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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
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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 AaDO_2. As the median of AaDO_2 improvement was 13 mmHg, we divide the participants in two groups, high responders ($\Delta AaDO_2 \geq 13$ mmHg, $n = 10$) and low responders ($\Delta AaDO_2 < 13$ 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 (× 10^4 cells/ml)	16		19.1 ± 3.2	17		29.0 ± 4.8	0.098[b]
Macrophages (× 10^4 cells/ml)	16		11.3 ± 2.0	17		20.9 ± 3.7	0.029[b]
Lymphocytes (× 10^4 cells/ml)	16		6.5 ± 1.5	17		7.6 ± 2.0	0.64[b]
Neutrophils (× 10^4 cells/ml)	16		0.47 ± 0.12	17		0.44 ± 0.15	0.84[b]
Eosinophils (× 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: $A - aDO_2 = (P_B - P_{H_2O}) \times F_1O_2 - PaCO_2/R + \{PaCO_2 \times F_1O_2(1 - R)/R\} - PaO_2$.
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,

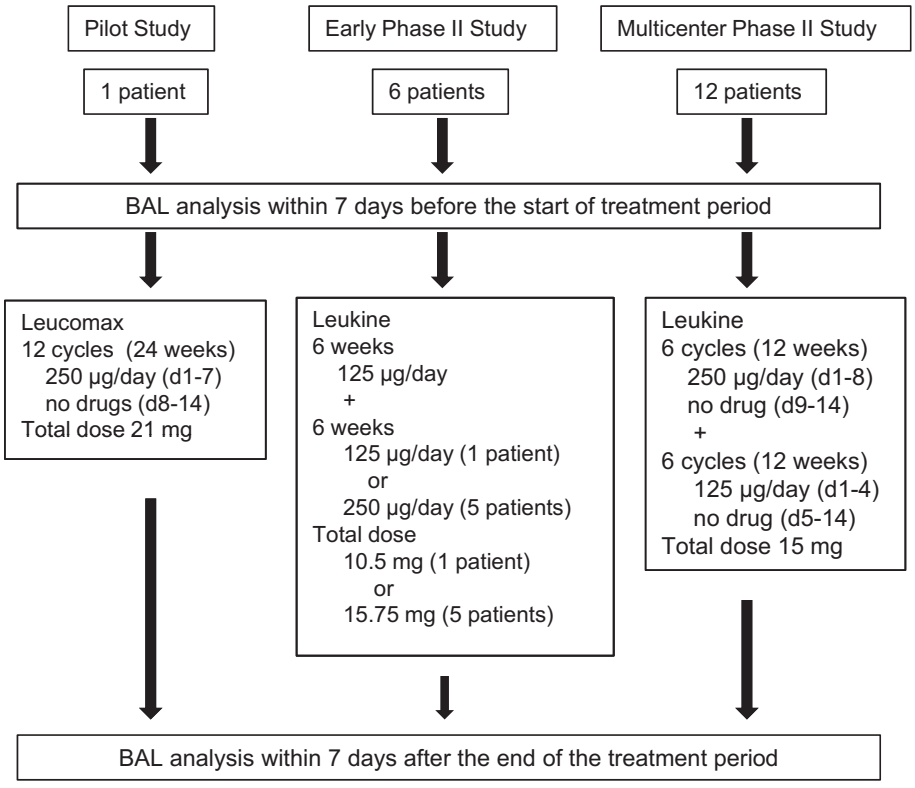

Figure 1 Profile of the study cohort.

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.

