Table 1

Association between log(VEGF-D) and clinical measurements at enrolment

	Log(VEGF-D)	p
FEV_1	−0·06 (0·05)	0·24
FVC	0·21 (0·08)	0·0064
DLCO	−2·38 (0·52)	<0·0001
6 min walk distance	−14·47 (13·19)	0·28
FEV_1/FVC	−0·06 (0·02)	0·0029
Total lung capacity	0·54 (0·14)	0·0002
Functional residual capacity	0·49 (0·12)	<0·0001
Residual volume	0·34 (0·11)	0·0031
Functional performance inventory	−0·16 (0·06)	0·0144
General wellbeing questionnaire	0·51 (0·61)	0·41
St George's respiratory questionnaire		
Symptoms	2·58 (2·39)	0·28
Activity	5·67 (2·25)	0·0136
Impact	3·96 (2·25)	0·08
Total	4·32 (1·96)	0·0307
36-item short form health survey		
Mental	−1·94 (1·37)	0·16
Physical	−0·91 (1·20)	0·45
European quality of life visual analogue scale		
Fatigue	2·77 (3·24)	0·39
Dyspnoea	1·69 (3·14)	0·59
Quality of life	−4·55 (2·46)	0·07

Data are parameter estimates for log(VEGF-D) and associated SE from the linear regression model with adjustment for height and age. FEV_1=forced expiratory volume in 1 s. FVC=forced vital capacity. DLCO=diffusing capacity of the lung for carbon monoxide.

Table 2

Effect of baseline VEGF-D concentrations on selected clinical measurements during the Multicenter International Lymphangioleiomyomatosis Efficacy of Sirolimus (MILES) trial

	12 month change per unit increase in baseline log(VEGF-D)			Per month change per unit increase in baseline log(VEGF-D)		
	Placebo	Sirolimus	p	Placebo	Sirolimus	p
FEV_1 (mL)	−59 (31)	75 (26)*	0·0007	−4 (2)	5 (2)†	0·0090
FVC (mL)	−41 (38)	137 (54)†	0·0003	−3 (3)	14 (4)*	0·0015
FEV_1/FVC	−0·006 (0·007)	0·008 (0·018)	0·40	−0·001 (0·001)†	−0·000 (0·001)	0·51
Total lung capacity (mL)	26 (107)	106 (105)	0·42	−11 (8)	−0·3 (9)	0·41
Functional residual capacity (mL)	−232 (119)	−13 (84)	0·36	−15 (8)	−7 (7)	0·45
Residual volume (mL)	3 (102)	7 (112)	0·87	−3 (7)	−7 (10)	0·78
DLCO (mL/min/mm Hg)	−0·840 (0·494)	0·301 (0·339)	0·38	0·026 (0·034)	0·050 (0·027)	0·52
6 min walk distance (m)	−6·038 (9·265)	15·27 (12·96)	0·15	−0·320 (0·971)	1·228 (1·019)	0·26
Functional performance inventory	−0·056 (0·044)	0·107 (0·080)	0·0306	−0·002 (0·004)	0·008 (0·006)	0·15
General wellbeing questionnaire	−0·898 (0·916)	−2·172 (0·965)†	0·36	−0·103 (0·072)	−0·207 (0·099)†	0·39
St George's respiratory questionnaire						
Symptoms	6·474 (2·369)†	−5·589 (3·762)	0·0056	0·398 (0·212)	−0·380 (0·340)	0·0436
Activity	1·900 (2·015)	−2·577 (1·990)	0·095	0·108 (0·167)	−0·313 (0·185)	0·10
Impact	1·051 (1·633)	−3·479 (2·474)	0·0463	−0·019 (0·170)	−0·356 (0·249)	0·21
Total	1·665 (1·263)	−2·926 (2·143)	0·0141	0·071 (0·127)	−0·343 (0·198)	0·05
36-item short form health survey						
Mental	0·442 (1·340)	1·778 (1·605)	0·41	0·077 (0·130)	0·215 (0·175)	0·53
Physical	−1·112 (0·993)	2·689 (1·362)	0·0143	−0·072 (0·098)	0·225 (0·125)	0·06
European quality of life visual analogue scale						
Fatigue	−2·884 (4·168)	−1·227 (5·063)	0·84	−0·442 (0·299)	−0·226 (0·435)	0·71

	12 month change per unit increase in baseline log(VEGF-D)			Per month change per unit increase in baseline log(VEGF-D)		
	Placebo	Sirolimus	p	Placebo	Sirolimus	p
Dyspnoea	8·291 (3·833)[†]	−3·883 (5·356)	0·057	0·131 (0·325)	−0·125 (0·458)	0·63
Quality of life	−0·003 (2·763)	3·996 (3·356)	0·27	0·084 (0·230)	0·264 (0·281)	0·59

A general linear model (adjusted for baseline concentration, age, and height) was the basis for calculation of p values for mean 12 month differences in lung function and patient-reported outcomes between the placebo and sirolimus groups depending on baseline log(VEGF-D). A mixed-effects linear model (adjusted for age and height) was used to calculate per month p values for no slope difference between the placebo and sirolimus groups depending on baseline log(VEGF-D). Data are parameter estimates for log(VEGF-D) and associated SE from the general linear and the linear mixed effects models. Functional performance inventory is scored from 1 to 4, the general wellbeing questionnaire from 0 to 110, and the European quality of life visual analogue scale from 0 to 100; low score suggests poor health status. St George's respiratory questionnaire is scored from 0 to 100; low score suggests good health status.

[*] p for no effect of log(VEGF-D) of less than 0·01.

[†] p for no effect of log(VEGF-D) less than 0·05.

CHEST

Original Research

DIFFUSE LUNG DISEASE

Duration of Benefit in Patients With Autoimmune Pulmonary Alveolar Proteinosis After Inhaled Granulocyte-Macrophage Colony-Stimulating Factor Therapy

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Background: **Treatment of autoimmune pulmonary alveolar proteinosis (aPAP) by subcutaneous injection or inhaled therapy of granulocyte-macrophage colony-stimulating factor (GM-CSF) has been demonstrated to be safe and efficacious in several reports. However, some reports of subcutaneous injection described transient benefit in most instances. The durability of response to inhaled GM-CSF therapy is not well characterized.**

Methods: **To elucidate the risk factors for recurrence of aPAP after GM-CSF inhalation, 35 patients were followed up, monitoring for the use of any additional PAP therapies and disease severity score every 6 months. Physiologic, serologic, and radiologic features of the patients were analyzed for the findings of 30-month observation after the end of inhalation therapy.**

Results: **During the observation, 23 patients remained free from additional treatments, and twelve patients required additional treatments. There were no significant differences in age, sex, symptoms, oxygenation indexes, or anti-GM-CSF antibody levels at the beginning of treatment between the two groups. Baseline vital capacity (% predicted, %VC) were higher among those who required additional treatment ($P < .01$). Those patients not requiring additional treatment maintained the improved disease severity score initially achieved. A significant difference in the time to additional treatment between the high %VC group (%VC ≥ 80.5) and the low %VC group was seen by a Kaplan-Meier analysis and a log-rank test ($P < .0005$).**

Conclusions: **These results demonstrate that inhaled GM-CSF therapy sustained remission of aPAP in more than one-half of cases, and baseline %VC might be a prognostic factor for disease recurrence.**

Trial registry: **ISRCTN Register and JMACCT Clinical Trial Registry; No.: ISRCTN18931678 and JMAIIA00013; URL: http://www.isrctn.org and http://www.jmacct.med.or.jp**

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Abbreviations: A-aDO_2 = alveolar-arterial oxygen difference; Ab = antibody; aPAP = autoimmune pulmonary alveolar proteinosis; AT = additional treatment; BALF = BAL fluid; CEA = carcinoembryonic antigen; DLCO = diffusing capacity of the lung for carbon monoxide; DSS = disease severity score; FR = free from additional treatment; GM-CSF = granulocyte-macrophage colony-stimulating factor; IQR = interquartile range; KL-6 = Krebs von den Lungen-6; LDH = lactate dehydrogenase; PAP = pulmonary alveolar proteinosis; ROC = receiver operating characteristics curve; SP = surfactant protein; VC = vital capacity; WLL = whole-lung lavage

Autoimmune pulmonary alveolar proteinosis (aPAP) is a rare lung disease characterized by the accumulation of surfactant protein (SP), which causes progressive respiratory insufficiency.[1-3] The pathogenesis has been attributed to the excessive production of a neutralizing autoantibody against granulocyte-macrophage colony-stimulating factor (GM-CSF) that impairs GM-CSF-dependent surfactant clearance mediated by

alveolar macrophages.[4-8] On pulmonary function testing, the most common pattern seen is that of a restrictive defect, with a disproportionate reduction in diffusing capacity of the lung for carbon monoxide (DLCO) relative to a modest impairment of vital capacity (VC).[2] The disease is usually treated by whole-lung lavage (WLL), which remains the standard therapy to date.

