating that IgM-GMAb was only a very weak neutralizer of GM-CSF (Fig. 3*B*).

Concentration of GMAb in BALF. To investigate the isotypic distribution of GMAb in BALF, which derived from the major involved lesion in aPAP, we measured IgG-, IgM-, and IgA-GMAb (Table 3). In nine patients tested, IgG-GMAb was consistently detected, whereas IgM- or IgA-GMAb was not detected in eight patients. Thus the result confirmed that IgG-GMAb is the causative isotype of aPAP.

Table 3. *Concentration of each isotype of GMAb in BALF*

Sample	IgG-GMAb, μg/ml	IgM-GMAb*, μg/ml	IgA-GMAb†, OD_{450}
Case 1	0.12	ND	ND
Case 2	0.02	ND	ND
Case 3	0.11	0.01	1.111
Case 4	0.41	ND	ND
Case 5	0.06	ND	ND
Case 6	0.15	ND	ND
Case 7	0.04	ND	ND
Case 8	0.15	ND	ND
Case 9	0.01	ND	ND

*The lower detection limit is 0.004 μg/ml. ND shows that datum was lower than detection limit. †The lower detection limit is 0.063 in optical density at 450 nm by ELISA. BALF; bronchovascular lavage fluid, OD_{450}; optical density at 450 nm.

DISCUSSION

In this study, we first evaluated the profile of PBMNCs population in aPAP comparing with healthy subjects. Then we reevaluated the isotypic distribution of GMAbs in aPAP and detected low concentrations of IgM- and IgA-GMAb for the first time. Our previous studies had paid little attention to the contribution of IgM-GMAbs to the pathogenesis of aPAP because their role is likely to be minor considering their low concentrations in patient sera. Using ELISA, the concentrations of IgM-GMAbs were found to be 1–10% of IgG-GMAb in patients. Noticeably, IgM-GMAb was present in more than 80% of patients but only 20% of healthy subjects. Moreover, there was a weak correlation in the patients, but not in the controls, between IgM-GMAb and IgG-GMAb concentrations. These results suggest that the occurrence of IgM-GMAb might be associated with the process of IgG-GMAb development. In contrast, the occurrence of IgA-GMAb was similar between patients and controls.

In general, immunoglobulins in BALF were lower than 0.1% of the serum level (16). The concentrations of IgG-GMAb in BALF with less than 1% of the serum level shown in the study were reasonable. In contrast to IgG-GMAb levels in BALF, both IgM- and IgA-GMAb were negligible in most patients. Taken together with high avidity and neutralizing capacities of IgG-GMAb, our present results reconfirm the pathogenic importance of IgG-GMAb.

Although the mechanism for production of GMAb is still unknown, the high retention of IgM-GMAb in patients compared with controls suggests the possibility of recurrent or persistent stimulus of B-lymphocytes by GM-CSF and expansion of the B cell repertoire reacting to self-GM-CSF in lymphatic organs in patients. Thus B cells expressing IgM-GMAb may have many chances for class-switching, differentiating to IgG-GMAb-producing B cells. In addition, as the avidity of IgG-GMAb is extremely high, affinity maturation by somatic hypermutation and clonal expansion may repeatedly proceed in germinal centers of lymphatic organs in the patients.

Table 2. *Binding avidity of IgM- and IgG-GMAb*

Sample	Kav, nM
IgM-GMAb	
Purified IgM-GMAb*	20.7
Case 1	6.54
Case 2	3.57
Case 3	2.34
Case 4	24.6
Case 5	4.34
Case 6	14.1
IgG-GMAb	
Purified IgG-GMAb*	0.05
Case 1	0.17
Case 2	0.04
Case 3	0.07

*Each sample was purified from pooled sera derived from 40 of the present 71 patients with aPAP (described in MATERIALS AND METHODS). GMAb, granulocyte-macrophage colony-stimulating factor autoantibody.

Previous reports demonstrated that nonneutralizing IgM-GMAb mainly developed in adult cancer patients during the early phase of exogenous rhGM-CSF administration (7, 9), followed by a predominance of IgG-GMAb after repeated injections. However, neutralizing IgG-GMAb occurs in only 40% of patients, whereas IgG-GMAbs in aPAP are consistently neutralizing (6, 19). Therefore, isotypic changes from IgM to IgG and phenotypic changes from nonneutralizing to neutralizing forms are critical events in the pathogenesis of aPAP. Taken together, we suppose that B cells self-reactive to GM-CSF that have escaped apoptosis by B cell receptor editing in bone marrow may undergo somatic hypermutation and class-switching in response to repeated or continuous stimulation by GM-CSF in secondary lymphatic tissues in patients with aPAP.

The present study has clarified the isotypic distribution and characterized IgM-GMAb in patients with aPAP. Although IgM-GMAb is etiologically a bystander, it is frequently present and increased in the patients, suggesting the occurrence of a continuous antigen pressure in vivo.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: T.N., J.T., R.T., K. Nakagaki, K.S.A., A.A., and K. Nakata conception and design of research; T.N., S.U., N.M., C.K., and H.K.

performed experiments; T.N., S.U., N.M., C.K., K.A., and T.I. analyzed data; T.N., S.U., N.M., J.T., C.K., K.A., and T.I. interpreted results of experiments; T.N. prepared figures; T.N., R.T., K. Nakagaki, K.S.A., and K. Nakata drafted manuscript; T.N., R.T., K. Nakagaki, K.S.A., and K. Nakata edited and revised manuscript; K. Nakata approved final version of manuscript.

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Direct evidence that GM-CSF inhalation improves lung clearance in pulmonary alveolar proteinosis

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Abbreviations: aPAP, autoimmune pulmonary alveolar proteinosis; BALF, broncho-alveolar lavage fluid; CA125, cancer antigen-125; GM-CSF, granulocyte-colony stimulating factor; GMAb, GM-CSF antibody; IL-17, interleukin-17; PAP, pulmonary alveolar proteinosis; SP-A, surfactant protein A.

