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Letter to the Editor

Modified eosinophil adhesion in pulmonary alveolar proteinosis caused by *CSF2RA* deletion

Dear Editor,

Pulmonary alveolar proteinosis (PAP) is characterized by impaired surfactant homeostasis and accumulation of lipoproteinaceous material within the alveolar space, resulting in the development of respiratory failure.¹ There are three distinct forms of PAP: autoimmune, secondary, and hereditary. Autoimmune PAP is caused by the disruption of GM-CSF signaling by autoantibodies to GM-CSF. Secondary PAP is related to underlying diseases such as hematologic diseases. Hereditary PAP is associated with a deficiency of gene encoding such as surfactant protein (SP)-B, SP-C, or ABCA3.

GM-CSF signaling plays important role(s) in the pathogenesis of PAP. GM-CSF receptor (GM-CSF-R) is composed of the binding α chain and the common β (β c) chain that is also utilized by IL-3 and IL-5. Recently, it was found that some cases of hereditary PAP show defects in GM-CSF-R expression.^{2–4} There are also cases of PAP caused by mutations in the coding region of the GM-CSF-R α gene (*CSF2RA*).^{2,3} In these PAP cases with GM-CSF receptor dysfunction, levels of GM-CSF in both BAL fluid and serum are increased. As autoimmune PAP is typically characterized by neutralization of GM-CSF in the lung, an elevated GM-CSF level is useful in distinguishing between autoimmune and GM-CSF receptor dysfunction PAP.

We previously reported a case of elderly-onset hereditary PAP caused by a *CSF2RA* deletion.⁵ Briefly, a previously healthy 77-year-old non-smoking woman was referred to our hospital because of gradually developed dyspnea on exertion.⁵ Her chest roentgenograph was normal at a medical check-up 3 years ago. She was diagnosed as having PAP by typical findings on a chest computed tomography scan and findings of BAL fluid. She tested negative for GM-CSF autoantibodies and had no underlying disease. The serum level of GM-CSF at admission was elevated to 488.3 pg/ml (<2 pg/mL). GM-CSF-stimulated phosphorylation of signal transducer and activator of transcription (STAT) 5 in PBMCs was not observed, and GM-CSF-R α expression was defective in the patient's blood cells.⁵ Genetic screening revealed a homozygous, single-base C > T mutation at nt 508 in exon 7 of *CSF2RA*, which gave rise to a stop codon.⁵ Further, high-resolution single nucleotide polymorphism array analysis confirmed a 22.8-Mb loss of heterozygosity region in Xp22.33p22.11, encompassing the *CSF2RA* gene.⁵ Important parts of the case presentation, including figures, are reproduced in the [Supplementary Materials](#). Further details are in the original report.⁵

GM-CSF plays important roles in the differentiation, cell survival, and activation of eosinophils.⁶ Therefore, eosinophil functions of PAP patients may be different from those of healthy volunteers. However, as far as we know, there is no study of eosinophil functions in PAP patients, although neutrophil dysfunctions in PAP caused by GM-CSF autoantibodies have already been reported.⁷ Further, eosinophil functions of PAP patients caused by *CSF2RA* deletions have not been investigated. So, in this study, we examined the adhesion of eosinophils obtained from a patient with PAP induced by a *CSF2RA* deletion.

Eosinophils were isolated from peripheral blood collected from the PAP patient caused by the *CSF2RA* deletion or non-atopic healthy volunteers using negative selection with immunomagnetic beads as described previously.^{8–10} We received approval from the Ethical Committee of Saitama Medical University, and informed consent was obtained before sample collection. We obtained blood eosinophils from the patient after whole lung lavage and improvement of symptoms. Eosinophils were incubated with IL-3, IL-5, or GM-CSF in intercellular adhesion molecule (ICAM)-1-coated plates for 20 min. In selected experiments, eosinophils were incubated with LTD₄ or platelet-activating factor (PAF) (10 nM for both) in ICAM-1-coated plates. Following washing the assay plates, eosinophil adhesion to ICAM-1 was assessed based on the residual eosinophil peroxidase (EPO) activity of adherent eosinophils.^{8–10} Results were compared using a one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons, or a paired *t* test for analysis of the differences between two groups. Values of *P* < 0.05 were considered statistically significant.