The first patient successfully treated with subcutaneously administered GM-CSF was reported in 1996.[9] In an international multicenter phase 2 trial study, 14 patients were treated with GM-CSF by subcutaneous injection in escalating doses over a 3-month period, with an overall response rate of 43%.[10,11] A subsequent single-center study of 21 patients with aPAP treated with GM-CSF by subcutaneous administration in escalating doses for 6 to 12 months reported an overall response rate of 48%.[12] Several single cases of subcutaneous GM-CSF therapy have reported similar outcomes.[13,14] However, local reaction at sites of injection and other minor toxicities occurred in 85% of patients receiving subcutaneous GM-CSF.[2]

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GM-CSF inhalation is a promising alternative therapy for aPAP that has been demonstrated to lead to functional, biologic, and radiologic improvement.[15-18] Our national, multicenter phase 2 study revealed that the therapy reduced alveolar-arterial oxygen difference (A-aDO_2) by 12.3 mm Hg in 35 patients who completed the therapy, resulting in 24 responders. No treatment-related side effects were noted. Of importance, our previous phase 2 study showed that there was no significant difference in serologic, physiologic, and CT scan testing, except for serum Krebs von den Lungen-6 (KL-6) levels, between the responders and the nonresponders.[18]

There is limited information regarding the duration of benefit after various treatments of aPAP. In the literature analysis of 55 cases with a therapeutic response to WLL, the median duration of clinical benefit from lavage was 15 months.[2] A phase 2 study of subcutaneous GM-CSF administration demonstrated that 45% of patients required WLL during follow-up observation of 39 ± 17.3 months.[12] In a retrospective analysis of inhaled GM-CSF therapy (250 μg bid), five of 12 patients manifest progressive disease during observation.[17] As the disease progresses very slowly and can fluctuate in some cases, it is necessary to evaluate the prognosis by monitoring prospectively at the same time points after the treatment and by disease severity score as well as the need for additional treatment. The aim of this study was to define the duration of benefit among patients who underwent GM-CSF inhalation therapy.

Materials and Methods

Patients and Protocols

The present study prospectively observed patients who participated in a multicenter phase 2 trial (35 patients, registered as ISRCTN18931678 and JMAIIA00013) of GM-CSF inhalation therapy described previously. In brief, patients who had lung biopsy or cytologic findings diagnostic for pulmonary alveolar proteinosis (PAP), including elevated serum anti-GM-CSF antibody (Ab) levels and no improvement during a 12-week observation period, entered the treatment phase. Recombinant human GM-CSF dissolved in 2 mL of sterile saline was inhaled using an LC-PLUS nebulizer (PARI International). The treatment consisted of high-dose GM-CSF administration (125 μg bid on days 1-8, none on days 9-14; sargramostim) for six repetitions of 2-week cycles, then low-dose administration (125 μg once daily on days 1-4, none on days 5-14) for six repetitions of 2-week cycles (for a total dose of 15 mg). The clinical information including physiologic, serologic, and radiologic features obtained[18] was compared with the results of the following 30-month observation.

Patients were regularly evaluated by their physicians at the network hospitals after the GM-CSF inhalation therapy. The worsening dyspnea was evaluated with pulse oximetry, arterial blood gas analysis, or both in outpatient settings. Disease severity in patients was evaluated using PAP disease severity score (DSS) described previously.[19] Patients underwent additional treatments based on

either of the following criteria: (1) DSS is 3 or 4 and symptoms are worsening or (2) DSS 5, as shown in Figure 1. The consortium office of Niigata University contacted the network hospitals every 6 months with a questionnaire regarding additional treatment and disease severity score of the patient. The follow-up clinical information obtained at each network hospital was entered into a database to be compared with the results of the baseline clinical evaluation of each patient. The data were collected from nine clinical research centers in Japan (Hokkaido University, Tohoku University, Chiba University, Kitasato University, Niigata University, Aichi Medical University, National Hospital Organization Kinki-Chuo Chest Medical Center, National Hospital Organization Yamaguchi-Ube Medical Center, and Nagasaki University Institute of Tropical Medicine).

The study was approved by institutional review board of Niigata University (approval No. NH17-006) and the institutional review boards at all participating centers. Informed consent was obtained from all control subjects. The clinical information obtained by the clinical studies was entered into a database to be compared with the results of the 30-month observation. The study was designed and monitored for data quality and safety by a steering committee composed of the principal investigator at each participating site. The steering committee held a conference twice a year, where the findings of the observation were monitored.

BAL Procedures and GM-CSF Autoantibodies

The steering committee edited a standard operational procedure for BAL, which was followed by all participating institutes and described previously.[18,20] The concentration of GM-CSF autoantibodies in BAL fluid (BALF) or in serum were measured using a sandwich enzyme-linked immunosorbent assay as described previously.[4,21]

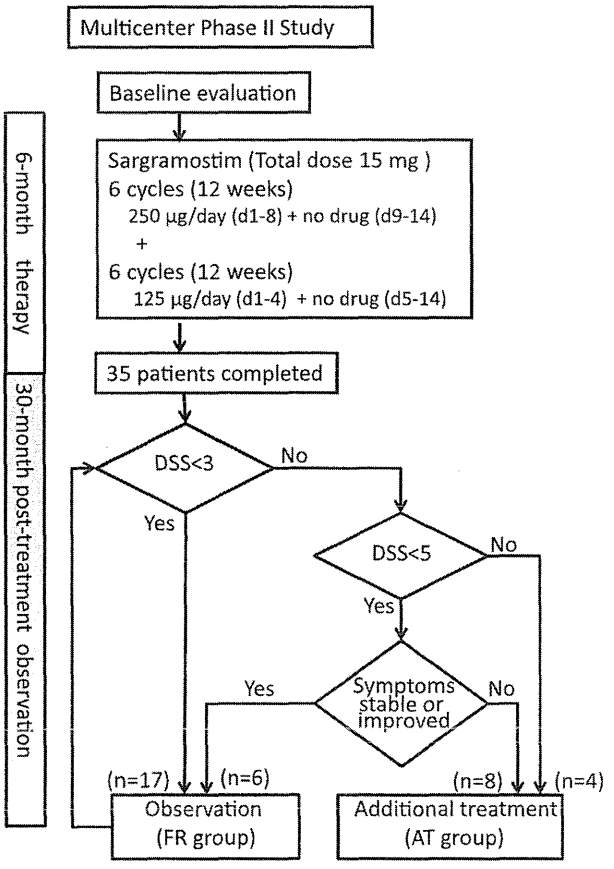

FIGURE 1. Profile of the study cohort. AT = additional treatment; DSS = disease severity score; FR = free from additional treatment.

Statistical Analysis

Numerical results are presented as the mean ± SE or the median and interquartile range (IQR). The χ^2 test was used to evaluate proportions for variables between high and low responders. The paired *t* test was used for comparisons between normally distributed data and the treatment periods. Comparisons of nonparametric data were made using the Wilcoxon signed-rank test. For group comparisons, unpaired *t* tests and Wilcoxon rank-sum tests were used. All *P* values were reported as two-sided. Analysis was performed using JMP software, version 8.0.2 (SAS Institute Inc).

RESULTS

Patient Characteristics and Requirements for Additional Treatments as an Indicator of Recurrence

Demographic data of patients are shown in Table 1. During the 30 months of observation after the end of GM-CSF inhalation, the need for treatments was monitored as an indicator of disease recurrence in each patient. Twenty-three patients were free from additional treatments during 30 months of observation and were designated as FR (free from additional treatment). Twelve patients who required additional treatments, including six patients with recurrence described in our previous study,[18] were designated as AT (additional treatment). Of those, two patients maintained most severe disease (DSS 5) even after the GM-CSF treatment and underwent subsequent WLL. One patient who had dyspnea, cough, and sputum production did not respond to the GM-CSF treatment and underwent subsequent WLL. One patient with cough and dyspnea showed worsening in PaO_2 and cough and had WLL 12 months after the GM-CSF inhalation. The other eight patients with dyspnea showed worsening in PaO_2/oxygen saturation by pulse oximetry (two patients worsened to DSS 5) and underwent additional therapy (e-Fig 1); five underwent additional GM-CSF inhalation treatments, two had WLL, and one patient, a nonresponder, declined WLL and underwent acetylcysteine inhalation, showing much improvement in hypoxia. Median time to additional treatment of the 12 patients was 50.5 weeks, with a range of 8.5 to 117.5 weeks. There was no significant difference in age, sex, symptoms, smoking status, history of dust exposure, arterial blood gas analysis, numbers of responders to GM-CSF inhalation, history of previous lung lavage, and anti-GM-CSF-Ab titer between the FR and AT groups (Table 1). There was no significant difference in disease markers, including baseline levels of PaO_2, A-aDO_2, %VC, %DLCO, CT scan scores, lactate dehydrogenase (LDH), and KL-6 between the patients who underwent WLL (n = 6, AT-WLL group) and those treated with GM-CSF inhalation (n = 5, AT-GM group)