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KEYWORDS
Pulmonary alveolar proteinosis;
Granulocyte/macrophage-colony stimulating factor;
Autoantibody;
Bronchoalveolar lavage;
Cancer antigen 125;
Interleukin-17

Summary
Background: Autoimmune pulmonary alveolar proteinosis (aPAP) is caused by granulocyte/macrophage-colony stimulating factor (GM-CSF) autoantibodies in the lung. Previously, we reported that GM-CSF inhalation therapy improved alveolar-arterial oxygen difference and serum biomarkers of disease severity in these patients. It is plausible that inhaled GM-CSF improves the dysfunction of alveolar macrophages and promotes the clearance of the surfactant. However, effect of the therapy on components in bronchoalveolar lavage fluid (BALF) remains unclear.
Objectives: To figure out changes in surfactant clearance during GM-CSF inhalation therapy.
Methods: We performed retrospective analyses of BALF obtained under a standardized protocol from the same bronchus in each of 19 aPAP patients before and after GM-CSF inhalation therapy (ISRCTN18931678, JMA-IIA00013; total dose 10.5–21 mg, duration 12–24 weeks). For evaluation, the participants were divided into two groups, high responders with improvement in alveolar-arterial oxygen difference $\geq$13 mmHg (n = 10) and low responders with that $<$ 13 mmHg (n = 9).
Results: Counts of both total cells and alveolar macrophages in BALF did not increase during the therapy. However, total protein and surfactant protein-A (SP-A) were significantly decreased in high responders, but not in low responders, suggesting that clearance of surfactant materials is correlated with the efficacy of the therapy. Among 94 biomarkers screened in bronchoalveolar lavage fluid, we found that the concentration of interleukin-17 and cancer antigen-125 were significantly increased after GM-CSF inhalation treatment.
Conclusions: GM-CSF inhalation decreased the concentration of total protein and SP-A in BALF, and increase interleukin-17 and cancer antigen-125 in improved lung of autoimmune pulmonary alveolar proteinosis.

Introduction

Pulmonary alveolar proteinosis (PAP) is a rare lung disease characterized by excessive accumulation of surfactant materials within alveolar spaces.[1] Patients with autoimmune PAP, which consists 90% of the disease with 0.49 and 6.04 cases per million for the incidence and prevalence in the general population of Japan, respectively,[2] present a high level of autoantibodies against granulocyte/macrophage-colony stimulating factor (GM-CSF) in the serum as well as in bronchoalveolar lavage fluid (BALF).[3] GM-CSF autoantibodies (GM-Ab) neutralize the biological activity of GM-CSF,[4–6] impairing alveolar macrophage(AM) mediated pulmonary surfactant clearance.[7–10] Recently, GM-Ab purified from a patient with autoimmune PAP was demonstrated to reproduce PAP after transfer into nonhuman primates treated with anti-CD20 monoclonal antibody and cyclophosphamide for blocking xenogrophic immune responses, indicating that GM-Ab directly causes PAP.[11]

Based on studies using GM-CSF knockout mice and a phase I pilot study of inhaled GM-CSF which demonstrated that inhaled delivery of GM-CSF improved PAP,[12–14] we previously conducted a national, prospective, multicenter, phase II trial evaluating inhaled GM-CSF in patients with unremitting or progressive PAP.[15] Of 35 patients who completed the 6-month inhalation, 24 patients (62%) improved with decrease in alveolar-arterial oxygen difference more than 10 mmHg. In these subjects, serum biomarkers including a mucin-like glycoprotein KL-6, carcinoembryonic antigen A (CEA), and surfactant protein A (SP-A), which are known to correlate with the disease severity,[12] decreased significantly during the therapy. The area of ground-glass-opacity (GGO) in pulmonary high-resolution CT also reduced.

As suggested by our pilot study,[14] inhaled GM-CSF may promote the terminal differentiation of AM, and thus, activate surfactant clearance, and improve the oxygen transfer. In the pilot study, we showed that the maturation level of AM proceeded and the function was restored after GM-CSF inhalation. However, no direct evidence for improvement in the surfactant clearance by the therapy has been shown in the previous studies. In this study, we investigated the components in BALF which were obtained from the same bronchus by the same operator of the same institute before the start of and after the end of the GM-CSF inhalation therapy period. Our study revealed that aerosolized GM-CSF therapy decreased the concentration of total protein and surfactant protein A in BALF, while other biological markers, including cancer antigen-125 (CA125) and interleukin-17 (IL-17), increased during the treatment.

Methods

Patients and protocols

The present study retrospectively utilized BALF which was collected as an optional evaluation procedure from the patients that participated in a pilot study (1 patient), an early phase II study (6 patients), and a multicenter phase II trial (12 patients, registered as ISRCTN18931678, JMA-IIA00013) of GM-CSF inhalation therapy described previously.[14] In brief, patients who had lung biopsy or cytology

findings diagnostic for PAP, including elevated serum GM-Ab levels and no improvement during twelve-week observation, entered the treatment periods. Recombinant human GM-CSF dissolved in 2 ml of sterile saline was inhaled using an LC-PLUS nebulizer with a manual interrupter valve connected to a portable compressor (PARI GmbH, Starnberg, Germany). For the first pilot study, treatment consisted of 12 treatment cycles (250 μg daily on days 1–7 and no drug on days 8–14 per cycle, Leucomax; Novartis AG, Switzerland, total dose of 21 mg). For the early phase II study, the treatment period consisted of two successive six-week periods. In the first period, patients received inhaled GM-CSF at a dose of 125 μg daily. In the second six-week period, patients received inhaled GM-CSF (Leukine; Berlex, Seattle, WA) at a dose of either 125 μg/day if the change in A-aDO_2 was >10 mmHg, or 250 μg daily if it was <10 mmHg. This corresponded to a total administration of either 10.5 mg or 15.75 mg of GM-CSF during the treatment period. For the multicenter phase II study, treatments included high-dose GM-CSF administration (125 μg twice daily on days 1–8, none on days 9–14, Leukine; Berlex, Seattle, WA) for six two-week cycles, then low-dose administration (125 μg once daily on days 1–4, none on days 5–14) for six two-week cycles (total dose of 15 mg). The clinical information that was obtained in each study was compared with the results of BAL analysis.

The study was approved by institutional review boards and the BAL procedures were performed after written informed consent was obtained. The clinical information obtained at the clinical studies was entered into a database to be compared with the results of BAL analysis. Each study was designed and monitored for data quality and safety by a steering committee composed of the principal investigator at each participating site.

BAL procedures

The steering committee edited a standard operational procedure for BAL which all participating institutes followed. Three 50 ml aliquots of normal saline were instilled into and suctioned sequentially through a bronchus of the right middle lobe under bronchoscope using standard procedures. Each patient underwent the BAL procedure at the same bronchus in the right middle lobe by the same operator of the same institute within one week before the start of, and after the end of the GM-CSF inhalation therapy period according to the unified standard procedure protocol. Three aliquots of retrieved BAL fluids were collected but only the second and the third aliquots were combined and sent to Niigata University Medical and Dental Hospital and subjected to the centralized analysis. Cells were stained by modified Wright-Giemsa staining (Diff Quick) and 400 nucleated cells were counted differentially in cytocentrifuge preparations. Two hundred alveolar macrophages were photographed and evaluated for their sizes using Image J software (NIH).