There was no difference in spontaneous eosinophil adhesion between the PAP patient with the *CSF2RA* deletion and healthy volunteers (Fig. 1A–C). IL-3 and IL-5, but not GM-CSF, induced eosinophil adhesion to ICAM-1 (Fig. 1A–C), suggesting that the response to GM-CSF is impaired in eosinophils of the patient, and the impairment is due to the defect of GM-CSF-R α , not of GM-CSF-R β (β c), of the eosinophils. Furthermore, IL-3- or IL-5-induced eosinophil adhesion in the patient was greater than that in healthy volunteers (Fig. 1A–C). Moreover, eosinophil adhesion was also induced by LTD₄ or PAF in the patient, and the adhesion of the patient was significantly greater than that of healthy volunteers (Fig. 2A, B), suggesting hyper-responsiveness of the patient eosinophils in response to other stimuli irrespective of the β c chain.

Our findings are consistent with the findings for leukocytes (mainly neutrophils) in the patient. CD11b expression of leukocytes from the patient with hereditary PAP was not increased by stimulation with GM-CSF.⁵ Although we did not examine the CD11b

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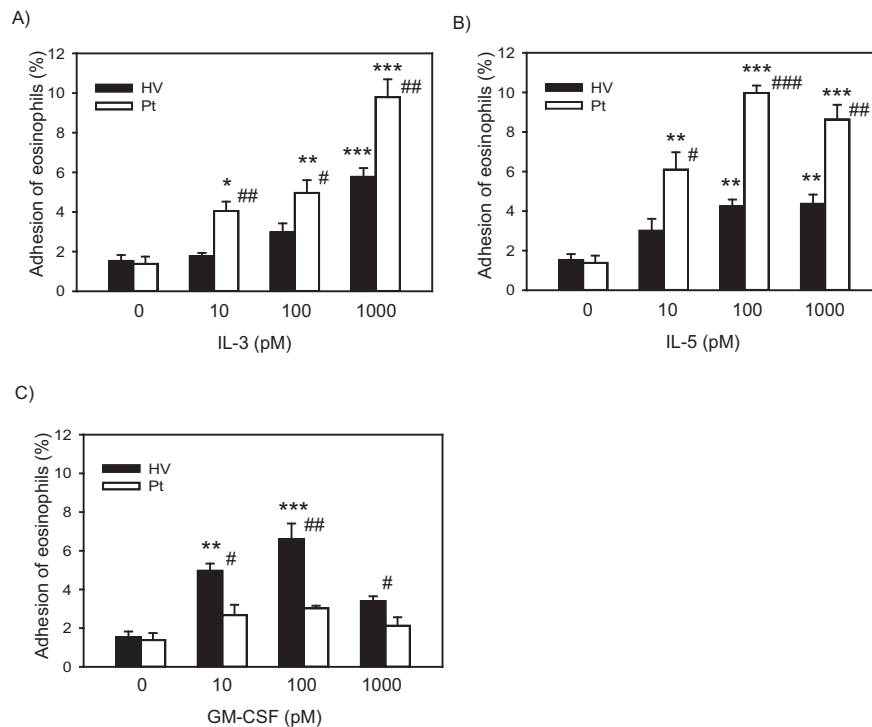