Table 1—*Baseline Clinical Characteristics of Patients Free From Additional Treatment and Those Who Required Additional Treatment After GM-CSF Inhalation*

Characteristic	FR (n = 23)			AT (n = 12)			*P* Value
	No.	%	Median (IQR) or Mean ± SE	No.	%	Median (IQR) or Mean ± SE	
Age, y	23	...	52.5 (48-61)	12	...	52.5 (41.75-58)	.33[a]
Sex			...			...	.54[b]
Female	9	39		6	50		
Male	14	61		6	50		
Responders	17	74	...	7	58	...	.35[b]
Duration of symptoms, mo	23	...	20 (11-61)	12	...	18 (7.75-72)	.78[a]
Symptoms			...			...	
Dyspnea	22	96		12	100		.36[b]
Cough	10	43		7	58		.65[b]
Sputum	8	35		4	33		.71[b]
Smoking status			...			...	.39[b]
Current smoker	8	35		2	17		
Ex-smoker	5	22		2	17		
Never smoked	10	43		8	67		
Dust exposure	22	...	...	11	...	...	.27[b]
Yes	8	36		3	18		
No	14	64		8	82		
Arterial blood gas analysis							
$PaCO_2$, Torr[c]	23	...	38.0 ± 0.7	12	...	39.0 ± 0.9	.40[d]
PaO_2, Torr[c]	23	...	60.6 ± 2.1	12	...	56.3 ± 3.0	.25[d]
A-aDO_2, Torr[e]	23	...	43.5 ± 2.4	12	...	46.2 ± 3.3	.51[d]
Disease severity score	23	...	3 (3-4)	12	...	3.5 (3-5)	.58[a]
GM-CSF autoantibody, μg/mL	23	...	22.8 (8.5-33.2)	12	...	23.1 (16.9-34.2)	.94[a]
Previous lung lavage (>6 mo prior to study)			...			...	.22[b]
Yes	5	22		5	42		
No	18	78		7	58		

Thirty-five patients completed both the high-dose and low-dose period of GM-CSF inhalation therapy. A-aDO_2 = alveolar-arterial oxygen difference; AT = additional treatment; FR = free from additional treatment; GM-CSF = granulocyte-macrophage colony-stimulating factor; IQR = interquartile range (range from the 25th to the 75th percentiles of the distribution).

[a]Calculated using the Wilcoxon rank sum test.

[b]Calculated using the χ^2 test.

[c]Measured with patient in a supine position and breathing room air.

[d]Calculated using Student *t* test.

[e]Calculated using the following equation: A-$aDO_2 = (PB - PH_2O) \times FIO_2 - PaCO_2/R + \{PaCO_2 \times FIO_2 \times (1 - R)/R\} - PaO_2$, where PB = barometric pressure measured by local observatories; PH2O = partial pressure of water vapor in inspired air (assumed to be 47 mm Hg); FIO_2 = fractional concentration of oxygen in dry gas (assumed to be 0.21); and R = respiratory quotient (assumed to be 0.8).

(e-Table 1). However, changes in A-aDO_2 during the GM-CSF treatment were significantly higher in the AT-GM group,

Association of Clinical Parameters With Requirement for Additional Treatment

There was no significant difference in baseline findings in terms of PaO_2, $PaCO_2$, FEV_1, and DLCO between AT and FR groups. Both %VC (% predicted value) and %FVC were higher in the FR group ($P < .01$) (Fig 2A, Table 2, e-Fig 2). There was no correlation between baseline %VC and age ($P = .97$), sex ($P = .41$), baseline PaO_2 ($P = .18$), or baseline %DLCO ($P = .34$). There was no significant difference in high-resolution CT scan scores and serum markers, including LDH, KL-6, carcinoembryonic antigen (CEA), SP-A, and SP-D (Table 2). As for differential blood cell counts, no significant difference was observed between FR and AT groups, except for numbers of basophils and platelets. The cell density of macrophages in BALF was lower in the FR group than those in the AT group ($P < .05$), whereas lymphocytes were lower in the AT group as compared with the FR group.

Next, clinical parameters at the end of treatment were evaluated. The %DLCO was lower in the AT group than that in the FR group, and serum markers (eg, LDH, KL-6, CEA, SP-D, SP-A) and CT scan scores were higher in the AT group than those in the FR group at the end of treatment ($P < .05$). However, there was no significant difference in A-aDO_2, blood cell counts, and cell differentials in BALF (Table 3). The patients free from additional treatment maintained the improved disease severity score initially achieved (e-Fig 3).

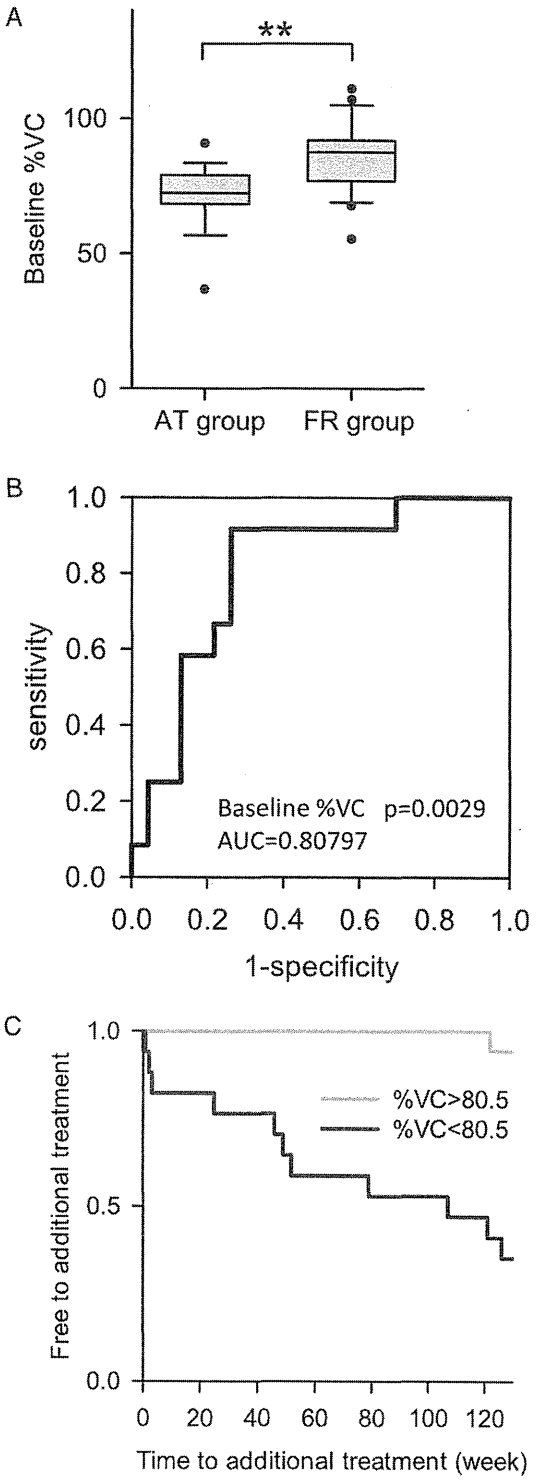

FIGURE 2. The association between VC (% predicted, %VC) and additional treatments during the 30-mo observation period (**$P < .01$). A, Baseline levels of %VC in FR and AT patient groups. B, Receiver operating curve of %VC. C, Kaplan-Meier plot showing patients of the high %VC group (%VC ≥ 80.5) and those of the low %VC group (%VC < 80.5). AUC = area under the receiver operating curve; VC = vital capacity. See Figure 1 legend for expansion of other abbreviations.

Predictive Value of VC for Prognosis After GM-CSF Inhalation

Because only %VC and %FVC differed between FR and AT groups among treatment-related pretreatment factors, the predictive value of parameters for recurrence after GM-CSF inhalation was evaluated using receiver operating characteristics curve (ROC) analysis and Kaplan-Meier analysis of time to additional treatment.

For ROC analysis, the area under the ROC curve was calculated nonparametrically, as proposed by Hanley and McNeil.[22] An additional therapy was defined as a positive indicator for disease recurrence. When the cutoff level of 80.5% was set for %VC, the baseline %VC predicted the additional therapy with a sensitivity of 92% and a specificity of 74% (Fig 2B).

For Kaplan-Meier analysis of time to additional treatment, we divided the patients into two groups, namely the high %VC group (%VC ≥ 80.5) and the low %VC group (%VC < 80.5). A significant difference in the time to additional treatment between the two groups was seen when the whole period of follow-up was compared ($P = .0001$) (Fig 2C). In the univariate Cox proportional analysis of baseline markers, %VC < 80.5% (hazard ratio, 18.42; 95% CI, 3.55-337.68; $P < .0001$) was associated with additional treatment, whereas no correlations were found between additional treatment and age, sex, baseline PaO_2, changes in A-aDO_2, and baseline levels of LDH, KL-6, SP-A, CEA, and anti-GM-CSF-Ab.