Analysis of biomarkers in BAL fluid proteins

BAL fluid aliquots were analyzed using a standard Multi-Analyte Profile (MAP) panel of 94 human analytes(Antigen Immunoassay; Rules-Based Medicine, Inc., Austin, TX). This assays permits simultaneous quantification of multiple analytes including chemokines and cytokines with minimal sample volume. Concentrations of IL-17 were also measured using Quantikine Human IL-17 Immunoassay kits according to the manufacturer's instructions (R&D Systems). Concentrations of CA125 were also measured with chemiluminescent enzyme immunoassay using Lumipulse Presto system (Fujirebio Inc., Tokyo). We measured total protein concentrations of BALF samples using the dye-binding Bradford method (Bio-Rad Laboratories, Inc.). The IL-17 levels and the CA125 levels were normalized to total protein levels in BALF and expressed as pg per μg of BALF protein, or U per μg of BALF protein, respectively.

Immunohistochemical localization of cancer antigen 125 (CA125) and IL-17

CA125 and IL-17 were localized in the lung by immunohistochemical staining on paraffin-embedded lung sections from one aPAP patient or a control using a mouse monoclonal anti-human CA125 (Dako, Inc.) and goat polyclonal anti-human IL-17(R&D Systems), as described previously.[4] Control lung tissues were obtained from the normal lung parenchyma of surgical specimens removed for the resection of lung cancer nodules. Color development was performed using 3-amino-9-ethyl carbazole (AEC) liquid substrate chromogen (DAKO) for IL-17 and diaminobenzidine (DAB) (Nichirei, Tokyo, Japan) for CA125.

Statistical analysis

Numerical results are presented as the mean ± standard error or the median ± interquartile range. The χ^2 test was used to evaluate proportions for variables between high responders and low responders. Analysis of variance and paired *t* test were used for comparisons between normally distributed data before and after the treatment periods. Comparisons of nonparametric data were made using the Wilcoxon's signed-rank test. For group comparisons, analysis of variance and Wilcoxon's rank-sum tests were used. The correlation coefficient was obtained using Spearman's correlation method. All *p* values reported are two-sided. Analysis was performed using JMP™ software version 6.0.3.

Results

Demographic data of participants before treatment

Nineteen patients whose BALF was subjected to the study did not differ from the 39 participants in the multicenter phase II study of inhaled GM-CSF[15] in clinical features including age, gender, symptoms, smoking status, history of dust exposure, pulmonary functions, and GM-Ab titer. The 19 participants improved significantly in various oxygenation indices including symptoms, oxygen supplement status, 6-min walking tests, and A-aDO_2. As the median of A-aDO_2 improvement was 13 mmHg, we divide the participants in two groups, high responders (ΔA-a$DO_2 \geq 13$ mmHg, $n = 10$) and low responders (ΔA-a$DO_2 < 13$ mmHg, $n = 9$),

based on the AaDO2 improvement to evaluate the correlation of therapeutic response with clinical parameters. There was no significant difference in demographic data between the two groups (Table 1).

Serum biomarkers including LDH, KL-6, CEA and SP-A were significantly improved, while SP-D were not altered (Table 2). The serum concentration of GM-CSF autoantibodies in the 19 participants remained at similar levels throughout the therapy. These results indicated that the patients in the present study had similar backgrounds to the participants of the previous phase II study[15] and also demonstrated similar improvement during GM-CSF inhalation.

Recovery rate of bronchoalveolar lavage

Recovery rate of saline instilled during branchoalveolar lavage did not differ significantly (−2.1 ± 4.1%, 95% confidence interval [CI]; −10.8 to 6.7) before and after GM-CSF inhalation therapy (61.3 ± 3.9% [95% CI 53.1–69.5] to 59.2 ± 3.7% [95% CI 51.3–67.2]; $n = 16$; $p = 0.62$; paired t test) (Table 2). The mean of the ratio between recovery rates of before and after GM-CSF inhalation therapy in each patient was 1.00 ± 0.08 [95% CI 0.83–1.17], suggesting intra-participant difference was not observed in recovery rate.

Cellular changes in BALF during the treatment

To evaluate the effects of GM-CSF inhalation on BALF, we first measured the cell counts. Due to the excessive accumulation of amorphous materials in BALF of autoimmune PAP, we managed to evaluate baseline cell counts in 16 out of 19 participants in whom the data of BALF were available. Total cell counts did not increase during the therapy.

Table 1 Clinical Characteristics of patients with PAP.

Characteristic	All patients ($n = 19$)			High responders ($AaDO_2 > 13$) ($n = 10$)			Low responders ($AaDO_2 < 13$) ($n = 9$)			P Value[a]
	n	%	Median (I.Q. range)[b] or mean ± SE	n	%	Median (I.Q. range)[b] or mean ± SE	N	%	Median (I.Q. range)[b] or mean ± SE	
Age, years	19		54 (45–61)	10		56 (47.5–59.5)	9		53 (32.5–62)	0.39[c]
Gender										0.81[d]
Female	9	47		5	50		4	44		
Male	10	53		5	50		5	56		
Duration of symptoms, months	19		17 (11–48)	10		14 (7–24.5)	9		32 (14–88.5)	0.06[c]
Symptoms										
Dyspnea	18	95		9	90		9	100		0.25[d]
Cough	6	32		4	40		2	22		0.40[d]
Sputum	4	21		3	30		1	11		0.30[d]
Smoking status										0.41[d]
Current smoker	4	21		3	30		1	11		
Ex smoker	6	32		2	20		4	44		
Never smoker	9	47		5	50		4	44		
Dust exposure										0.40[d]
Yes	6	32		4	40		2	22		
No	13	68		6	60		7	78		
Past lung lavage (>6 mo prior to study)										0.26[d]
Yes	8	42		3	30		5	56		
No	11	58		7	70		4	44		
Pulmonary Function										
VC, % predicted	18		82.6 ± 4.7	9		86.3 ± 7.8	9		79.0 ± 5.5	0.45[e]
FEV1/FVC, %	18		86.1 ± 1.5	9		87.6 ± 2.3	9		84.8 ± 2.1	0.38[e]
DLCO, % predicted	18		57.5 ± 4.4	9		55.2 ± 6.0	9		59.9 ± 6.6	0.60[e]
PaO_2, torr[f]	19		55.0 ± 1.9	10		52.5 ± 2.2	9		57.7 ± 3.1	0.18[e]
$PaCO_2$, torr[f]	19		38.2 ± 0.7	10		38.7 ± 1.0	9		37.6 ± 1.1	0.45[e]
GM-CSF autoantibody, μg/ml	19		21.5 (12.6–39.6)	10		20.4 (6.5–39.6)	9		24.2 (14.4–38.5)	0.54[c]

[a] Comparison between high responders and low responders.
[b] Interquartile range is the range from the 25th to the 75th percentiles of the distribution.
[c] Calculated using the Wilcoxon's rank-sum test.
[d] Calculated using the χ^2 test.
[e] Calculated using Student's t test.
[f] Measured with patient in a supine position and breathing room air.