Fig. 1. Effect of IL-3, IL-5, or GM-CSF on adhesion of eosinophils obtained from the PAP patient with the *CSF2RA* deletion. Eosinophils ($100 \mu\text{l}$ of 1×10^5 cell/ml) from the PAP patient with the *CSF2RA* deletion (Pt) or healthy volunteers (HV) were incubated with IL-3 (10 pM/100 pM/1000 pM) (A), IL-5 (10 pM/100 pM/1000 pM) (B), or GM-CSF (10 pM/100 pM/1000 pM) (C) at 37°C for 20 min. The adhesiveness of eosinophils to recombinant human ICAM-1 was assessed using residual EPO assays ($n = 4$) * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. eosinophil adhesion without stimulation. # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ vs. eosinophil adhesion of HV (A, B) or that of the patient (C). The results presented are representative data from three independent experiments with different healthy volunteers. Experiment (Ex) 1; eosinophil adhesion; IL-3 (100 pM): HV $3.0 \pm 0.5\%$, the patient $5.0 \pm 0.6\%$; IL-5 (100 pM): HV $4.2 \pm 0.3\%$, the patient $10.0 \pm 0.4\%$; GM-CSF (100 pM): HV $6.6 \pm 0.8\%$, the patient $3.0 \pm 0.1\%$. Ex 2; IL-3 (100 pM): HV $1.3 \pm 0.1\%$, the patient $2.7 \pm 0.4\%$; IL-5 (100 pM): HV $2.5 \pm 0.3\%$, the patient $3.9 \pm 0.4\%$; GM-CSF (100 pM): HV $3.4 \pm 0.4\%$, the patient $1.7 \pm 0.4\%$. Ex 3; IL-3 (100 pM): HV $2.6 \pm 0.3\%$, the patient $3.5 \pm 0.5\%$; IL-5 (100 pM): HV $4.2 \pm 0.4\%$, the patient $8.5 \pm 0.9\%$; GM-CSF (100 pM): HV $4.7 \pm 0.3\%$, the patient $2.0 \pm 0.2\%$.

expression of eosinophils in this study, our results suggested that GM-CSF did not induce adhesion and CD11b expression not only in neutrophils but also in eosinophils of the PAP patient with the *CSF2RA* deletion.

The mechanism for the delay of the clinical onset of PAP in the present case remains unknown. One possibility is that some factors other than GM-CSF may have compensated for the functions of

immune cells, including macrophages. As for eosinophils, we found that IL-3- or IL-5-induced eosinophil adhesion was enhanced in the patient (Fig 1A, B), suggesting that IL-3 and IL-5 can compensate for eosinophil differentiation and activation through βc signaling. Furthermore, LTD₄ or PAF-induced eosinophil adhesion was also increased in the patient, indicating alternative pathways of compensation for eosinophil functions. Another possibility is that

