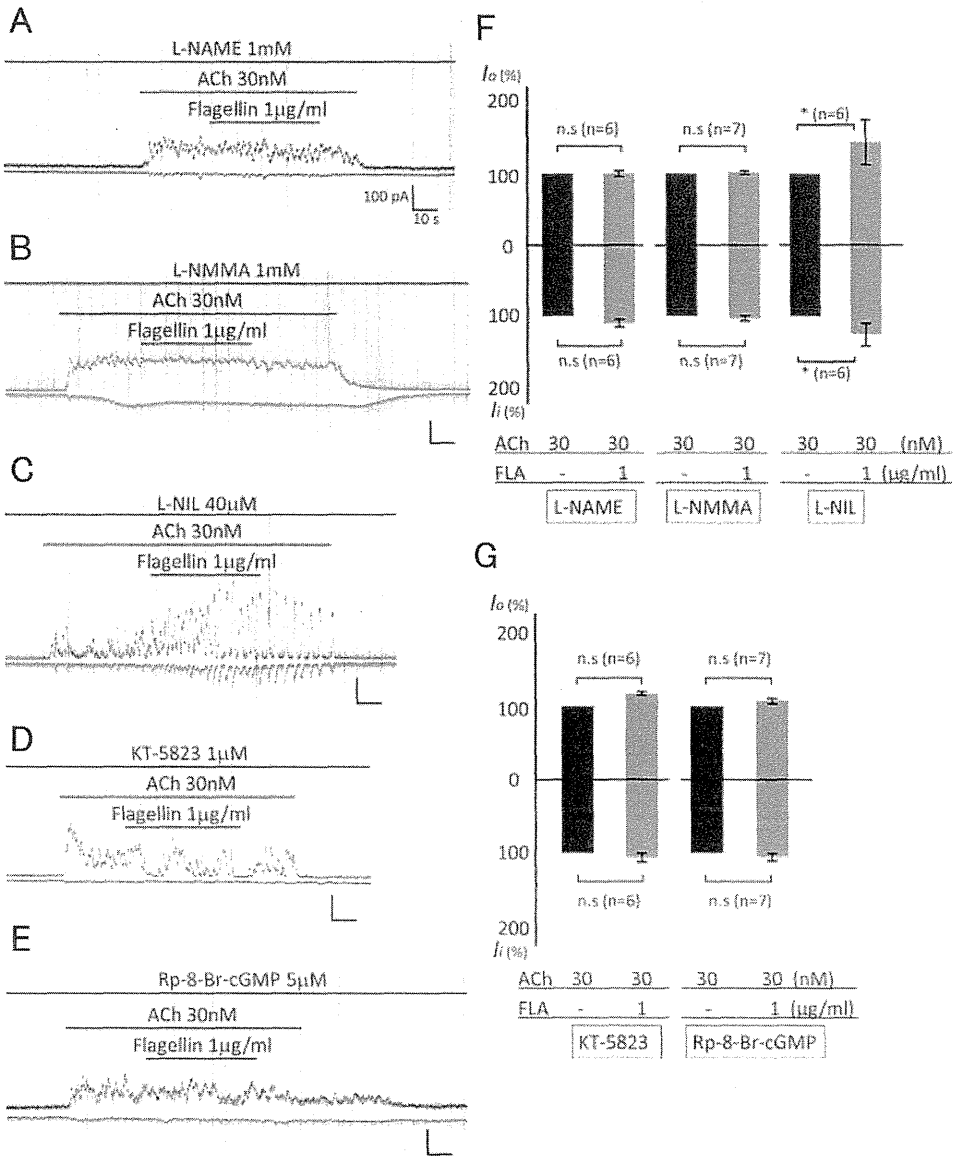

Fig. 5. Representative original recordings showing the effects of nitric oxide synthase (NOS) inhibitors (*A*, *B*, *C*, and *F*) and cGK inhibitors (*D*, *E*, and *G*) on the flagellin-induced potentiation in ACh (30 nM)-evoked ionic currents. *A*: nitro-L-arginine methyl ester (L-NAME) (1 mM), a nonspecific NOS inhibitor, abolished the flagellin-induced potentiating effects on ACh (30 nM)-evoked I_o and I_i. B: Nω-monomethyl-L-arginine acetate (L-NMMA) (1 mM), another nonspecific NOS inhibitor, also abolished the flagellin-induced potentiating effects on ACh (30 nM)-evoked I_o and I_i. *C*: under the presence of *N*6-(1-iminoethyl)-L-lysine hydrochloride (L-NIL) (40 μM), a specific iNOS inhibitor, flagellin still showed significant potentiating effects on ACh (30 nM)-evoked I_o and I_i. *D*: KT-5823 (1 μM), a cGK inhibitor, abolished the flagellin-induced potentiating effects on ACh (30 nM)-evoked I_o and I_i. *E*: Rp-8-Br-cGMP (5 μM), another competitive cGK inhibitor, also abolished the flagellin-induced potentiating effects on ACh (30 nM)-evoked I_o and I_i. *F*: summary of the effects of NOS inhibitors on the flagellin-induced potentiating effects to ACh (30 nM)-evoked I_o and I_i. Both L-NAME and L-NMMA nearly abolished the flagellin-induced potentiation of ACh-evoked I_o and I_i, but L-NIL did not. *G*: summary of the effects of cGK inhibitors on the flagellin-mediated potentiation of ACh (30 nM)-evoked I_o and I_i. Both KT-5823 and Rp-8-Br-cGMP nearly abolished the flagellin potentiation of ACh-evoked I_o and I_i. $^*P < 0.05$.