Table 2 Symptom, oxygen supplement, Exercise Tolerance, pulmonary function, serum biomarkers, and findings in bronchoalveolar lavage fluid in patients with PAP before and after inhaled GM-CSF therapy.

Characteristic	Before therapy			After therapy			*P* value
	n	%	Mean ± SE	*n*	%	Mean ± SE	
Dyspnea							<0.0001[a]
Yes	18	95		12	63		
No	1	5		7	37		
Oxygen supplement							0.023[a]
Yes	8	42		2	11		
No	11	58		17	89		
6 min walking test[c]							
Walking distance (m)	12		418 ± 37	12		474 ± 24	0.10[b]
Minimal SpO_2 (%)	12		83.5 ± 1.8	12		89.8 ± 1.9	0.005[b]
A–aDO_2 mmHg[d]	19		48.2 ± 1.8	19		32.0 ± 2.9	<0.0001[b]
Serum biomarkers of PAP							
LDH (IU/l)	19		347 ± 32.5	19		297 ± 31.3	0.009[b]
CEA (ng/ml)	19		7.6 ± 1.7	18		3.4 ± 0.7	0.033[b]
KL-6 (U/l)	19		12527 ± 2400	18		5521 ± 1176	0.014[b]
SP-A (ng/ml)	19		138 ± 18	18		101 ± 15	0.011[b]
SP-D (ng/ml)	19		304 ± 40	18		231 ± 37	0.19[b]
GM-CSF autoantibody (μg/ml)	19		24.5 ± 3.5	18		25.0 ± 3.4	0.92[b]
BALF findings							
Recovery rate (% of 150 ml saline)	16		61.3 ± 3.9	16		59.2 ± 3.7	0.62[b]
Cell Count ($\times 10^4$ cells/ml)	16		19.1 ± 3.2	17		29.0 ± 4.8	0.098[b]
Macrophages ($\times 10^4$ cells/ml)	16		11.3 ± 2.0	17		20.9 ± 3.7	0.029[b]
Lymphocytes ($\times 10^4$ cells/ml)	16		6.5 ± 1.5	17		7.6 ± 2.0	0.64[b]
Neutrophils ($\times 10^4$ cells/ml)	16		0.47 ± 0.12	17		0.44 ± 0.15	0.84[b]
Eosinophils ($\times 10^4$ cells/ml)	16		0.039 ± 0.021	17		0.063 ± 0.035	0.54[b]
Macrophage size (μm^2)							
High Responders	6		545 ± 76	6		531 ± 83	0.90[b]
Low Responders	8		555 ± 60	8		715 ± 79	0.13[b]

[a] Calculated using the χ^2 test.
[b] Calculated using Student's *t* test.
[c] Optional evaluation including 7 high responders and 5 low responders, of which change in $AaDO_2$ was −15.5 ± 2.9 and did not significantly differ from that of the total 19 patients.
[d] Calculated using the following equation: $A - aDO_2 = (P_B - P_{H_2O}) \times F_IO_2 - PaCO_2/R + \{PaCO_2 \times F_IO_2(1 - R)/R\} - PaO_2$.
PB; barometric pressure measured by local observatories, P_{H_2O}; partial pressure of water vapor in inspired air (assumed to be 47 torr), F_IO_2; fractional concentration of oxygen in dry gas (assumed to be 0.21), $PaCO_2$; partial pressure of arterial CO_2 measured in arterial blood, R; respiratory quotient (assumed to be 0.8), PaO_2; partial pressure of arterial oxygen measured in arterial blood.

However, macrophages significantly increased after the therapy in the whole group ($p < 0.05$, $n = 16$), but not significantly in high responders ($n = 8$) (Table 2). Base-line counts of lymphocytes and neutrophils in high responders were significantly higher than those in low responders (Fig. 1A, B). However, the numbers of both neutrophils and lymphocytes remained unchanged during the therapy (Fig. 1A, B). Eosinophil numbers remained at baseline levels during the therapy (Table 1) and no difference was observed between high and low responders.

Changes in components in BALF

Subsequently, we characterized various markers in BALF for the state of surfactant accumulation in the respiratory tracts including total protein, phospholipids, and SP-A. Total protein in high responders significantly decreased (Fig. 1C). Phospholipids in BALFs showed a tendency to decrease in high responders after the therapy, while remaining at higher levels in low responders after the therapy (Fig. 1D). Similarly, SP-A levels were higher in low responders compared to high responders after the therapy (Fig. 1E). Interestingly, SP-A in BALF improved significantly in high responders during the therapy, although the serum levels did not differ between high and low responders (Fig. 1F). These results demonstrated that markers for the state of surfactant accumulation were associated with the improvement in oxygenation.

Changes of biomarkers in BALF

To evaluate the effects of GM-CSF inhalation on other markers such as cytokines and epithelial markers in BAL fluids, we performed a preliminary screening of 94 biomarkers on BALF from 10 patients using a microanalyte system which revealed several candidates that could predict the response to GM-CSF inhalation. The patients comprised five high-responders and five low-responders,

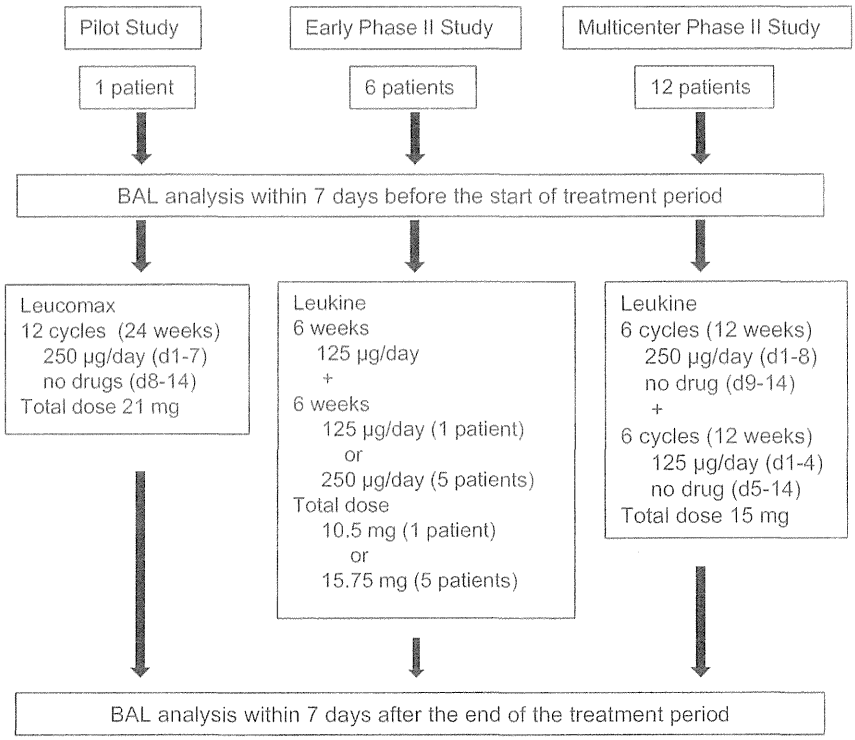

Figure 1 Profile of the study cohort.