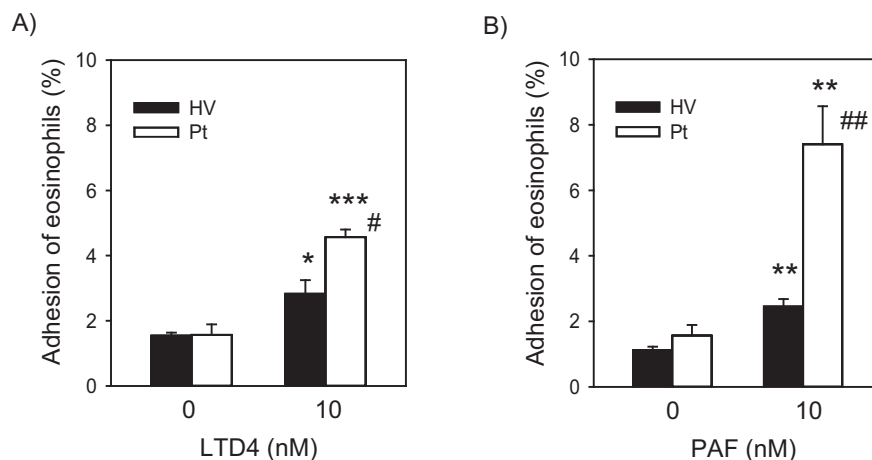


Fig. 2. Effect of LTD₄ or PAF on adhesion of eosinophils obtained from the PAP patient with the *CSF2RA* deletion. Eosinophils ($100 \mu\text{l}$ of 1×10^5 cell/ml) from the PAP patient with the *CSF2RA* deletion (Pt) or healthy volunteers (HV) were incubated with LTD₄ (10 nM) (A) or PAF (10 nM) (B) at 37°C for 20 min. The adhesiveness of eosinophils to recombinant human ICAM-1 was assessed using residual EPO assays ($n = 4$) * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. eosinophil adhesion without stimulation. # $P < 0.05$ and ## $P < 0.01$ vs. eosinophil adhesion of HV. The results presented are representative data from three independent experiments with different healthy volunteers. Ex 1; eosinophil adhesion, LTD₄ (10 nM): HV $2.8 \pm 0.4\%$, the patient $4.6 \pm 0.2\%$; PAF (10 nM): HV $2.5 \pm 0.2\%$, the patient $7.4 \pm 1.2\%$. Ex 2; LTD₄ (10 nM): HV $3.2 \pm 0.3\%$, the patient $5.3 \pm 0.3\%$; PAF (10 nM): HV $3.2 \pm 0.3\%$, the patient $5.2 \pm 0.4\%$. Ex 3; LTD₄ (10 nM): HV $1.9 \pm 0.2\%$, the patient $5.5 \pm 0.2\%$; PAF (10 nM): HV $2.8 \pm 0.3\%$, the patient $9.4 \pm 0.7\%$.

GM-CSF works to some degree in spite of the *CSF2RA* deletion. In this study, although it was not significant, GM-CSF slightly upregulated the eosinophil adhesion in the patient (Fig. 1C). The serum concentration of GM-CSF in the patient was extremely elevated (488 pg/ml; about 33 pM), which was similar to that used in this study *in vitro*.

One limitation in interpreting the findings of our study is that we have not examined other eosinophil functions such as superoxide production or degranulation. As for neutrophil functions in PAP by GM-CSF autoantibodies, not only adhesion but also phagocytosis and hydrogen peroxide production are impaired.⁷ So we speculate that other functions of eosinophils are similar to adhesion; GM-CSF does not activate eosinophils, and other stimuli such as IL-5 or cysLTs compensate for eosinophil functions. Unfortunately, we could not do that experiment. Another limitation is that the mechanisms for upregulation of eosinophil adhesion in the patient are still unknown. We examined the expressions of IL-3R α or β chain on PBMCs by western blotting or PCR in our previous study.⁵ We found that the expression levels of IL-3R α or β chain in patient PBMCs were comparable with those of healthy volunteers, although the expression of GM-CSF R α was not detected in the patient.⁵ Therefore, we speculate that the increase of eosinophil adhesion by IL-3 in the patient was not by IL-3R α or β chain upregulation. However, we checked neither the expressions of IL-3R α or β chain on eosinophils nor IL-5R α expression on PBMCs or eosinophils in this study. In addition, we could not compare the eosinophil functions in PAP by *CSF2RA* deletion with that in PAP by GM-CSF autoantibodies. Considering the impaired neutrophilic functions in PAP by GM-CSF autoantibodies,⁷ eosinophil functions may also be impaired, which should be elucidated in future studies.

In conclusion, GM-CSF did not induce eosinophil adhesion in the PAP patient with the *CSF2RA* deletion, while IL-3 or IL-5 enhanced the adhesion, suggesting that the deficiency of GM-CSF R α also occurs in eosinophils. In contrast, eosinophil adhesions by IL-3, IL-5, LTD₄, or PAF were all enhanced as compared with healthy volunteers, indicating alternative pathways to compensate for eosinophil differentiation and activation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.alit.2019.05.015>.

Conflict of interest

The authors have no conflict of interest to declare.