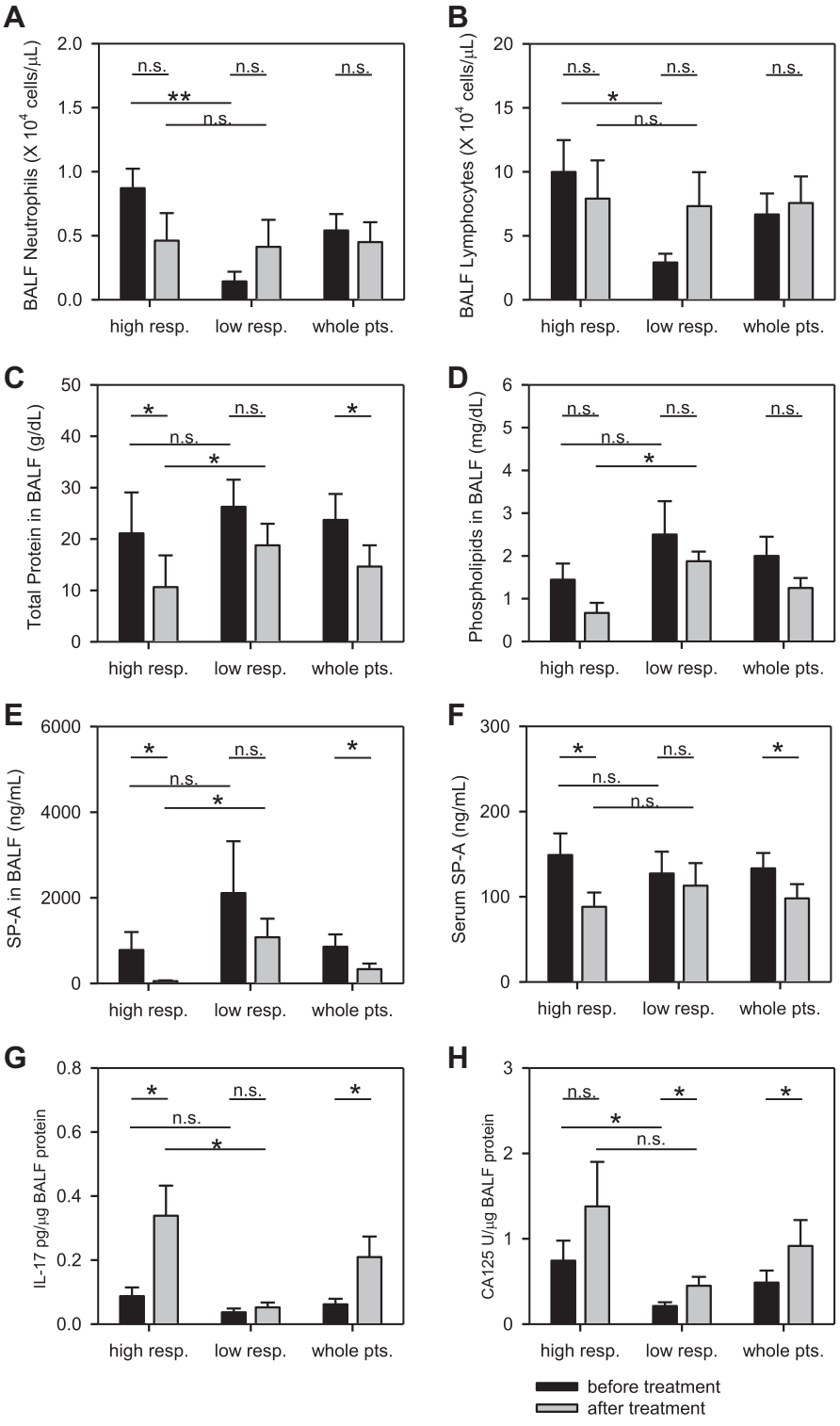

Figure 2 The findings of BAL fluid obtained from high responders (high resp.) and low responders (low resp.) before therapy (black bars) and after therapy (gray bars). The cell counts (A, B) and markers of clearance including total protein (C), phospholipid (D), surfactant protein A (E) compared with 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].

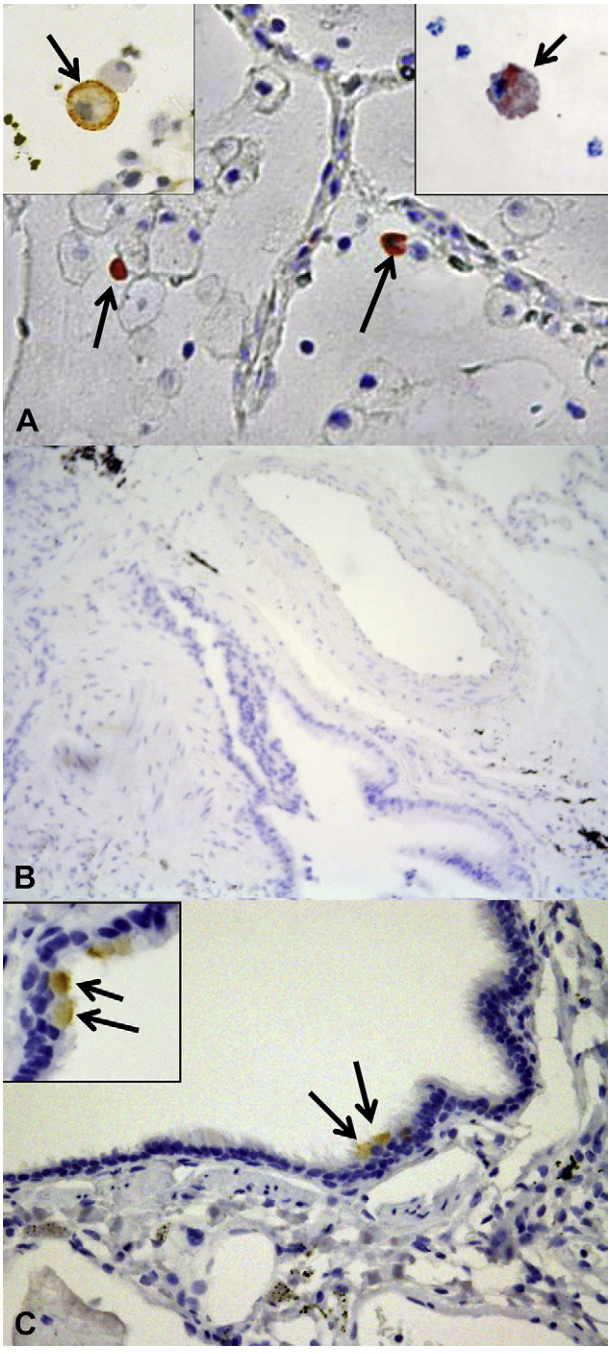

Figure 3 Immunohistochemical detection of IL-17 (panel A and B) and CA125 (panel C) in the lungs of autoimmune PAP (panel A and C) and the normal lung (panel B). The arrows mean the positively stained cells. Insets show higher magnification of the cells of the lungs (left) and a BALF sample (right) (X400).

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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