including one patient of the pilot study, five from the early phase II study, and four from the multicenter phase II study. Out of 94 biomarkers, levels of 62 markers were within detectable ranges of the microanalyte system. Seventeen markers increased more than two folds during the treatment, but were not statistically significant probably due to small scale of samples. The levels of other 45 markers did not change during the treatment (Table 3). Base-line levels of nine markers demonstrated significant correlation with the improvement in $AaDO_2$ ($\Delta AaDO_2$) (Table 4), from which IL-17 and cancer antigen-125 (CA125) with correlation coefficient of 0.756 and 0.739, respectively, were chosen for further analyses. To confirm the production and localization of both IL-17 and CA125 in the lung of autoimmune PAP, we first performed immunohistochemistry on paraffin embedded lung sections from a patient and a control. AM and lymphocyte-like mononuclear cells in the alveolar spaces were frequently stained with anti-IL-17 antibody (Fig. 3A), whereas no positive cell was observed in the control lung (Fig. 3B). On the other hand, CA125 positive staining was observed in the ciliated bronchial epitherial cells in the autoimmune PAP, as in normal lungs of a previous report[27] (Fig. 3C). Then we determine the level of these markers in the BALF using commercialized ELISA kits. The levels of IL-17 tended to be higher in BALF of high responders compared to low responders at baseline (0.083 and 0.037 pg/μg BALF protein for high and low responders, respectively) and became significantly higher after the therapy (0.34 and 0.052 pg/μg BALF protein for high and low responders, respectively, Fig. 2G). Similarly, the levels of CA125 were significantly higher in BALF of high responders compared to low responders at baseline and substantially increased after the therapy (Fig. 2H).

Discussion

The present study demonstrated that GM-CSF inhalation therapy decreased markers of surfactant accumulation, including total protein and SP-A in the BALF of high responders. Base-line CA125 levels and the counts of lymphocytes and neutrophils were higher in high responders than in low responders, while IL-17 levels were higher in high responders after treatment, suggesting that these markers may be candidates to predict the response to GM-CSF inhalation. These results were based on the BALF data which were normalized using total protein concentration. As total protein decreased during GM-CSF therapy, we attempted to undertake normalization using urea and IgA concentrations, which produced comparable results.

There have been few previous reports on BALF of patients with PAP who have undergone GM-CSF therapy. Case reports showed that total protein and GM-CSF antibody (GM-Ab) decreased in BALF obtained from a PAP patient who was treated with GM-CSF administered subcutaneously.[16,17] However, none of the open-labeled trials of PAP patients treated with subcutaneous GM-CSF administration have studied components in BALF.[18–20] When examining GM-CSF inhalation, neither a report of a child case,[21] nor a retrospective study of 12 patients treated with inhaled GM-CSF[22] studied the change of markers in BALF. We have previously characterized BALF

Table 3 Biomerkers which increased more than two fold during GM-CSF inhalation treatment.

	Post-therapy/ pre-therapy ratio
β-2 Microglobulin	2.18
Endothelin-1	2.11
Haptoglobin	2.69
IgA	5.18
IgM	2.48
IL-15	3.36
IL-16	2.46
IL-17	2.40
IL-18	4.18
IL-23	2.76
MCP-1 (monocyte chemoattractamt protein 1; CCL2)	3.91
MDC (macrophage-derived chemokine; CCL22)	6.27
MIP-1β (macrophage inflammatory protein-1β; CCL4)	2.86
Myoglobin	3.24
OSM (Oncostatin M)	2.23
SHBG (sex hormone-binding globulin)	3.72
TNF RII (tumor necrosis factor receptor II)	2.92

Following biomarkers did not change during the treatment: Alpha-1 Antitrypsin, Adiponectin,α-2 Macroglobulin, α-Fetoprotein, Apolipoprotein A1, Apolipoprotein CIII, Apolipoprotein H, Complement 3, Cancer Antigen 125, Cancer Antigen 19–9, CD40, CD40 Ligand, Carcinoembryonic Antigen, C Reactive Protein, EGF(epidermal growth factor), EN-RAGE(extracellular newly identified RAGE(receptor for advanced glycation end products)-binding protein), Fatty Acid Binding Protein, Factor VII, Ferritin, basic FGF(fibroblast growth factor), Fibrinogen, Glutathione S-Transferase, ICAM-1(inter-cellular adhesion molecule 1), IGF-1(insulin-like growth factor 1)IL-17E, IL-1beta, IL-1ra, IL-4, IL-8, Lipoprotein (a), MIP-1alpha, Myeloperoxidase, PAI-1(plasminogen activator inhibitor-1), Prostatic Acid Phosphatase, RANTES(regulated upon activation, normal T-cell expressed, and secreted; CCL5), Serum Amyloid P, Stem Cell Factor, Thyroxine Binding Globulin, Tissue Factor, TIMP-1(tissue inhibitor of metalloproteinase 1), TNF-alpha, Thyroid Stimulating Hormone, VEGF(Vascular endothelial growth factor), and von Willebrand Factor.

Table 4 Biomerkers which demosntrataed correlation with the improvement in $AaDO_2$ ($\Delta AaDO_2$).