In the airway mucosal innate immunity system, the importance of flagellin/TLR5 signaling has become increasingly clear (35). Actually, it was reported that flagellin derived from *P. aeruginosa* caused significant expression changes in hundreds of different genes, including the cytokines IL-8, IL-1β, and TNF-α, as well as genes associated with antibacterial factors and NF-κB signaling in human nasal cystic fibrosis epithelial cells (13). Additionally, it has also been reported that flagellin/TLR5 signaling induced MUC5AC overproduction via Duox2/ROS/TACE/TGF-α/EGFR signaling cascade in cultivated 16HBE cells (51). Both reports focused on the pathophysiological significance of flagellin/TLR5 signaling and mucus overproduction in chronic *P. aeruginosa* infections. We also showed the abundant expression of TLR5 on, not only tracheal submucosal gland cells, but also ciliated epithelial cells on the surface of trachea (Fig. 4*B*). These findings are likely to indicate that flagellin/TLR5 signaling in ciliated epithelial cells also plays an important role in airway defenses. Indeed, *P. aeruginosa* infection with mucus overproduction in the airway is a leading cause of morbidity and mortality in patients with chronic inflammatory airway diseases, such as severe COPD and cystic fibrosis. However, airway serous secretion is also important for hydrating airway surfaces and flushing mucin glycoproteins out of the ducts, resulting in the upregulation of mucociliary transport (2, 4, 10, 24, 48). There are many reports concerning the role of TLR5 in the airway mucus secretion, but those in serous secretion are quite limited in number (14, 27). Kunzelmann and colleagues (27) reported that flagellin inhibited Na^+ absorption by the epithelial Na^+ channel without an elevation in $[Ca^{2+}]_i$ and might promote mucociliary clearance of pathogens in airway epithelia (27). Because human airway epithelium is likely to be primarily absorptive (6, 18, 48) and because a major fraction of the airway fluid appears to be derived from the submucosal glands (3, 20, 28, 37), we think it is rather important to understand the effects of flagellin/TLR5 signaling on Cl^- secretion from submucosal glands. Notably, Illek and colleagues (14) reported a very interesting study showing that flagellin activated p38,