Subgroup Analysis: To test whether VC is an independent predictive factor for the time to additional therapy, we did subgroup analyses because of the small number of the AT patients. The patients were divided into two groups of an upper one-half and a lower one-half regarding age; sex; baseline PaO_2; change in A-aDO_2; baseline levels of LDH, KL-6, SP-A, CEA; and anti-GM-CSF-Ab. In these subgroups, a significant difference in the time to additional treatment between the high %VC group (%VC ≥ 80.5) and the low %VC group (%VC < 80.5) was still evident, suggesting that VC might be an independent factor predicting the time to additional therapy (e-Fig 4).

Time Course of Autoantibody Levels: In our previous reports, serum levels of anti-GM-CSF-Ab levels did not change during treatment.[16] To study longitudinal changes of serum levels of anti-GM-CSF-Ab after the inhaled GM-CSF therapy, serum samples were collected for anti-GM-CSF-Ab testing as an optional evaluation after the 30-month observation period. The serum levels were unchanged during the observation period except for three cases (e-Fig 5). In two cases, the serum levels increased by > 100 μg/mL, and one case required an additional treatment, whereas

Table 2—*Baseline Pulmonary Function, Radiologic Appearance, Serum Biomarkers, Hematologic Indexes, and BALF Cell Findings in Patients With PAP in FR and AT Groups Before GM-CSF Inhalation Treatment*

Measure	FR		AT		*P* Value
	No.	Mean ± SE or Median (IQR)	No.	Mean ± SE or Median (IQR)	
Pulmonary function					
VC, % predicted	23	85.9 ± 2.7	12	71.6 ± 3.8	.0045[a]
FVC, % predicted	23	85.3 ± 2.8	12	71.4 ± 3.9	.0064[a]
FEV_1/FVC	23	87.1 ± 2.0	12	84.9 ± 2.7	.51[a]
DLCO, % predicted	23	57.0 ± 3.4	10	46.0 ± 5.1	.082[a]
HRCT scan scores[b]					
Upper lung region	23	3 (2-5)	12	4.5 (2-5)	.12[c]
Middle lung region	23	4 (3-5)	11	4 (3-5)	.38[c]
Lower lung region	23	4 (3-5)	12	5 (4-5)	.36[c]
Serum biomarkers of PAP					
LDH, IU/L	23	287 ± 19	12	325 ± 26	.26[a]
CEA, ng/mL	23	6.2 ± 1.0	12	8.0 ± 1.4	.30[a]
KL-6, U/L	23	10,038 ± 1,531	12	9,434 ± 2,120	.81[a]
SP-A, ng/mL	23	127 ± 15	12	153 ± 20	.29[a]
SP-D, ng/mL	23	227 ± 25	12	290 ± 34	.14[a]
Hematologic indexes					
WBC count, cells/μL	23	5,608 ± 267	12	6,358 ± 370	.11[a]
Neutrophils, cells/μL	22	3,428 ± 200	12	3,596 ± 271	.62[a]
Monocytes, cells/μL	22	344 ± 21	12	396 ± 28	.15[a]
Lymphocytes, cells/μL	22	1,730 ± 147	12	2,122 ± 198	.12[a]
Eosinophils, cells/μL	22	107 ± 28	12	199 ± 38	.058[a]
Basophils, cells/μL	22	18.3 ± 4.3	12	45.3 ± 5.9	.0008[a]
Hemoglobin, g/dL	23	15.4 ± 0.3	12	14.4 ± 0.4	.058[a]
Platelets, × 10^3 cells/μL	23	224 ± 9.1	11	271 ± 13	.0046[a]
BALF cell classification, %					
Alveolar macrophages	17	63 ± 3.6	5	38 ± 6.7	.0036[a]
Neutrophils	17	5.2 ± 1.5	5	10.8 ± 2.7	.082[a]
Eosinophils	17	0.84 ± 0.32	5	0.40 ± 0.60	.52[a]
Lymphocytes	17	31.2 ± 3.8	5	50.4 ± 7.1	.027[a]

BALF = BAL fluid; CEA = carcinoembryonic antigen; DLCO = diffusing capacity of the lung for carbon monoxide; HRCT = high-resolution CT; KL-6 = Krebs von den Lungen-6; LDH = lactate dehydrogenase; PAP = pulmonary alveolar proteinosis; SP = surfactant protein; VC = vital capacity. See Table 1 legend for expansion of other abbreviations.

[a]Calculated using Student *t* test.

[b]Described previously,[16] left lung.

[c]Calculated using the Wilcoxon rank sum test.

the others did not. In the third case, the serum levels decreased to 0.47 μg/mL, and additional treatments were not required.

Discussion

In the present study we have prospectively analyzed, for the time to our knowledge, the requirements of additional therapy and disease severity scores in 35 patients who completed GM-CSF inhalation therapy. The results demonstrate that 23 patients were free from administration of additional treatment during the 30-month observation period, indicating the enduring nature of the therapy. VC could be a useful predictive parameter for the recurrence of disease after GM-CSF therapy. This study contributes to the promotion of GM-CSF inhalation for initial therapy of aPAP.

WLL remains the standard of care today. A retrospective analysis of 231 cases found clinically significant improvement in Pao_2, FEV_1, VC, and DLCO and reported that the median duration of clinical benefit from lavage was 15 months.[2] In a report of 21 patients with PAP who underwent WLL in an experienced center, > 70% of patients remained free from recurrent PAP during 7-year observation.[23] In our study, the median time to application of additional therapy was 30 months after GM-CSF therapy, suggesting the effects of GM-CSF inhalation may be comparable to those of WLL. Notably, the difference in changes in $A\text{-}aDO_2$ during the GM-CSF treatment between the AT-WLL group patients and the AT-GM group patients suggests that nonresponders to the first GM-CSF treatment might be likely to undergo WLL when disease recurred.

In a single-center, phase 2 study for subcutaneous administration of GM-CSF for PAP, Venkateshiah et al[12]

Table 3—*Pulmonary Function, Radiologic Appearance, Serum Biomarkers, Hematologic Indexes, and BALF Cell Findings in Patients With PAP in FR and AT Groups at the End of GM-CSF Inhalation Treatment and Before the 30-Mo Observation*

Measure	FR		AT		*P* Value
	No.	Mean ± SE or Median (IQR)	No.	Mean ± SE or Median (IQR)	
Pulmonary function					
VC, % predicted	23	93.4 ± 3.0	12	74.2 ± 4.2	.0007[a]
FVC, % predicted	23	80.5 ± 3.3	12	72.2 ± 4.5	.0025[a]
FEV_1/FVC	23	85.6 ± 1.6	12	84.7 ± 2.2	.73[a]
DLCO, % predicted	23	68.4 ± 3.4	11	46.8 ± 4.7	.0006[a]
HRCT scan scores[b]					
Upper lung region	23	2 (2-3)	12	3.5 (2-4)	.036[c]
Middle lung region	23	3 (2-3)	12	4 (2.25-4.75)	.023[c]
Lower lung region	23	2 (2-3)	12	4 (2.25-5)	.0039[c]
Serum biomarkers of PAP					
LDH, IU/L	23	242 ± 13	12	308 ± 18	.0064[a]
CEA, ng/mL	23	2.7 ± 0.6	12	5.7 ± 0.8	.0075[a]
KL-6, U/L	23	3,675 ± 735	12	6,565 ± 1,017	.028[a]
SP-A, ng/mL	23	80 ± 12	12	131 ± 16	.015[a]
SP-D, ng/mL	23	170 ± 34	12	304 ± 47	.027[a]
Hematologic indexes					
WBC count, cells/μL	23	5,213 ± 306	12	5,797 ± 424	.27[a]
Neutrophils, cells/μL	22	2,961 ± 205	12	3,026 ± 277	.85[a]
Monocytes, cells/μL	22	320 ± 30	12	338 ± 41	.74[a]
Lymphocytes, cells/μL	22	1,755 ± 131	12	2,153 ± 177	.080[a]
Eosinophils, cells/μL	22	145 ± 40	12	233 ± 55	.20[a]
Basophils, cells/μL	22	27.4 ± 5.9	12	43.7 ± 8.4	.12[a]
Hemoglobin, g/dL	23	14.8 ± 1.3	12	14.4 ± 1.4	.52[a]
Platelets, × 10^3 cells/μL	23	214 ± 9.0	12	235 ± 12	.17[a]
BALF cell classification, %					
Alveolar macrophages	13	67 ± 4.1	5	58 ± 6.7	.28[a]
Neutrophils	13	6.6 ± 2.2	5	7.4 ± 3.5	.86[a]
Eosinophils	13	0.90 ± 0.46	5	0.82 ± 0.75	.93[a]
Lymphocytes	13	25.6 ± 4.8	5	33.2 ± 7.7	.41[a]

See Table 1 and 2 legends for expansion of abbreviations.

[a]Calculated using Student *t* test.

[b]Described previously,[18] left lung.