	R^a	P Value[b]
IL-17	0.756	0.012
Cancer Antigen 125	0.739	0.015
C Reactive Protein	0.731	0.016
CD40 Ligand	0.712	0.021
IL-8	0.698	0.025
Complement 3	0.688	0.028
von Willebrand Factor	0.681	0.030
IL-15	0.653	0.041
Endothelin-1	0.652	0.041

[a] Spearman correlation coefficient.
[b] Values calculated using the Spearman correlation test.

and alveolar macrophages of three patients treated successfully with inhaled GM-CSF, which was a predecessor of this study.[14]

Although GM-Ab level remained stable,[15] oxygenation indices and clearance markers significantly improved during the GM-CSF inhalation. To consider the mechanism of the improvement of oxygenation, we should note that the total amount of inhaled GM-CSF would be far less than the total amount of GM-Ab. Using the Pari LC plus nebulizer, 10–20% of inhaled GM-CSF was estimated to reach the peripheral airspace in the lungs (12.5–25 μg/day).[23] GM–CSF–inhibitory activity in BALF of aPAP was −24.9 ± 16.4 ng/mL,[4] which was estimated to be equivalent to more than 150 μg of GM-CSF for both lungs.Consequently, the amount of inhaled GM-CSF was far less than the putative amount of GM-CSF to neutralize the whole GM-Ab in a patient with PAP. In this regard, it is notable PAP lesions are not evenly distributed, as indicated by the geographic distribution of ground glass opacity in high-resolution CT.[24] Inhaled GM-CSF might first reach the mildly impaired region in the lungs, rather than severely impaired regions, and improve the function of the macrophages present in those locations. The restored function of these alveolar macrophages may contribute to improving the clearance in the adjacent regions and the microstructure of the lungs, such as pores of Kohn, could permit such a process.

This study suggested that IL-17 in BALF might be associated with the clinical response to GM-CSF inhalation. In this regard, alveolar macrophages were reported to be a cellular source of IL-17 in asthma.[25] The report suggested that IL-17 is mainly produced by macrophages and not Th17 cells in allergic inflammation related to asthma. GM-CSF inhalation may stimulate macrophages to augment the production of IL-17, and thus, may be utilized as a marker of macrophage function. Lymphocytes are known to be another source of IL-17 in the lung[26] and more lymphocytes were observed in baseline BALF of high responders than that of low responders. In addition, epithelial cells are likely to be indirectly involved in the clearance of surfactant material by stimulating the maturation and function of alveolar macrophages, because CA125, reported to be produced by airway epithelial cells,[27] was associated with the improvement in oxygenation. Alternatively, regenerating broncho-epithelial cells might be associated with the clearance of surfactant materials in lower respiratory tracts, thus, CA 125 might be related to the treatment effectiveness.

In our previous pilot study, oxygenation indices improved and total cell numbers in BALF were increased after the GM-CSF inhalation. The functional evaluation of macrophages, including measures of phagocytic ability and the expression of PU.1 and surface mannose receptors, were restored to control levels after GM-CSF inhalation.[14] In contrast, the number of cells, especially alveolar macrophages, was not changed after inhalation in high responders of this study. Furthermore, there was no significant difference in the BALF data between participant of the 12-week early phase II study and those of the 24-week multicenter phase II study. The discrepancy between the previous and present studies might be due to the limited dose of GM-CSF (total 15 mg) compared to our pilot study (total 21 mg). It will be worthy to evaluate macrophage function in a future study using a randomized trial comparing a high-dose regimen with a low-dose one.

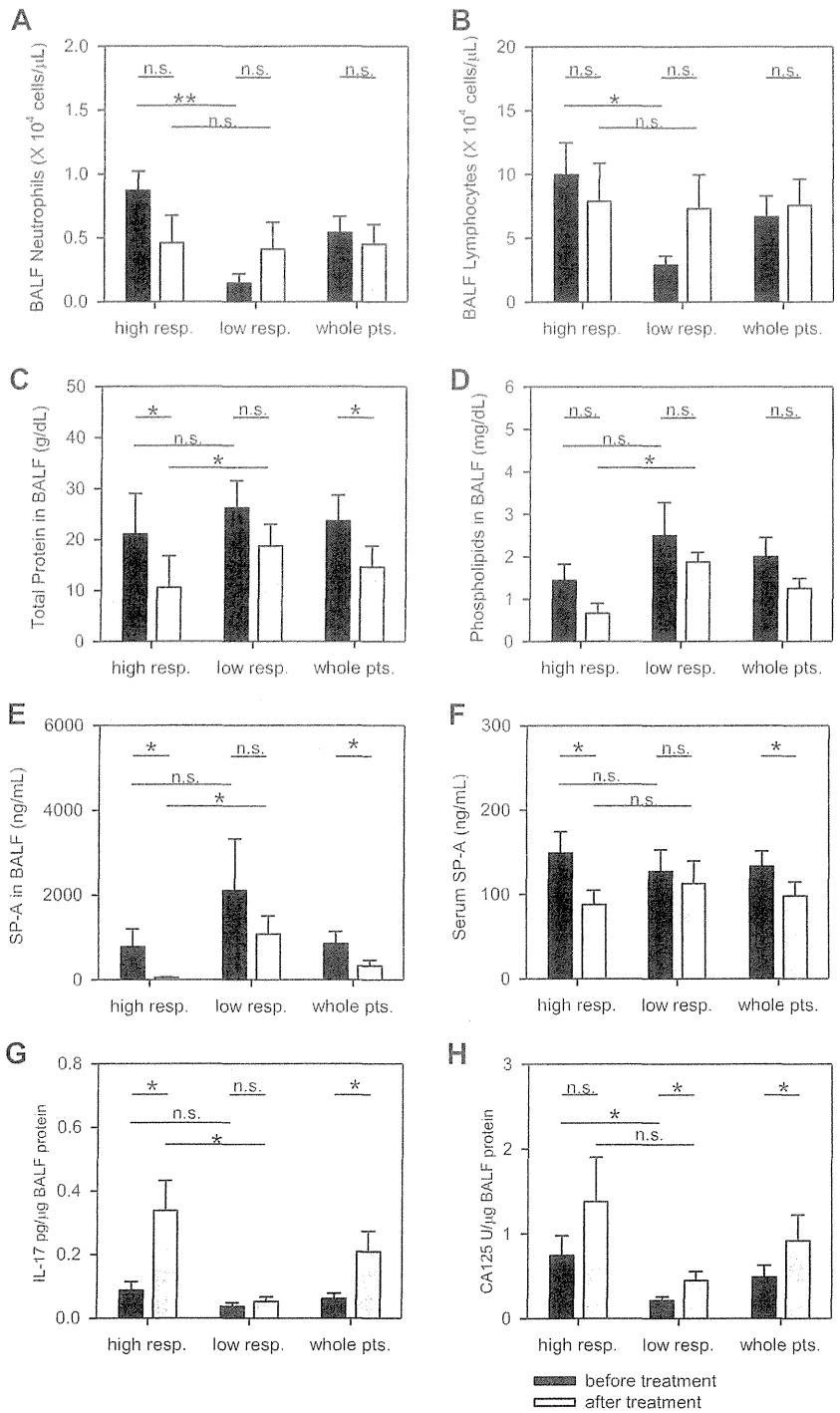

Figure 2 The findings of BAL fluid obtained from high responders (high resp.) and low responders (low resp.) before therapy (black bars) and after therapy (gray bars). The cell counts (A, B) and markers of clearance including total protein (C), phospholipid (D), surfactant protein A (E) compared with serum levels of SP-A (F), IL-17(G) and CA125(H) are shown. Each bar represents the mean (±SE) for the designated patient [$p < 0.05$ (asterisk) and <0.01 (double asterisk) calculated using Wilcoxon's signed-rank test or Wilcoxon's rank-sum test].