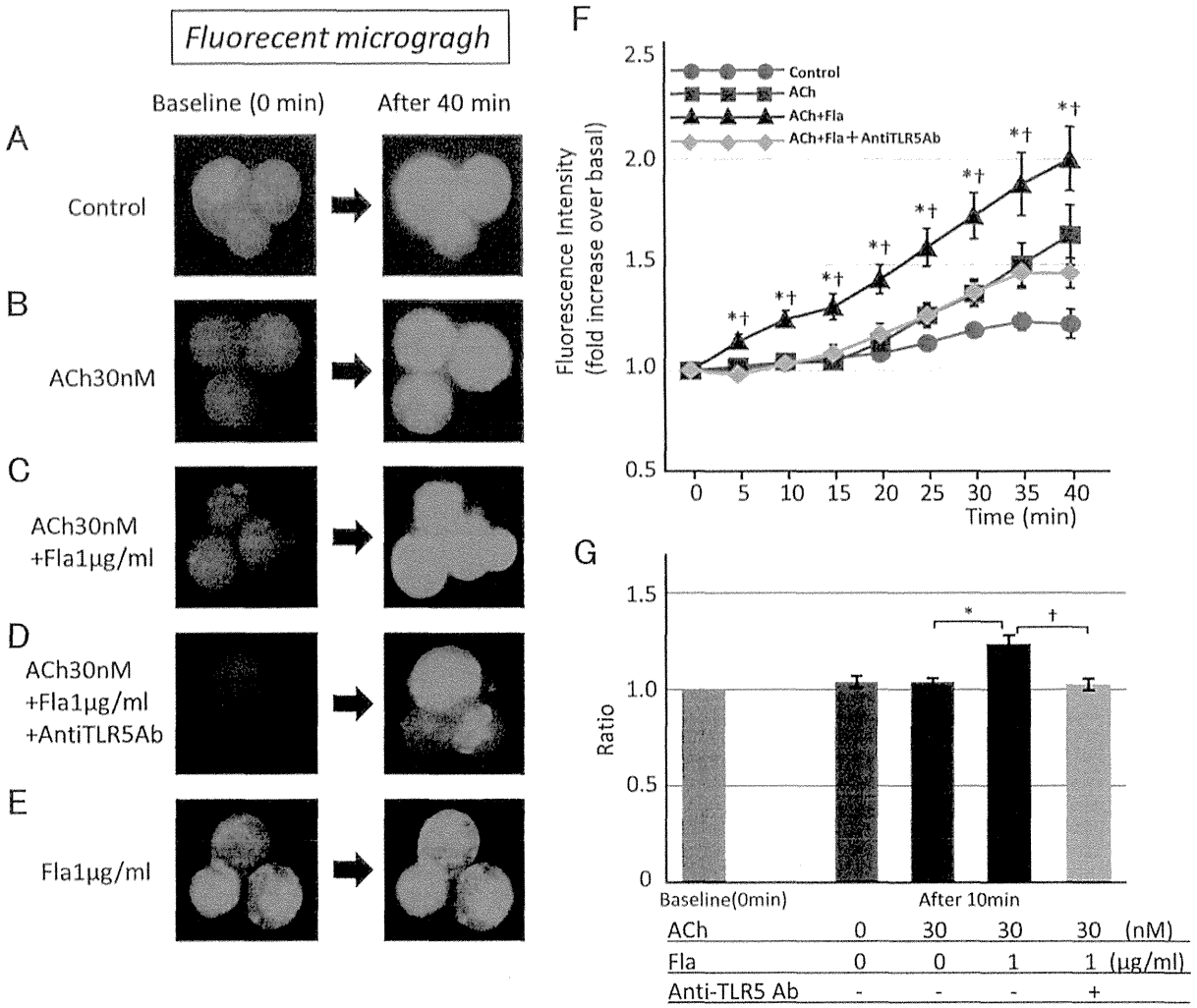

Fig. 6. Detection of endogenous NO synthesis on swine tracheal gland acinar cells by using the NO-dependent fluorescence intensities. *A–E*: representative fluorescence micrograph of NO imaging. Because these clusters of cells became fixed to the surface of the slide glasses spontaneously and tightly, we could observe the time course changes in fluorescence intensities in the same cells. The fluorescence intensities indicate the integrated amount of synthesized NO in the cytosol. *A*: unstimulated control cells did not apparently change in fluorescence intensities even after 40-min observation. *B*: when cells were stimulated by ACh (30 nM), the fluorescence intensities at 40 min clearly and diffusely increased compared with those at baseline. *C*: when cells were stimulated by flagellin (1.0 μg/ml) in combination with ACh (30 nM), the increases in the fluorescence intensities at 40 min were much stronger than those stimulated by ACh alone. *D*: under the presence of 10 μg/ml of anti-TLR5 Ab, these increases in fluorescence intensities diminished to almost the same degree as those stimulated by ACh (30 nM) alone. *E*: cells stimulated by flagellin (1.0 μg/ml) alone did not show significantly increased fluorescence intensities. *F*: time courses of changes in synthesized NO in tracheal gland cells. The fluorescence intensities were calculated by measuring the intensities per unit area from cytosol and compared by estimating the mean intensities of prestimulation (0 min) as 1.0. When cells were stimulated by flagellin in combination with ACh, the fluorescence intensities significantly increased at 5 min or later compared with those stimulated by ACh alone. However, these increases in the fluorescence intensities were completely abolished under the presence of anti-TLR5 Ab, and the intensity curves were almost at the same levels as those when stimulated by ACh alone. $n = 7$, $^*P < 0.05$ to ACh alone. $n = 6$, $\dagger P < 0.05$ to ACh/flagellin/antiTLR5Ab. *G*: summary of the increases in fluorescence intensities of each indicated stimulation at 10 min after measurement. Flagellin (1.0 μg/ml) significantly increased the intensities up to 1.2-fold compared with ACh (30 nM)-stimulated cells, but these upregulations were completely abolished under the presence of anti-TLR5 Ab. $n = 7$, $^*P < 0.05$ to ACh alone. $n = 6$, $\dagger P < 0.05$ to ACh/flagellin/antiTLR5Ab.

NF-κB, IL-8, and CFTR-dependent Cl^- secretion without altering the tight-junction permeability in Calu-3 cells, a serous-like cell line. They mentioned that, during *P. aeruginosa* infection, flagellin could be expected to increase CFTR-dependent Cl^- secretion and promote bacterial clearance from the airways. Indeed, these conclusions are basically in line with ours, but there are several fundamental differences between their report and ours. First, we focused on Ca^{2+}-dependent Cl^- secretion, not cAMP/CFTR-dependent Cl^- secretion. Second, flagellin itself did not cause any ionic currents in our experiments. Third, the flagellin-induced potentiating effect was reproduced only when the cells were weakly stimulated by physiologically relevant low doses of ACh (30 nM) but not by robust doses of ACh (1 μM) in our experiments. At present, we do not have a clear explanation for these discrepancies, but they could be due to differences between freshly isolated gland cells and cultivated epithelial cell lines and also those between CaCC and CFTR.

Generally, it is known that there are two major pathways in the Cl^- secretion from tracheal submucosal glands. One is characterized by the CFTR Cl^- channel, which is mainly activated by cAMP; the other is characterized by CaCC.

Indeed, Vaadnrager and colleagues (47) reported that cGMP could phosphorylate and activate the CFTR Cl^- channel. However, in our experiments, using proper channel inhibitors and ion substitution experiments, we have confirmed that the ACh-induced I_o and I_i were dependent on $[Ca^{2+}]_i$ (17, 38, 39). Additionally, we have reported that maneuvers anticipating a rise in cAMP failed to potentiate and rather decreased ionic currents in tracheal gland cells (45). These findings suggest that the Cl^- currents observed in our experiments are due mainly to CaCC, not the CFTR Cl^- channel. However, our considerations do not mean to exclude the involvement of CFTR in airway defenses. Further investigations will be needed to make it clear in the future. Concerning the importance of CaCC, it is noteworthy that TMEM16A has recently been identified as an essential component of Ca^{2+}-dependent Cl^- secretion and widely exists in the airway surface epithelium and submucosal glands (28, 33). Additionally, it was also reported that human, swine, and ferret tracheal submucosal glands respond much more strongly to cholinergic stimulants than to other agents (19, 20, 49). An in vivo study has also demonstrated that cholinergic agents were much more potent stimulators of gland secretion than were adrenergic agonists, as ascertained by the hillock formations from a powdered tantalum layer coating the airway surface (32). Furthermore, it was reported that Ca^{2+}-dependent Cl^- secretion is upregulated as a compensation mechanism in cystic fibrosis airways, where cAMP-dependent Cl^- secretion is completely attenuated (29). From these considerations, we believe that CaCC must be very important in the physiological basal airway secretion, which is stimulated by vagal nerve tonus in vivo. As shown in previous reports (16, 17, 31, 38, 39, 44, 45), the airway basic serous secretion could be carried by intracellular calcium oscillations induced by physiologically relevant, low concentrations of ACh (30 nM) on freshly isolated tracheal submucosal glands. In the case of cultivated cells, strong stimulation by a considerably high dose of the agonist would be needed to obtain electrophysiological responses. However, we have also shown that excessively robust stimulation by ACh (300 nM or more) caused desensitization and that the cells did not respond to further stimulations in the case of freshly isolated cells (17, 31, 44, 45). One of our experimental advantages is that we are able to detect the basal secretions as delicate oscillatory ionic currents induced by very low concentrations of ACh and investigate the effects of various physiological exogenous regulators. In several other kinds of exocrine glands, such as parotid gland, salivary gland, and pancreatic gland, it is also known that these calcium oscillations are essential for basic secretions (5, 21, 36, 43, 46). We think that flagellin likely enhances the ACh-activated signaling pathway through Ca and NOS but does not activate the same pathway. Our study showed that flagellin alone did not generate any ionic currents (Fig. 1*D*). Additionally, the intracellular calcium assays revealed that flagellin did not increase the $[Ca^{2+}]_i$ after the stimulation by 30 nM of ACh (Fig. 2). Without any increase in $[Ca^{2+}]_i$, flagellin (0.5–1.0 μg/ml) significantly potentiated both the ionic currents (Fig. 1*A*) and NO synthesis (Fig. 6*G*) induced by 30 nM of ACh. These findings strongly suggested that flagellin increased the sensitivity of NOS to Ca^{2+}, which was properly raised by low doses of ACh. When the cells were stimulated by very high concentrations of ACh, they were desensitized, and we think that flagellin could no longer show any potentiation (Fig. 1*G*).