[c]Calculated using the Wilcoxon's rank sum test.

reported that nine of 21 patients (43%) required WLL. In a retrospective study of 12 patients who underwent aerosolized GM-CSF therapy, Wylam et al[17] reported that five of 11 responders had recurrence of disease. In four of five patients, the mean time to relapse was 6.3 months and ranged from 5.5 to 12 months.[15] It is notable that the dose of GM-CSF used in their study was twice that used in our study, although the prognosis of our cases was comparable to that of their study.

PAP is often described as a lung disorder with restrictive physiology. In the present study, 18 of 35 patients were in the normal range (≤ 80) in %FVC, whereas the other 17 patients were mildly to moderately restricted, which was comparable to previous studies.[24] Seymour et al[25] investigated 14 patients who underwent subcutaneous GM-CSF administration and suggested that higher VC before treatment was one marker to define responsiveness to GM-CSF therapy. In the present study, VC did not correlate with responsiveness to GM-CSF therapy, but it showed significant association with the requirement for additional treatment. Although limited by the small number of cases, the subgroup analyses suggested that VC is an independent factor from age, sex, baseline PaO_2, change in A-aDO_2, and baseline levels of serum markers, including anti-GM-CSF-Ab. However, there is a possibility that some clinical variables might be intrinsically related to VC. The physicians' decision for retreatment might be influenced by such clinical markers. Notably, a recent study of a series of patients with PAP followed in a reference center reported that the need for lavage was significantly associated with FVC.[26]

Reduction of VC might be due to two different factors: accumulation of surfactant-derived materials in the alveolar space and fibrotic changes of lung tissue. In a study of a quantitative CT scan analysis of patients with PAP who underwent WLL and showed improvements in %DLCO and %FVC, Perez et al[27] demonstrated that there was a reduction in lung weight

following lavage, which correlated with the dry weight of the lavage effluent. The study demonstrated a shift in the regional lung inflation toward more inflated lung with a corresponding increase in the mean lung inflation. Surfactant accumulation might be associated with an elevated ventilation-perfusion mismatch and disproportionately impaired DLCO in patients with aPAP.[2] Seymour et al[25] demonstrated serum levels of SP-A correlated with VC in 14 patients at baseline. The present study also showed that serum levels of SP-A correlated with VC at baseline as well as after treatment. However, requirement of additional therapy was not significantly associated with SP-A at baseline. Surfactant materials might be easily redistributed in alveolar spaces and may not be related to the impairment of lung tissue that might lead to additional treatment.

The other factor, fibrotic changes of lung tissue, might be maintained even after GM-CSF therapy or WLL. Pulmonary fibrosis has been reported to be associated with PAP, and exposure to oxygen or repeated WLL have been suggested as potential contributors to fibrosis. Although irreversible scarring of the lung is rarely associated with PAP, a small fraction of patients with PAP demonstrated substantially impaired %VC and rather poor prognosis. To investigate this possibility, two radiologists reevaluated baseline CT scans of 32 of the 35 participants for findings other than PAP without knowing the study results regarding responsiveness and prognosis of the GM-CSF inhalation. They only pointed out traction bronchiectasis in one patient (responder, FR), bronchiectasis in one patient (responder, FR), and multiple bullae in one patient (responder, AT). Thus, we failed to find any significant association between fibrotic change in CT scan and requirement of additional treatments. In the present study, the mean %VC levels of patients in the FR group improved from 85.9% to 93.4%, whereas those of patients in the AT group changed from 71.6% to 74.2%. The difference in improvement between the groups might be associated with the balance of surfactant accumulation and lung fibrosis in the lungs of patients.

For future studies, it would be useful to explore novel treatment regimens for patients with moderately impaired VC. As shown in this study, inhaled GM-CSF therapy did not change serum levels of anti-GM-CSF-Ab. However, the BALF titers of anti-GM-CSF-Ab were reduced in responders, which was likely due to the improved clearance in alveolar spaces. The future treatments might include a combination of GM-CSF inhalation with WLL to improve the environment of airway/alveolar spaces or with administration of rituximab to reduce the systemic production of anti-GM-CSF-Ab.

In conclusion, this study demonstrated that VC might be clinically useful in predicting the need for additional therapy in patients with aPAP who were treated with inhaled GM-CSF therapy. We believe this study contributes to improving the quality of life and treatments for patients with aPAP.

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Additional information: The e-Figures and e-Table can be found in the "Supplemental Materials" area of the online article.

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Mycoplasma pneumoniae Extract Induces an IL-17-Associated Inflammatory Reaction in Murine Lung: Implication for Mycoplasmal Pneumonia

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Abstract—*Mycoplasma pneumoniae* (Mp) may cause immune cell reactions as pivotal aspects of this clinically common respiratory pathogen. Our aim is to determine if Mp extract induces a cellular immune response associated with interleukin (IL)-17, leading to lung inflammation and lung injury. BALB/c mice were immunized with Mp extract intraperitoneally followed by its intratracheal administration, to mimic repeated Mp infection found in humans (repeated inoculation, RI group). Those with a single inoculation were compared as single inoculation group (SI group). Analysis of bronchoalveolar lavage fluid (BALF) demonstrated that keratinocyte-derived cytokine, tumor necrosis factor-α, and IL-6 were produced and peaked on days 0.5 or 1, followed by IL-17 on day 2. Levels of these mediators in BALF were higher in RI group than SI group ($P<0.05$). Further, significantly more neutrophils were recruited to the lungs of the RI group ($P<0.05$). These observations suggest that IL-17 is involved in the prolonged induction of neutrophils in mice treated with Mp extract.

KEY WORDS: *Mycoplasma pneumoniae*; IL-17; IL-23; neutrophil.

INTRODUCTION

Mycoplasma pneumoniae (Mp) is a well-known cause of community-acquired pneumonia that can induce a cellular immune response, leading to inflammation and lung injury [1]. Even cases of pulmonary diseases caused by macrolide-resistant Mp have been successfully treated with macrolide, an agent that has an immunomodulatory effect [2], and cellular host defense reactions are enhanced by repeated Mp stimulation in animal models [3]. The clinical manifestation of Mp infection in humans varies with age, as younger children infected with Mp do not tend to develop pneumonia, whereas older children and adults do tend to develop pneumonia [4–7].

The symptoms of Mp pneumonia are more severe than expected in individuals who have been immunized with Mp vaccine [8]. Studies have demonstrated that interleukin (IL)-4 in bronchoalveolar lavage fluid (BALF) and IL-5 in blood were elevated in patients with Mp pneumonia [9, 10]. Several reports further demonstrated that corticosteroid was clinically beneficial for patients with Mp pneumonia, suggesting that anti-immune and anti-inflammatory actions of corticosteroid were effective against these cellular host defense reactions [11, 12]. Taken together, it has been speculated that cellular immune reactions play an important role in induction of lung inflammation and lung injury by Mp.

In order to mimic the effect of Mp on such immune cell reactions, we previously established a murine model

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of Mp pneumonia for which we employed Mp extract, rather than live Mp, in order to avoid the infectious aspect of the disease [13, 14]. Histological analysis of lung tissues of this mouse model, which had an immune cell reaction against Mp, showed lymphoplasmacytic inflammation in the peri-bronchial area, which is the same pathology as that observed in humans suffering from severe Mp pneumonia [14]. Interestingly, comprehensive analysis of inflammatory mediators in these mice demonstrated that IL-17 levels increased in BALF following Mp inoculation [13]. IL-17 concentration and neutrophil counts in BALF were elevated in parallel when mice were inoculated with live Mp [15]. Similar IL-17-linked signal activation was observed in patients suffering from Mp pneumonia who showed significantly higher levels of serum IL-17 than patients with streptococcal pneumonia [16]. Since it has been reported that IL-17 plays a role in the transition from innate immunity to adaptive immunity [17, 18], these observations led to the hypothesis that some components of the Mp extract induce IL-17, which then results in excessive inflammatory cell reactions in Mp pneumonia.

The mouse models of Mp pneumonia that we previously established [13, 14] were pretreated with alum-adjuvant, which may affect the expression of IL-17 and associated molecules. In the present study, a novel mouse model of Mp pneumonia that did not employ alum-adjuvant treatment was prepared. Furthermore, BALB/c mice were immunized with Mp extract intraperitoneally followed by its intratracheal administration, to mimic repeated Mp infection found in humans (repeated inoculation, RI group). Those with a single inoculation were compared as single inoculation group (SI group) in order to clarify whether IL-17 levels are enhanced by injection of Mp extract.

MATERIALS AND METHODS

Preparation of Mp Extract

Mp extract was prepared from cultured *M. pneumoniae* (ATCC 29342) in a pleuropneumonia-like organism liquid broth containing 20 % horse serum and 10 units/mL penicillin G (Nikkenkagaku, Kyoto, Japan), according to a previously published method with some modification [13]. In brief, cultured Mp was centrifuged and repeatedly washed with Hanks' balanced salt solution (HBSS) (Invitrogen, Grand Island, NY). The sediment was suspended in HBSS and sonicated. This suspension was centrifuged and the supernatant was defined as the Mp extract. The amount of Mp extract was defined in terms of protein concentration, and the Mp extract was prepared in HBSS at a concentration of 1.0 μg/μL.