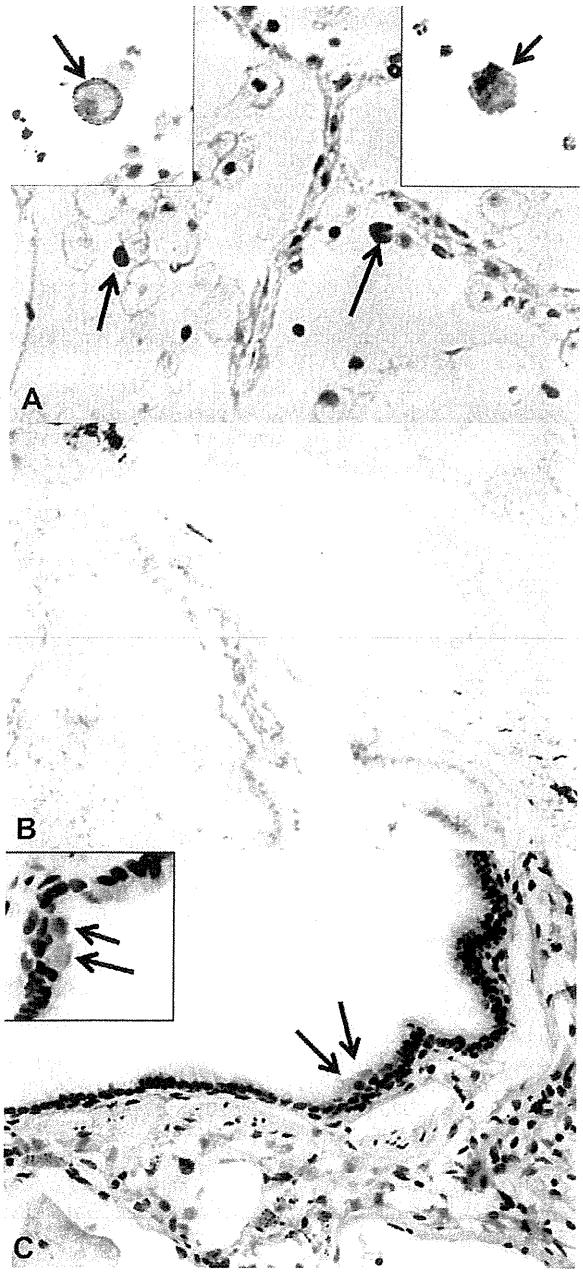

Figure 3 Immunohistochemical detection of IL-17 (panel A and B) and CA125 (panel C) in the lungs of autoimmune PAP (panel A and C) and the normal lung (panel B). The arrows mean the positively stained cells. Insets show higher magnification of the cells of the lungs (left) and a BALF sample (right) (X400).

Conclusions

We confirmed that GM-CSF inhalation decreased the concentration of total protein and surfactant protein A in BALF. We believe the data presented in this study will help to delineate the mechanism of efficacy of GM-CSF inhalation therapy.

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Conflict of interest statement

None of the authors has any conflict of interest related to the manuscript.

Author's contributions

All authors have made substantial contributions to: (1) the conception and design of the study, acquisition, analysis and interpretation of the data, (2) drafting the article and revising it critically for important intellectual content, and (3) gave final approval of the version submitted.

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Cell Metabolism

Production of Functional Classical Brown Adipocytes from Human Pluripotent Stem Cells using Specific Hemopoietin Cocktail without Gene Transfer

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SUMMARY

Brown adipose tissue is attracting much attention due to its antiobestic effects; however, its development and involvement in metabolic improvement remain elusive. Here we established a method for a high-efficiency (>90%) differentiation of human pluripotent stem cells (hPSCs) into functional classical brown adipocytes (BAs) using specific hemopoietin cocktail (HC) without exogenous gene transfer. BAs were not generated without HC, and lack of a component of HC induced white adipocyte (WA) marker expressions. hPSC-derived BA (hPSCdBA) showed respiratory and thermogenic activation by β-adrenergic receptor (AdrRβ) stimuli and augmented lipid and glucose tolerance, whereas human multipotent stromal cell-derived WA (hMSCdWA) improved lipid but inhibited glucose metabolism. Cotransplantation of hPSCdBA normalized hMSCdWA-induced glucose intolerance. Surprisingly, hPSCdBAs expressed various hemopoietin genes, serving as stroma for myeloid progenitors. Moreover, AdrRβ stimuli enhanced recovery from chemotherapy-induced myelosuppression. Our study enhances our understanding of BA, identifying roles in metabolic and hemogenic regulation.

INTRODUCTION

Brown adipose tissue (BAT) is involved in nonshivering thermogenesis during cold exposure (Enerbäck et al., 1997) and diet-induced thermogenesis (Feldmann et al., 2009). It also contributes to the prevention of aging-associated obesity, as demonstrated in *Ucp1* null mice (Kontani et al., 2005). In large-sized mammals, the majority of BAT disappears within a few days after birth; however, some portions remain and function through adulthood. ^{18}Fluorodeoxyglucose-positron emission tomography in combination with computed tomography (^{18}F-FDG-PET/CT) along with histological and gene expressional studies has shown the presence of functional BAT in adult humans in supraclavicular and paravertebral regions (Cypess et al., 2009; Virtanen et al., 2009; van Marken Lichtenbelt et al., 2009; Saito et al., 2009; Yoneshiro et al., 2011). Although accumulating evidence has shown an inverse correlation between the amounts of active BAT and the development of metabolic syndrome in humans (Ouellet et al., 2011; Jacene et al., 2011), the cause-and-effect relationship between BAT and metabolic improvement remains unsubstantiated. Moreover, the whole picture of the development of human BAT is not clarified yet; for example, it remains elusive whether BAT derives from a common progenitor with myoblast, immature mesenchymal cells from which white adipocyte (WA) is also generated or from vascular components such as endothelial and perivascular cells (Tran et al., 2012; Gupta et al., 2012), and whether bone morphogenic protein 7 (BMP7) (Tseng et al., 2008) is sufficient or additional cytokines are required for BA differentiation. The newly proposed concept of brite adipocytes, WA-derived BA-like cells (*br*own + wh*ite* = brite) (Petrovic et al., 2010), makes the situation complex, often encumbering an understanding of classical BAT development. For an advanced understanding of BAT, establishing a method to generate BAs from pluripotent stem cells, including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), is of great use. Recently, a trial to program human iPSCs (hiPSCs) into BA via transferring of exogenous genes was reported (Ahfeldt et al., 2012); however, biological effects of the programmed BA on lipid/glucose metabolism remains unevaluated. Moreover, artificially programmed

cells are not applicable to the investigations on natural developmental pathways of human BA.