Concerning the relationship between flagellin/TLR5 and NO/cGMP/cGK signaling, there are some useful reports describing the importance of iNOS. Mizel and coworkers (30) reported that flagellin induced NO synthesis via an increase in iNOS mRNA and activation of the iNOS promoter in HeNC2 cells, a murine macrophage cell line. They mentioned that this flagellin-induced upregulation of iNOS required signaling via heteromeric TLR5/TLR4 complexes (30). Kumer and coworkers (26) reported that flagellin enhanced bacterial clearance and significantly improved the disease outcome via an upregulation of iNOS in the C57BL/6 mouse model of *P. aeruginosa* keratitis. Unlike these two reports, we do not think iNOS was involved in the flagellin-induced rapid potentiation of basal secretion investigated here. As shown in Fig. 5, *A*, *B*, *C*, and *F*, the iNOS inhibitor L-NIL did not abolish the flagellin-induced potentiating effects although the nonspecific NOS inhibitors L-NAME and L-NMMA did. Additionally, the response time seems be too short to upregulate iNOS transcription because our studies were focused only on the very rapid (milliseconds to seconds) effects of flagellin/TLR5 on triggered-ionic currents. These findings suggested that constitutive NOS, not iNOS, should be involved in the flagellin/TLR5-mediated potentiation of tracheal gland secretion. However, we cannot exclude the possibility that iNOS has additively important roles in the potentiation of airway serous secretion at a much later phase in airway chronic inflammation.

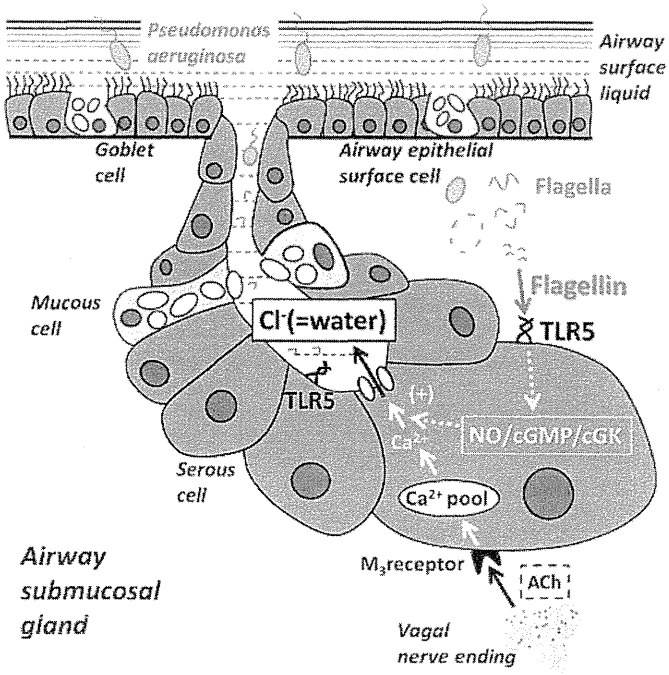

Fig. 7. Drawing delineating the proposed pathways of flagellin/TLR5 signaling in electrolyte secretion in tracheal submucosal gland acinar cells. In the airways with chronic airway *Pseudomonas aeruginosa* (*P. aeruginosa*) infection, if flagellin can reach both around the submucosal glands and the lumen of the gland ducts, TLR5 is likely to be activated and show potentiating effects on Cl^- secretion induced by physiologically relevant low doses of ACh from vagal nerve ending in a NO/cGMP/cGK-dependent manner. Thus we believe that, not only LPS/TLR4 signaling, but also flagellin/TLR5 signaling is likely involved in hypersecretion, resulting in exacerbations in chronic inflammatory airway diseases with repeated *P. aeruginosa* infection.

Finally, there remains the clinical significance of flagellin/TLR5 in chronic airway *P. aeruginosa* infection. Differing from a report describing the importance of heteromeric TLR5/TLR4 complexes (30), our present study showed that the cross reaction between flagellin and TLR4 or between LPS and TLR5 was not involved in these potentiations (Fig. 3). We believe that, not only LPS/TLR4 signaling, but also flagellin/TLR5 signaling is likely involved in hypersecretion, resulting in exacerbations in chronic inflammatory airway diseases with repeated *P. aeruginosa* infection (Fig. 7). If our data can be recapitulated in vivo, we think that these findings will advance the field in the future. Furthermore, it will be complementary if tracheal submucosal glands could be treated with wild-type *P. aeruginosa* compared with an isogenic flagellin-deficient strain (such as PAK/fliC) *P. aeruginosa* and show the significance of flagellin in airway secretion. This will provide some in vivo relevance to the potential role of flagellin in chronic airway diseases.

In conclusion, we revealed a novel potentiating effect of flagellin/TLR5 signaling in airway serous secretion that was independent of LPS/TLR4 signaling. These findings suggest that pathogens with these ligands for TLRs are able to cause hypersecretion in the airway even in cases of colonization. We believe that, not only TLR4, but also TLR5 could be a new therapeutic candidate for controlling airway secretion in chronic inflammatory airway diseases, such as COPD and even cystic fibrosis.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: S.M., T.T., and M.N. conception and design of research; S.M. and T.T. performed experiments; S.M., T.T., and K.M. analyzed data; S.M. prepared figures; S.M. and T.T. drafted manuscript; M.N., T.K., M.K., Y.M., M.E., T.N., and M.I. interpreted results of experiments.