Primary Culture of Lung-Derived Cells

Cells were prepared from lung tissues originating from four 12-week-old BALB/c female mice (Charles River Laboratories, Kanagawa, Japan), which were anesthetized and then sacrificed after their blood was replaced with saline. Their lungs were removed and cut into pieces, and the tissues were incubated for 2 h at room temperature in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Grand Island, NY, USA) that contained 300 U/mL collagenase type 1 (Worthington Biochemical Corporation, NJ, USA), 0.01 % gentamicin (GM), and 0.1 % amphotericin-B (AMPH-B). The suspended cells were then harvested, washed with DMEM containing 0.01 % GM, 0.1 % AMPH-B, and 10 % fetal calf serum (FCS, Invitrogen), passed five times through 40-μm nylon mesh cell strainers (BD FALCON, Franklin Lakes, NJ, USA), seeded at a concentration of 5.0×10^4 cells/well in a 96-well plate, and incubated at 37 °C in 5 % CO_2. After 24 h, the culture media were replaced with DMEM containing 0.01 % GM, 0.1 % AMPH-B, 10 % FCS, and 100 U/mL polymyxin B. To assess the time-dependent release of cytokines, the cells were treated with or without Mp extract (50 μg/mL). After incubation of the cells for 24, 48, and 72 h with the Mp extract, the media were collected for measurement of IL-23 and IL-17 levels. Media were also collected after 72 h incubation with different concentrations of the Mp extract (0, 1.9, 5.6, 16.7, and 50 μg/mL) to evaluate dose-dependent stimulating effects of Mp extract. Each assay was performed in quadruplicates.

Mp Inoculation and Sampling

The protocol of Mp inoculation and sampling is shown in Fig. 1. Twelve-week-old BALB/c female mice (Charles River Laboratories, Kanagawa, Japan) were assigned to one of two groups. Mice in the SI group were intratracheally inoculated with 50 μg/50 μL of Mp extract alone on day 0. Mice in the RI group were intraperitoneally injected twice with 50 μg/250 μL of Mp extract 28 and 21 days before intratracheal injection with 50 μg/50 μL of the Mp extract (day 0). In RI group, the Mp extract was administered intraperitoneally

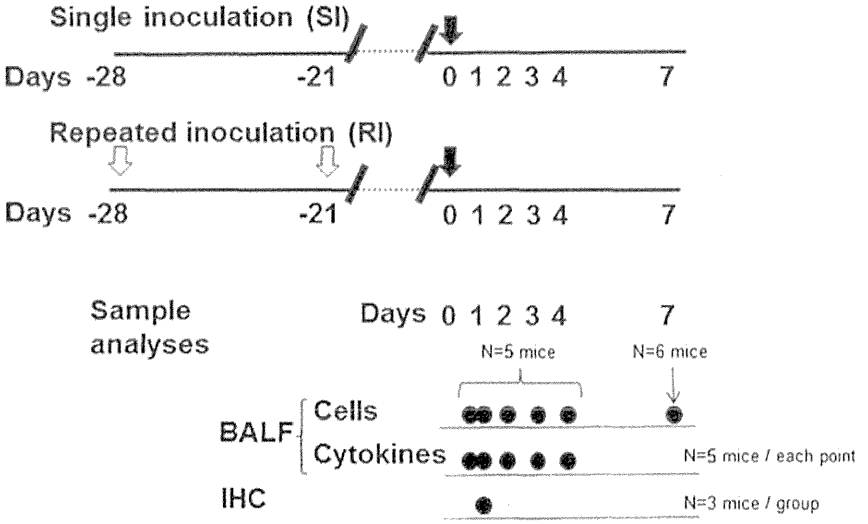

Fig. 1. Mp extract inoculation and sampling protocol. Mice that were SI were administered with Mp extract intratracheally on day 0. Mice that were RI were administered with Mp extract intraperitoneally on days −28 and −21 and were then inoculated intratracheally with the Mp extract on day 0. BALF was obtained on days 0.5, 1, 2, 3, 4, and 7 for analysis of cells (n=5 per each on days 0.5, 1, 2, 3, and 4; n=6 on day 7) and cytokines (n=5 per each point). Lungs were obtained on day 1 for IHC analysis (n=3 per each group).

in order to avoid damaging the trachea by repeated inoculations and to obtain reproducible results. Mice were anesthetized with pentobarbital and lavaged twice with 1 mL of HBSS through a catheter *via* the trachea. BALF was subsequently collected and was centrifuged at 400×*g* for 5 min. Total and differential cell counts were calculated for BALF (number of mice tested was indicated in each figure) collected on days 0.5, 1, 2, 3, 4, and 7. Cytokine levels were measured in BALF collected on days 0.5, 1, 2, 3, and 4 (number of mice tested was indicated in each figure). In preliminary experiments, any of cytokines tested were not detected in BALF collected on day 7. Immunohistochemical analysis of IL-23 expression in lungs (number of mice tested was indicated in each figure) was conducted on day 1 because preliminary experiments demonstrated that the percentage of IL-23-positive cells in lung tissue was higher on day 1 than on day 0.5. All animal experiments were performed in accordance with the Institutional Animal Care and Research Advisory Committee at Kyorin University.

Cytokine Measurements

The levels of IL-17, keratinocyte-derived cytokine (KC), tumor necrosis factor-α (TNF-α), IL-6, interferon-γ (IFN-γ), IL-4, and IL-12 in BALF were measured using a protein multiplex immunoassay kit (Biosource International, Camarillo, CA, USA) and a multiplex bead array (Luminex 200, Luminex, Austin, TX, USA), according to the manufacturer's instructions. The concentrations of IL-17 and IL-23 in the primary cell cultures were measured using an enzyme-linked immunosorbent assay kit (Quantikine mouse IL-17, Quantikine mouse IL-23, R&D Systems, Minneapolis, MN, USA).

IL-23 Immunohistochemistry

Mouse lungs were removed on day 1, fixed in 4 % paraformaldehyde, embedded in paraffin, and sectioned. After deparaffinization, the specimens were stained with an anti-IL-23 rabbit polyclonal antibody to IL-23p19 (Abcam, Cambridge, UK) at a dilution of 1:100, followed by staining with a peroxidase-conjugated secondary antibody at a dilution of 1:100. The number of IL-23-positive and IL-23-negative cells in ten microscope fields (330×430 μm) per mouse (N=3) was counted in a blinded fashion, and the percentage of the IL-23-positive cells to the total cells was calculated.

Statistical Analysis

All data are expressed as means ± standard deviation. Statistic analysis was performed using the Statistical Package for Social Sciences (SPSS) software (SPSS, Chicago, IL, USA). The Kruskal–Wallis test was

used to evaluate variance among all groups. If a significant variance was found, the Mann–Whitney test was used to determine significant differences between individual groups. $P<0.05$ was considered to represent a statistically significant difference.

RESULTS

Secretion of IL-23 and IL-17 by Primary Cultured Cells

Incubation of mouse lung-derived primary cell cultures with Mp extract (50.0 μg/mL) resulted in an increase in the concentration of IL-23 and IL-17 in the culture media when compared with control (Mp extract 0 μg/mL). The IL-23 concentration peaked at 24 h, whereas the IL-17 concentration peaked at 72 h (Fig. 2a). The IL-23 concentration was significantly higher at 24 and 48 h than that at 72 h. The IL-17 concentration at 48 and 72 h was significantly higher than that at 24 h.

Incubation of the lung-derived primary cultures with increasing concentrations of Mp extract (from 0 to 50 μg/mL) for 72 h resulted in a dose-dependent increase in IL-23 concentration in the media, which peaked at Mp extract concentration of 5.6 μg/mL (Fig. 2b). The IL-17 concentration in the media also significantly increased at an Mp extract concentration of no less than 5.6 μg/mL; to show a concentration dependency up to the highest Mp concentration of 50 μg/mL (Fig. 2c).