The existence of BAT in bone marrow (BM), which attenuates with aging and diabetes, was reported in mice (Krings et al., 2012). Also, a nontumorous infiltration of BAT in human BM was reported in a case of essential hyperthrombocythemia (Thorns et al., 2008). A functional link between BM fat and hematopoiesis was first reported by Dexter et al. (Dexter et al., 1977), who showed that BM adipocytes with mitochondria-attached multilocular lipid droplets were essential for the maintenance of colony-forming units-spleen (CFU-S), a short-term repopulating hematopoietic progenitor cell (HPC) distinct from hematopoietic stem cell (HSC). Although the microenvironment for HSC (reviewed in Kiel and Morrison, 2006; Arai and Suda, 2007) and B cells (Nagasawa, 2007) has been intensively studied, that for myeloid progenitor cells (MPCs) remains poorly understood. Moreover, controversial findings have been reported regarding the effects of "BM adipocyte" on the committed HPCs: some reports showed its capacity to support lymphopoiesis (Gimble et al., 1990) and granulopoiesis (Gimble et al., 1992), while others showed its inhibiting effects for hematopoiesis (Ookura et al., 2007, Naveiras et al., 2009). The controversy may come from, at least in part, the heterogeneity of bone marrow fat cells including BAT versus white adipocyte tissue (WAT).

During our research into the feeder-free hematopoietic differentiation of hPSCs, we serendipitously found the existence of BA-like cell clusters surrounding the hematopoietic centers and an induction of BA-selective gene, *PRDM16* (see Figure S1 online). Because the hematopoietic differentiation was achieved under a completely feeder-free condition, a de novo hematopoietic stroma must be generated from hPSCs per se. Eventually, murine C3H10T1/2 line, a commonly used feeder for the hematopoietic differentiation of monkey (Hiroyama et al., 2006) and human (Takayama et al., 2008) ESCs, can differentiate into functional BA (Tseng et al., 2008). Thus, an association between BA development and hemopoiesis has been suggested.

After a process of trial and error, we established a high-efficiency method to produce functional BAs from hPSCs including human ESCs (hESCs) and hiPSCs. Involvement of hPSC-derived BAs (hPSCdBAs) in metabolic improvement, its service as a stroma for MPCs, and the existence of BA in vertebral BM are also shown.

RESULTS

Directed Differentiation of Human PSC into Functional BA

By utilizing a specific hemopoietin cocktail (HC) composed of KIT ligand (KITLG), fms-related tyrosine kinase 3 ligand (FLT3LG), interleukin-6 (IL-6), and vascular endothelial growth factor (VEGF) along with the previously reported BA inducer BMP7 (Tseng et al., 2008), we successfully established a highly efficient BA differentiation method for hESCs and hiPSCs (Supplemental Information). The differentiated cells exclusively contained multilocular lipid droplets (Figure 1A), as confirmed by oil red O staining (Figure 1B). Quantitative RT-PCR studies demonstrated the induction of BAT-specific genes of *UCP1* and *PRDM16*, which were not detected in human multipoint stromal cell-derived WA (hMSCdWA) (Figure 1C). Although depletion of BMP7 significantly lowered BA differentiation efficiency as reported by Tseng et al. (Tseng et al., 2008) (Figure S2), BA differentiation was completely abolished by HC depletion even in the presence of BMP7 (Figure 1D). Expression of a series of BAT-selective and BAT/WAT-common genes, but not WAT-selective genes, was also determined (Figure 1E). UCP1 protein expression was confirmed by immunostaining studies, showing that over 95% of the BA differentiated cells expressed UCP1 at mitochondria (Figure 2A), and also western blotting, showing the presence of a 32 kDa band in the differentiated cells (Figure 2B). Lipid staining illustrated the wide distribution of mitochondria within the cytosol, some of which resided close to lipid droplets (Figure 2C). Electron micrographs confirmed the presence of multilocular lipid droplets and abundant mitochondria rich in transverse cristae (Figure 2D and Figure S3A), some of which located in close vicinity to lipid droplets (Figure S3B), in contrast to hMSCdWA, which showed meager mitochondria (Figure S3C).

We next evaluated the functional maturation of hESC/hiPSC-derived BAs. First, thermogenic potential was evaluated. Treatment with a β-adrenergic receptor, isoproterenol, augmented the expression of *UCP1*, a major contributor to thermogenesis, and *PRDM16*, a major inducer of *UCP1* expression, in hPSCdBAs (Figures 3A and 3B, left). Isoproterenol-responsive thermogenic activation (Jackson et al., 2001) was further confirmed in vivo by subcutaneous transplantation of hPSCdBAs into mice (Figures 3A and 3B, right). Respiratory activation was also assessed in vitro: hPSCdBAs showed considerably higher basal and maximum OCRs than hMSCdWA as demonstrated by a standard Mito Stress Test (Figure 3C). Responsiveness to a β3-adrenergic receptor-selective agonist, CL316,243, was also determined: statistically significant upregulation in oxygen consumption rates (OCRs) was determined in the cases of hPSCdBAs in response to CL316,243, whereas no significant changes were observed in the cases of hMSCdWA and immature hPSCs (Figure 3D). Upregulation of OCR was further determined in isoproterenol-treated hPSCdBAs (data not shown).

Together, these findings support the production of functional BAs from hPSCs.

Effects of hPSC-Derived BA on Lipid and Glucose Metabolism

Because endogenous BAT reportedly reduces blood triglyceride (TG) levels in response to cold stimuli (Bartelt et al., 2011), we examined the effects of transplantation of hPSCdBA on lipid metabolism. Compared to immature hPSC-transplanted mice, hPSCdBA-transplanted mice (Figure 4A, middle column) and hMSCdWA-transplanted mice (Figure 4A, right column) showed reduced fasting TG levels. Olive oil tolerance tests further confirmed that hPSCdBA transplantation augmented resistance to oral lipid loading (Figure 4B).

Next, effects of hPSCdBAs on glucose metabolism were evaluated. Ten-week-old mice were subcutaneously injected with saline, hESC-derived BA (hESCdBA), or hMSCdWA, and blood glucose levels were measured over time (Figure 4C). Fasting blood glucose levels were significantly lowered in hESCdBA-transplanted mice compared to saline-injected mice ($p = 0.0032$; $n = 3$) and to hMSCdWA-transplanted mice ($p = 0.0030$;