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

Kazumasa Ohashi [a], Atsuyasu Sato [b], Toshinori Takada [c], Toru Arai [d], Takahito Nei [a,e], Yasunori Kasahara [f], Natsuki Motoi [a], Masayuki Hojo [g], Shinya Urano [a], Haruyuki Ishii [h], Masanori Yokoba [i], Ryosuke Eda [j,k], Hideaki Nakayama [c], Yasuyuki Nasuhara [l], Yoshiko Tsuchihashi [m], Chinatsu Kaneko [a], Hiroko Kanazawa [a], Masahito Ebina [n], Etsuro Yamaguchi [o], Jacqueline Kirchner [p], Yoshikazu Inoue [b], Koh Nakata [a,*], Ryushi Tazawa [a]

[a] *Bioscience Medical Research Center, Niigata University Medical and Dental Hospital, 1-754 Asahimachi-dori, Niigata 951-8520, Japan*
[b] *Department of Pulmonary Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA*
[c] *Division of Respiratory Medicine, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan*
[d] *National Hospital Organization (NHO) Kinki-Chuo Chest Medical Center, Osaka, Japan*
[e] *Division of Pulmonary Medicine, Nippon Medical School, Tokyo, Japan*
[f] *Department of Respirology, Graduate School of Medicine, Chiba University, Chiba, Japan*
[g] *Division of Respiratory Medicine, National Center for Global Health and Medicine, Tokyo, Japan*
[h] *Department of Respiratory Medicine, Kyorin University School of Medicine, Kanagawa, Japan*
[i] *Department of Clinical Physiology, Kitasato University School of Allied Health Sciences, Tokyo*
[j] *NHO Yamaguchi-Ube Medical Center, Ube, Japan*
[k] *Kurashiki Municipal Hospital, Kurashiki, Japan*
[l] *First Department of Medicine, Hokkaido University School of Medicine, Sapporo, Japan*
[m] *Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan*
[n] *Department of Respiratory Medicine, Tohoku University Medical School, Sendai, Japan*
[o] *Division of Respiratory Medicine and Allergology, Department of Medicine, Aichi Medical University School of Medicine, Aichi, Japan*
[p] *Amgen, Inc, Seattle, WA, USA*

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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.

* Corresponding author. Tel.: +81 25 227 0847; fax: +81 25 227 0377.
E-mail address: radical@med.niigata-u.ac.jp (K. Nakata).

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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,

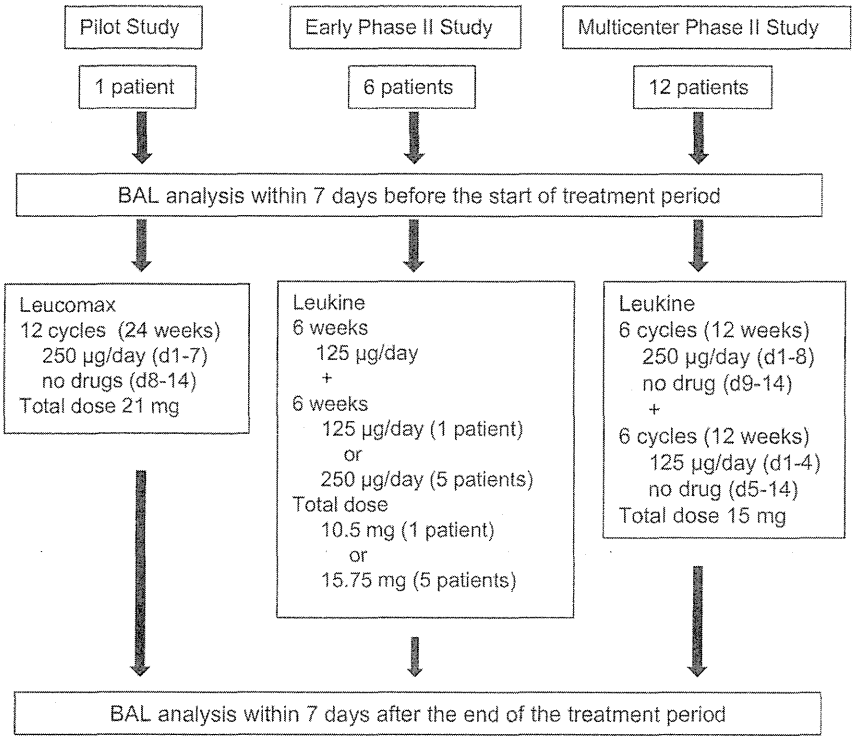

Figure 1 Profile of the study cohort.

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.