Analysis of the Cytokines in BALF

We measured the level of IL-17 and its associated cytokines in the BALF collected from mouse groups that were singly or repeatedly inoculated. IL-17 was detected

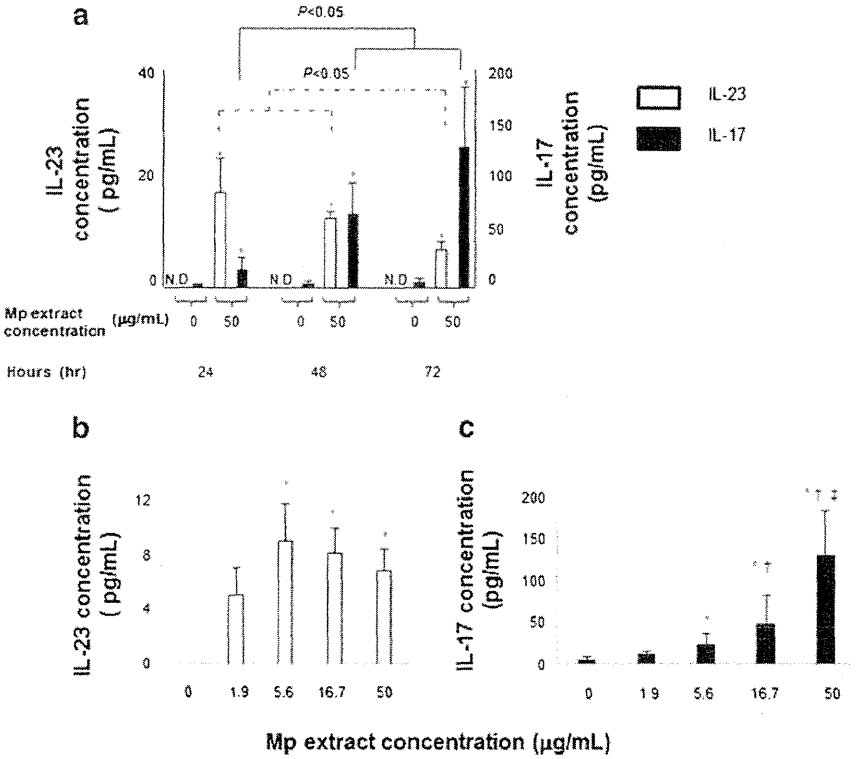

Fig. 2. Effect of incubation of primary lung cultures with Mp extract on the concentration of IL-23 and IL-17 in the culture media. a The concentration of IL-23 (*open bars*) and IL-17 (*solid bars*) in primary lung culture media was measured at 24, 48, and 72 h after the addition of *M. pneumonia* (*Mp*) extract at a final protein concentration of 0 and 50.0 μg/mL. The concentration of IL-23 (b) and IL-17 (c) in primary lung culture media was measured at 72 h after addition of the indicated concentration of the Mp extract. Significant differences are indicated by the following symbols: $^{*}P<0.05$ compared with no added Mp extract (0 μg/mL); $^{\dagger}P<0.05$ compared with the 1.9-μg/mL Mp extract; and $^{\ddagger}P<0.05$ compared with the 5.6-μg/mL Mp extract. Data are expressed as means ± standard deviation. Each assay was performed in quadruplicates. *N.D.* indicates "not detected."

on days 1 and 2 and peaked on day 2 in both the SI and the RI groups. However, on day 3, IL-17 was only detected in the RI group ($P<0.01$) (Fig. 3a). KC levels were significantly higher in the RI group than in the SI group on day 1 ($P<0.05$) (Fig. 3b). TNF-α levels were significantly higher in the RI group than in the SI group on days 0.5 and 1 ($P<0.01$) (Fig. 3c). IL-6 levels were significantly higher in the RI group than in the SI group on days 1 ($P<0.05$) and 2 ($P<0.01$) (Fig. 3d). The IFN-γ levels were low in both groups, and no difference was observed between the SI and RI groups (Fig. 3e). IL-4 levels were significantly higher in the RI group than in the SI group on day 0.5 ($P<0.05$) (Fig. 3f). IL-12 was not detected in either group on any day (data not shown). In preliminary experiments, these cytokines were not detected in the BALF collected on day 7.

Immunohistochemical Analysis of IL-23

Immunohistochemical analysis of lung tissue collected on day 1 indicated the focal distribution of IL-23-positive cells in the alveolar lumen in both groups (SI, Fig. 4a; RI, Fig. 4b). These IL-23-positive cells were morphologically identified as macrophages. The percentage of these cells among the total cell counts tended to be higher in the RI group (16.0±12.5 %) than in the SI group (5.9± 10.3 %).

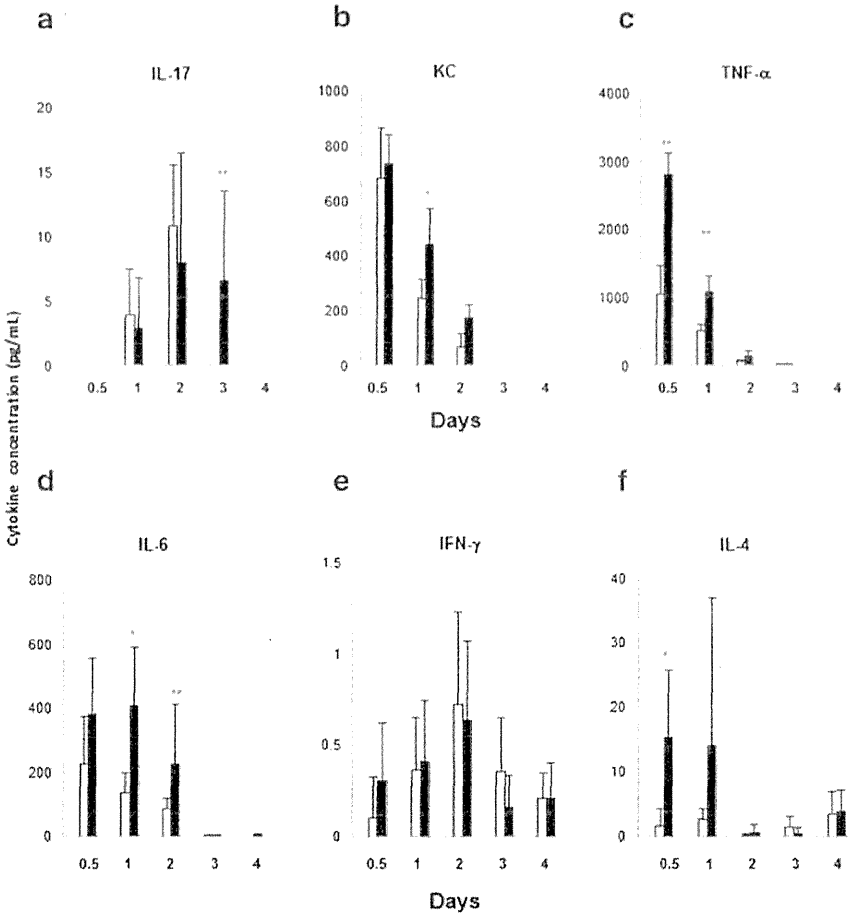

Fig. 3. Cytokine levels in the BALF of the mice. Cytokines in the BALF collected from SI (*open bars*) and RI (*solid bars*) mice on the indicated days following the final Mp extract inoculation were analyzed using a multiplex bead array. The concentrations of IL-17 (a), KC (b), TNF-α (c), IL-6 (d), IFN-γ (e), and IL-4 (f) are shown ($n=5$ per each point). Significant differences between SI and RI groups at specific times are indicated by asterisks: $^{*}P<0.05$, $^{**}P<0.01$. Data are expressed as means ± standard deviation.

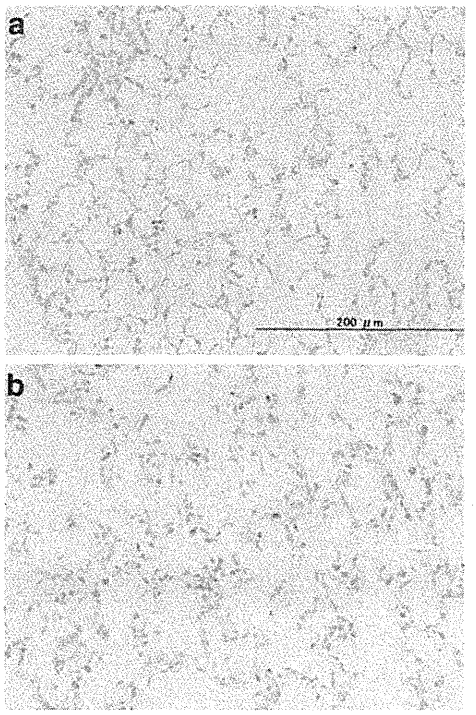

Fig. 4. Immunohistochemical analysis of IL-23 expression. Typical sections of the lungs for IL-23 expression are shown from **a** mice that were SI (n=3) or **b** RI (n=3) with Mp extract.

Analysis of the Cells in BALF

We assayed the effect of single or repeated inoculation of Mp extract into mice on the cell types present in BALF. Total cell counts were significantly higher in the RI group than in the SI group on days 2 (P <0.05), 3 (P<0.01), and 4 (P<0.01) (Fig. 5a). The number of each cell type in BALF is shown in Fig. 5b–d, and the percentages of each cell type comprising the total cell counts are shown in Fig. 5e. Neutrophil counts peaked on day 2 in both groups but were significantly higher in the RI group than in the SI group on days 2 (P <0.05), 3 (P<0.01), and 4 (P<0.01) (Fig. 5b). Macrophage counts were significantly higher in the RI group than in the SI group on days 0.5 and 1 (P<0.05) (Fig. 5c), and lymphocyte counts were significantly higher in the RI group than in the SI group on days 4 and 7 (P<0.05) (Fig. 5d). Neutrophils were the predominant cell type in both the SI and RI groups until day 3 (Fig. 5e).