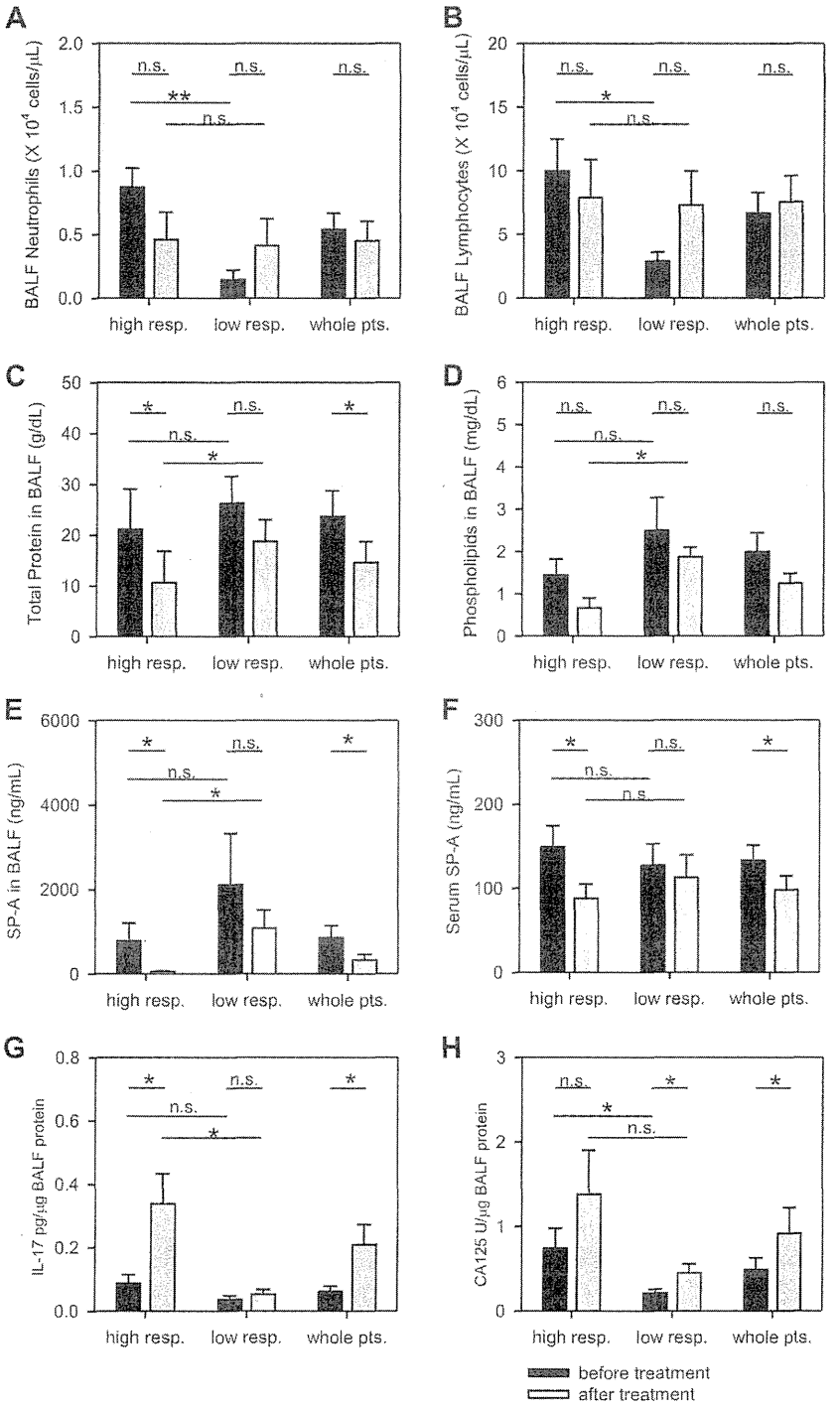

Figure 2 The findings of BAL fluid obtained from high responders (high resp.) and low responders (low resp.) before therapy (black bars) and after therapy (gray bars). The cell counts (A, B) and markers of clearance including total protein (C), phospholipid (D), surfactant protein A (E) compared with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

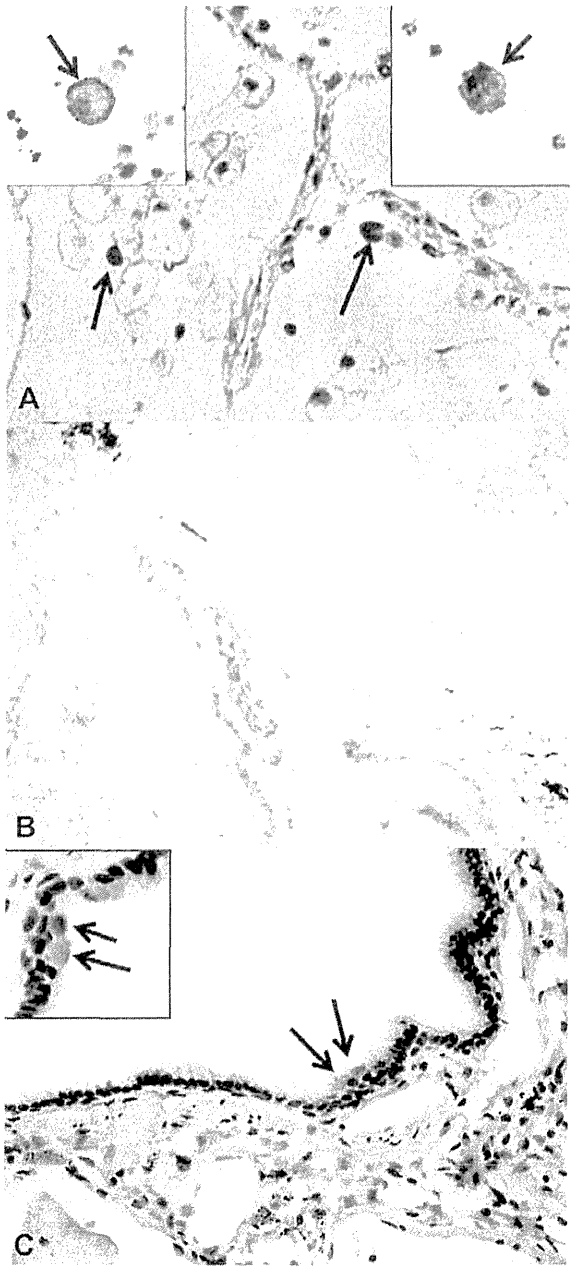

Figure 3 Immunohistochemical detection of IL-17 (panel A and B) and CA125 (panel C) in the lungs of autoimmune PAP (panel A and C) and the normal lung (panel B). The arrows mean the positively stained cells. Insets show higher magnification of the cells of the lungs (left) and a BALF sample (right) (X400).

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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We believe that our study is the first to identify different proportions of obstructive and restrictive airway disease between the main ethnic groups in sarcoidosis, and also the first to report an association of functional impairment with age. Despite a weak evidence base, sarcoidosis patients are often prescribed inhaled corticosteroids and β-agonists for respiratory symptoms regardless of their pattern of lung function at presentation [12]. Large airway obstruction in sarcoidosis is associated with increased mortality [13] and our data suggest that these patients may represent an under-diagnosed group. Further studies are needed to confirm these demographic associations, as well as to assess disease progression and response to inhaled therapies in this particular clinical phenotype of sarcoidosis.

M. Thillai*, L. Potiphar*, C. Eberhardt*, M. Pareek*, R. Dhawan#, O.M. Kon#, M. Wickremasinghe#, A. Wells", D. Mitchell" and A. Lalvani*

*National Heart and Lung Institute, Imperial College London, #St Mary's Hospital, and "Interstitial Lung Unit, Imperial College London NHS Healthcare Trust, London, UK.

Correspondence: M. Thillai, Wright-Fleming Institute, National Heart and Lung Institute, Imperial College London, Praed Street, London W2 1NY, UK. E-mail: m.thillai@imperial.ac.uk

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Reduced GM-CSF autoantibody in improved lung of autoimmune pulmonary alveolar proteinosis

To the Editors:

Pulmonary alveolar proteinosis (PAP) is a rare lung disease characterised by excessive accumulation of surfactant materials within alveolar spaces [1]. Patients with autoimmune PAP (aPAP) present a high level of granulocyte-macrophage colony-stimulating factor (GM-CSF) autoantibodies (GM-Ab) in the serum as well as in bronchoalveolar lavage fluid (BALF) [2]. GM-Ab neutralise the biological activity of GM-CSF in the lung [3], impairing terminal differentiation of alveolar macrophages and macrophage-mediated pulmonary surfactant clearance [4].

Based on the aetiology, clinical trials of exogenous GM-CSF supplementation have been carried out by a number of physicians with variable response rates ranging from 40 to 62% [5–9]. Previously, we reported that in three patients who received a pilot GM-CSF inhalation therapy, oxygenation was improved and the concentration of GM-Ab in BALF was reduced [7]. BONFIELD *et al.* [8] also reported that the serum titre of GM-Ab was reduced during successful treatment of aPAP with subcutaneously injected GM-CSF. However, our recent phase II trial of GM-CSF inhalation involving 35 patients revealed that serum levels of GM-Ab remained unchanged throughout the therapy, suggesting that GM-CSF inhalation therapy did not affect the production of GM-Ab [9]. Thus, the effect of exogenous GM-CSF administration on GM-Ab levels in the serum remains controversial. This discrepancy may be due to differences in the route

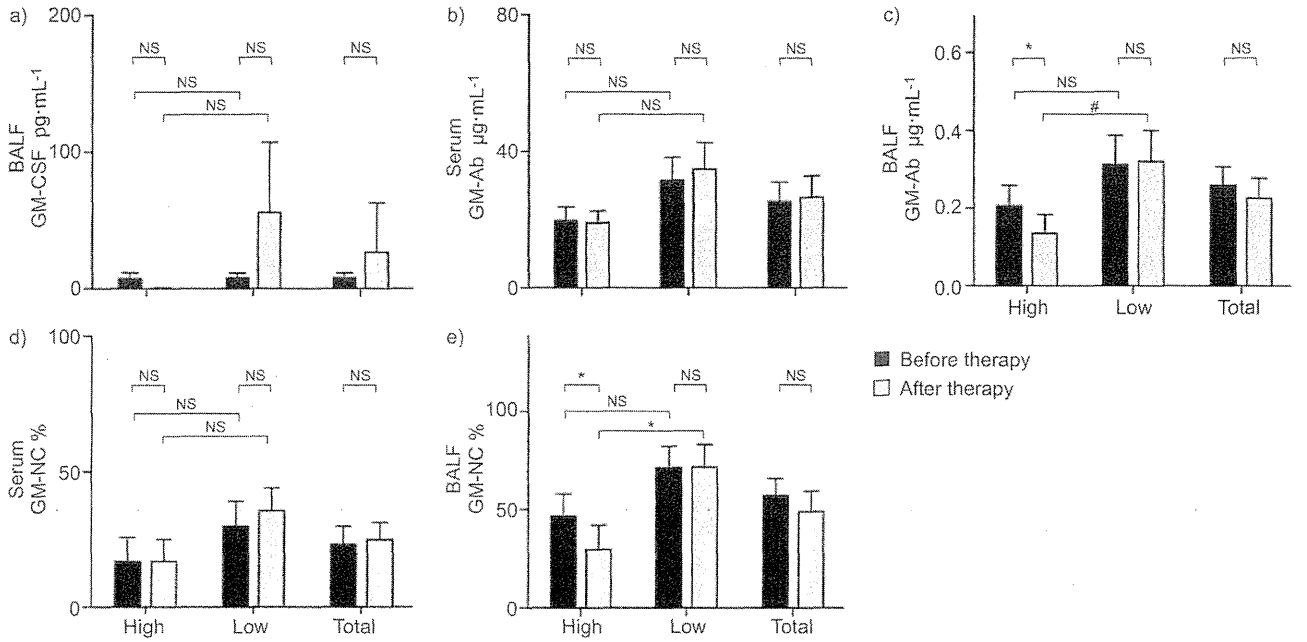

FIGURE 1. The titres of granulocyte-macrophage colony-stimulating factor (GM-CSF) and GM-CSF autoantibodies (GM-Ab) in bronchoalveolar lavage fluid (BALF) and serum obtained from high and low responders and total patients before and after GM-CSF inhalation therapy. a) GM-CSF in BALF. GM-Ab b) in serum and c) in BALF, and GM-CSF-neutralising capacity (GM-NC) d) in serum and e) in BALF are shown. Data are presented as mean and SE. *: $p<0.05$; #: $p=0.054$; NS: nonsignificant; p-values calculated using paired t-test for comparison between normally distributed data before and after therapy, and unpaired t-test for group comparison between high and low responders.

of administration and/or the dose of GM-CSF. Aerosolised GM-CSF reaches the lower respiratory tract and may stimulate immature alveolar macrophages directly to promote terminal differentiation and improve the local clearance of the accumulated surfactant and GM-Ab, although it does not affect the production of systemic GM-Ab.

To test this hypothesis, we performed a retrospective study using preserved BALF obtained through an optional evaluation procedure from the patients that participated in a pilot study (n=1) [7], an early phase II trial (n=6) [9] and a multicentre phase II trial (n=12) [9] of GM-CSF inhalation therapy. Importantly, each patient underwent the bronchoalveolar lavage procedure on the same bronchus of the right middle lobe by the same operator of the same institute within 1 week of the start of, and after the end of, the GM-CSF inhalation therapy period according to the unified standard procedure protocol described previously [9]. BALF was sent to the Niigata University Medical and Dental Hospital (Niigata, Japan) and subjected to centralised analysis. As the median alveolar–arterial oxygen tension difference ($P_{A\text{-}a,O_2}$) improvement was 13 mmHg, we classified the participants into two groups, high responders with an improvement >13 mmHg (n=10) and low responders with an improvement <13 mmHg (n=9), in order to evaluate the relationship between therapeutic response and changes in the level of GM-CSF or GM-Ab in the serum and BALF. There were no significant differences between the two groups in symptoms, including cough and dyspnoea, demographic data, lung function tests, except for $P_{A\text{-}a,O_2}$, or BALF recovery rates.

TABLE 1 Correlation between levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) autoantibodies (GM-Ab) and GM-CSF-neutralising capacity (GM-NC) in serum and bronchoalveolar lavage fluid (BALF)

Variable	Before treatment		After treatment	
	r#	p-value¶	r#	p-value¶
BALF GM-NC and BALF GM-Ab	0.7556	0.0005	0.8430	<0.0001
BALF GM-Ab and serum GM-Ab	0.8616	<0.0001	0.7780	0.0002
BALF GM-NC and serum GM-NC	0.5345	0.0271	0.6087	0.0073

#: Pearson's correlation coefficient; ¶: values calculated using the Pearson's correlation test.