DISCUSSION

This study established a novel mouse model of Mp pneumonia without the use of alum-adjuvant treatment. We analyzed this mouse model, as well as primary cultured cells prepared from mouse lungs that were stimulated with Mp extract, in order to address our hypothesis that some components of the Mp extract induce IL-17, which is associated with excessive inflammatory cell reactions in Mp pneumonia. We used Mp extract instead of live Mp in order to avoid the infectious aspect of Mp pneumonia.

We confirmed that levels of IL-23 and IL-17 were elevated at 72 h after treatment of mouse lung-derived primary cell cultures with Mp extract (Fig. 2b, c). This is the first report to demonstrate that levels of IL-23 in the media of cultured cells increased in response to the Mp extract (50 μg/mL), followed by an increase in IL-17 levels (Fig. 2a). Although we did not define which kind of cells of lung-derived primary cells produced IL-17, several reports [19–21] have demonstrated that lymphocytes and epithelial cells are the possible sources of IL-17. Previous reports indicated that some components of Mp induced IL-8 production in A549 and BEAS-2B cells [22, 23], which are derived from human alveolar or bronchial epithelial cells. Mp extract also induced IL-6 and TNF-α in RAW 264.7 cells that were derived from murine macrophages [13].

We also demonstrated that the levels of IL-17, KC, TNF-α, IL-6, and IL-4 were higher in the BALF from the RI group of mice than that from the SI group of mice (Fig. 3). IL-17 appears to be mainly generated by Th17 cells, which differentiate from naïve T cells in response to IL-6 and are maintained by IL-23 [19, 20]. Our results suggested that Mp extract might increase the levels of IL-23-expressing cells in the lungs as assessed by immunohistochemistry (IHC) and IL-6 production in BALF from RI mice, and both cytokines led to a Th17-skewed condition. KC stimulation potently induces neutrophil recruitment to the lungs. IL-17 was reported to increase the release of KC [24], and a combination of IL-17 and TNF-α appeared to synergistically enhance KC production [25]. In the present study, the KC levels in BALF were higher in the RI mice than in the SI mice. IL-17 and TNF-α levels were also higher in the RI mice. These data appear to be consistent with a scenario in which Mp increases IL-17, together with KC and TNF-α

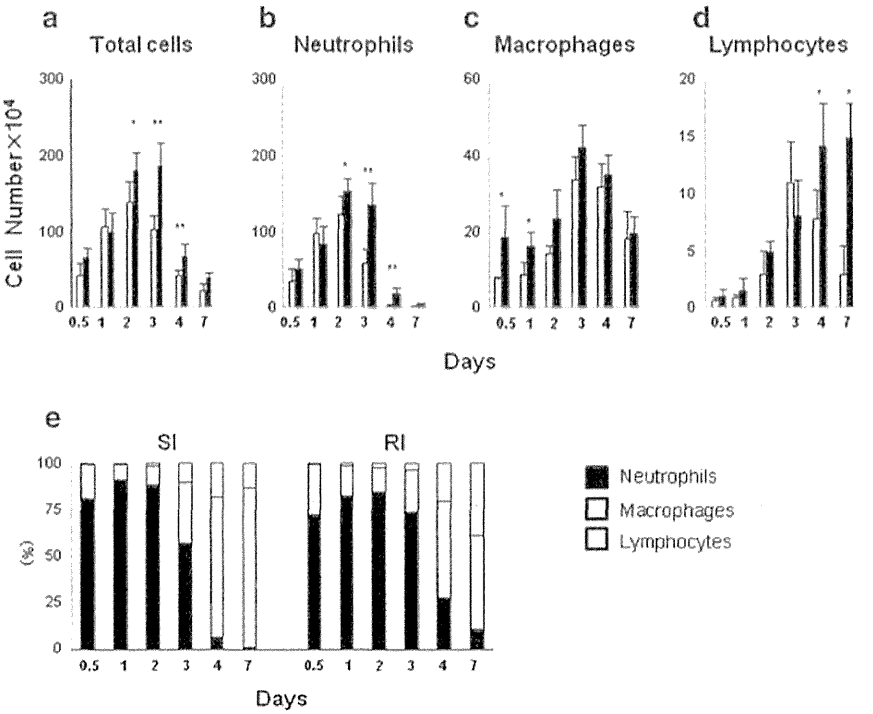

Fig. 5. Cell populations in the BALF of mice. Cell populations in the BALF of SI (*open bars*) and RI groups (*solid bars*) on days 0.5, 1, 2, 3, 4, and 7 after the final Mp extract inoculation are shown. The *vertical axis* represents average cell counts (n=5 each on days 0.5, 1, 2, 3, and 4; n=6 on day 7). Total cell counts (**a**), neutrophil counts (**b**), macrophage counts (**c**), and lymphocyte counts (**d**) are shown. Percentages of neutrophil (*black column*), macrophage (*gray column*), and lymphocyte (*white column*) are shown (**e**). Significant differences are indicated by asterisks: $^{*}P<0.05$, $^{**}P<0.01$. Data are expressed as means ± standard deviation.

and results in the accumulation of neutrophils in the lungs.

The total cell count in the BALF collected from RI mice was significantly higher than that in SI mice (Fig. 5a). In particular, the number of macrophages was higher in the RI mice than in the SI mice on day 0.5 and day 1 (Fig. 5c). This increase in macrophage number was followed by an increase in the number of neutrophils. The number of neutrophils was higher in the RI mice than in the SI mice at the later stages (days 2, 3, and 4) following tracheal inoculation (Fig. 5b). A previous report showed that repeated inoculation of live Mp induced neutrophilic inflammation in BALF similar to that observed in our experiment [26]. In that report, the accumulation of neutrophils in the BALF of mice repeatedly inoculated with live Mp persisted for a longer time when compared with that in mice inoculated with a single live Mp. Thus, repeated live Mp inoculation induced persistent neutrophilic inflammation similar to that observed in the RI mice in the present study.

Neutrophilic accumulation in BALF was observed in humans suffering from Mp pneumonia [9] and in mice inoculated with either live Mp [15] or with Mp extract. Few studies have assessed IL-17 levels in human BALF, although IL-17 levels in the serum of patients suffering from Mp pneumonia were higher than those suffering from streptococcal pneumonia [16]. The mechanisms of how Mp extract induced excessive infiltration of neutrophils in the lung remain not elucidated. In the mice inoculated with the Mp extract in the present study, elevation of IL-17 and associated neutrophil accumulation was observed. Previous studies of mice inoculated with live Mp reported that Mp induced an increase in the concentration of IL-17, KC, TNF-α, IL-6, IFN-γ, and IL-12 in BALF [15, 26–29]. Our current findings are generally consistent with the inflammatory reactions found in humans and mice with different experimental designs and thus suggest that lung inflam-

mation in the RI group, similar to the lung inflammation in human Mp pneumonia [5], seemed exacerbated by repeated Mp stimulation. Some of these reports [28, 29] have also suggested that IL-12 (and the IFN-γ production that it stimulated) plays a role in the increase in neutrophilic alveolar infiltration in mice infected with live Mp, whereas we did not observe an elevation in IL-12 levels.

This study had several limitations. Firstly, it was based on the evaluation of sequential changes of inflammatory mediators and cells. It should be borne in mind that very small concentrations of pro-inflammatory cytokines and mediators may produce biologic actions in a variety of different cell types. Thus, although our result failed to detect IL-12 in BALF perhaps due to dilution effect, IL-12 may still play a role in pro-inflammatory reactions induced by Mp extract.

Secondly, the initial aim of the investigation was to elucidate the noninfectious effect of modulating neutrophilic reactions by Mp extract, in association with IL-17. Thus, we focused on the levels of IL-17 and its related mediators on days 0.5, 1, 2, and 3, which is early in the time frame at which neutrophils were recruited to the lungs of RI mice. Although we did not define which cells of lung-derived primary cells produced IL-17, several reports have demonstrated that lymphocytes and epithelial cells are the possible sources of IL-17 [19–21]. Our result also demonstrated that lymphocytes were recruited to the lung on day 7, which could be linked to the modulatory effects on immune and inflammatory reactions induced by Mp extract. It was possible that increased lymphocytes in BALF after inoculation of Mp extract were Th17 cells, but we did not analyze subpopulations of BALF lymphocytes. Further analysis of the role of IL-17 may help elucidate the role of lymphocytes in this disease.

In summary, we have established a novel mouse model of Mp pneumonia by repeated inoculations of Mp extract without the use of alum-adjuvant treatment. Our current data further indicated that repeated inoculation of the Mp extract (RI group mice) results in increased levels of inflammatory cytokines including IL-17, associated with exacerbated neutrophilic lung inflammation. Although our study did not address the mechanism by which the IL-17 was activated by the Mp extract, or which component of the Mp extract exacerbates the lung lesions, the inflammatory reactions observed upon repeated inoculation of the Mp extract might help in understanding some of the important phenomena in the cellular and molecular pathogenesis of Mp pneumonia in humans.

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