To determine the stoichiometry of GM-CSF during the inhalation treatment, we measured the concentrations of total GM-CSF (*i.e.* autoantibody-bound plus free GM-CSF) in BALF according to a method described previously [10] in order to rule out the possibility that GM-CSF inhalation may augment the production of intrinsic GM-CSF in the lung. The concentration of total GM-CSF did not change during the inhalation therapy in both high and low responders (fig. 1a). Thus, GM-CSF inhalation was not associated with an increase of GM-CSF in the lung. Notably, BALF of low responders tended to contain a high level of GM-CSF that might derive from GM-Ab–GM-CSF complex residing in the alveolar space, as >99% of serum GM-CSF was bound to

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 4.9×10^3, 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.

K. Ohashi[*#], A. Sato[♦], T. Takada[#], T. Arai[+], Y. Kasahara[§], M. Hojo[ƒ], T. Nei[*], H. Nakayama[#], N. Motoi[*], S. Urano[*], R. Eda[,##], M. Yokoba[••], Y. Tsuchihashi[++], Y. Nasuhara[§§], H. Ishii[ƒƒ], M. Ebina[***], E. Yamaguchi[###], Y. Inoue[+], K. Nakata[*] and R. Tazawa[*]**

[*]Bioscience Medical Research Center, Niigata University Medical and Dental Hospital, [#]Division of Respiratory Medicine, Niigata University Graduate School of Medical and Dental Sciences, Niigata, [+]National Hospital Organization (NHO) Kinki-Chuo Chest Medical Center, Osaka, [§]Dept of Respirology, Graduate School of Medicine, Chiba University, Chiba, [ƒ]Division of Respiratory Medicine, National Center for Global Health and Medicine, [ƒƒ]Dept of Respiratory Medicine, Kyorin University School of Medicine, Tokyo, [**]NHO Yamaguchi-Ube Medical Center, Ube, [##]Kurashiki Municipal Hospital, Kurashiki, [••]Dept of Respiratory Medicine, Kitasato University School of Medicine, Kanagawa, [++]Institute of Tropical Medicine, Nagasaki University, Nagasaki, [§§]First Dept of Medicine, Hokkaido University School of Medicine, Sapporo, [***]Dept of Respiratory Medicine, Tohoku University Medical School, Sendai, [###]Division of Respiratory Medicine and Allergology, Dept of Medicine, Aichi Medical University School of Medicine, Aichi, Japan, and [♦]Dept of Pulmonary Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA.

Correspondence: K. Nakata, Bioscience Medical Research Center, Niigata University Medical and Dental Hospital, 1-754 Asahimachi-dori, Niigata 951-8520, Japan. E-mail: radical@med.niigata-u.ac.jp

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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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ORIGINAL ARTICLE

Drug-induced lung injury associated with sorafenib: analysis of all-patient post-marketing surveillance in Japan

Yuka Horiuchi-Yamamoto · Akihiko Gemma · Hiroyuki Taniguchi · Yoshikazu Inoue · Fumikazu Sakai · Takeshi Johkoh · Kiminori Fujimoto · Shoji Kudoh

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Abstract
Background Sorafenib is a multi-kinase inhibitor currently approved in Japan for unresectable and/or metastatic renal cell carcinoma and unresectable hepatocellular carcinoma. Although drug-induced lung injury has recently been the focus of interest in Japanese patients treated with molecular targeting agents, the clinical features of patients receiving sorafenib remain to be completely investigated.
Methods All-patient post-marketing surveillance data was obtained within the frame of Special Drug Use Investigation; between April 2008 and March 2011, we summarized the clinical information of 62 cases with drug-induced lung injury among approximately 13,600 sorafenib-treated patients in Japan. In addition, we summarized the results of evaluation by a safety board of Japanese experts in 34 patients in whom pulmonary images were available. For the calculation of reporting frequency, interim results of Special Drug Use Investigation were used.
Results In the sets of completed reports (2,407 in renal cell carcinoma and 647 in hepatocellular carcinoma), the reporting frequency was 0.33 % (8 patients; fatal, 4/8) and 0.62 % (4 patients; fatal, 2/4), respectively. Major clinical symptoms included dyspnea, cough, and fever. Evaluation of the images showed that 18 cases out of 34 patients had a pattern of diffuse alveolar damage. The patients with hepatocellular carcinoma showed a greater incidence and earlier onset of lung injury than those with renal cell carcinoma.
Conclusion Although the overall reporting frequency of sorafenib-induced lung injury is not considered high, the radiological diffuse alveolar damage pattern led to a fatal

Y. Horiuchi-Yamamoto (✉)
Pharmacovigilance, Medical Affairs, Bayer Yakuhin, Ltd.,
4-9 Umeda 2-chome, Kita-ku, Osaka 530-0001, Japan
e-mail: yuka.yamamoto@bayer.com

A. Gemma
Department of Pulmonary Medicine/Infection and Oncology,
Internal Medicine, Nippon Medical School,
Tokyo 113-8603, Japan

H. Taniguchi
Department of Respiratory Medicine and Allergy,
Tosei General Hospital, Seto, Aichi 489-8642, Japan

Y. Inoue
Department of Diffuse Lung Diseases and Respiratory Failure,
Clinical Research Center, National Hospital Organization Kinki-Chuo Chest Medical Center, Sakai, Osaka 591-8555, Japan

F. Sakai
Department of Diagnostic Radiology, International Medical Center, Saitama Medical University, Hidaka,
Saitama 350-1298, Japan

T. Johkoh
Department of Radiology, Kinki Central Hospital of Mutual Aid Association of Public School Teachers,
Itami, Hyogo 664-8533, Japan

K. Fujimoto
Department of Radiology, Kurume University
School of Medicine, and Center for Diagnostic Imaging,
Kurume University Hospital, Kurume, Fukuoka 830-0011, Japan

S. Kudoh
Fukujuji Hospital, Japan Anti-tuberculosis Association as Public Interest Incorporated Foundation, Kiyose,
Tokyo 204-8522, Japan