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Supplementary Materials

Modified eosinophil adhesion in pulmonary alveolar proteinosis caused by *CSF2RA* deletion

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Case presentation

We reported a case of elderly-onset hereditary pulmonary alveolar proteinosis (PAP) caused by a *CSF2RA* deletion previously.¹ In this study, we examined the adhesion of eosinophils obtained from this patient and compared it with that of healthy volunteers.

Here, we reproduce the case presentation as described previously ("Elderly-onset hereditary pulmonary alveolar proteinosis and its cytokine profile" in *BMC Pulm Med* by Ito M, et al. (2017)

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"A previously healthy, 77-year-old, non-smoking woman, with normal chest radiograph at a medical check-up three years earlier, was referred to our hospital for dyspnea on exertion, which occurred one month earlier. She was a homemaker with no remarkable family history.

A physical examination revealed no abnormality. A chest X-ray and computed tomography (CT) scan indicated ground glass opacity in both lower lungs, of which crazy-paving appearance was confirmed upon high-resolution CT scan

(Supplementary Fig. 1A, B). Laboratory studies revealed elevated levels of serum

KL-6, a mucin-like protein (4314 U/ml), surfactant protein D (SP-D; 400 ng/ml), and carcinoembryonic antigen (CEA; 13.2 ng/ml); and negative test results for beta-D glucan and *Aspergillus* antigen. Analysis of arterial blood gas while breathing room air revealed a low partial pressure of oxygen (PaO₂) of 47 mmHg just before whole lung lavage (WLL). Bronchoalveolar lavage fluid (BALF) presented a milky appearance with lymphocytosis (macrophages, 64% of total cells; lymphocytes, 26%; neutrophils 9%; eosinophils, 1%), foamy macrophages, and amorphous materials (Supplementary Fig. 1C, D).

The patient was diagnosed with PAP, based on typical findings from chest CT scan and bronchoalveolar lavage. She had no underlying disease. GM-CSF autoantibodies were not detected in either the serum or the BALF. On the other hand, a high level of serum GM-CSF was detected (91.3 pg/ml; before treatment), indicating disrupted GM-CSF signaling and genetic defects in the GM-CSF receptor."

Further information about allergy status and laboratory findings on admission are provided here. She had no allergic disease and had no specific IgE antibodies to environmental allergens such as house dust mites, molds, or Japanese cedar pollen.

Complete blood counts with eosinophil counts and blood biochemistry on admission are presented in Supplementary Table 1. Her peripheral blood eosinophil counts did not increase during the clinical course (maximum eosinophil counts 179/ μ L (3.7%)). BALF findings on admission are presented in Supplementary Table 2. Concentrations of IL-3 and IL-5 before treatment (WLL) were 44.9 pg/ml and 8.2 pg/ml, respectively.¹ Methods for measurement of GM-CSF concentrations and GM-CSF autoantibodies were previously described.¹

GM-CSF-stimulated phosphorylation of signal transducer and activator of transcription (STAT) 5, GM-CSFR α expression, genetic screening, and single nucleotide polymorphisms (SNPs)

We reported a case of elderly-onset hereditary PAP caused by a *CSF2RA* deletion previously.¹ Here, we reproduce the description of GM-CSF-stimulated phosphorylation of STAT5 in PBMC, GM-CSFR α expression, genetic screening, and SNPs from before.¹

("Elderly-onset hereditary pulmonary alveolar proteinosis and its cytokine profile" in

BMC Pulm Med by Ito M, et al. (2017)

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"We decided to screen for GM-CSF signaling abnormalities because GM-CSF concentration was extremely high in her BALF.

To investigate signaling activated by GM-CSF in the PBMCs, we first probed the presence of phosphorylated STAT5 upon stimulating the PBMCs with increasing concentrations of GM-CSF (0–1000 ng/mL). Phosphorylated STAT5 was observed in the control but not in the patient PBMCs, indicating defective GM-CSF signaling in the latter (Fig. 2 in Ref. 1). To confirm this result, we performed flow cytometry.

The results also showed that expression of phosphorylated STAT5 was not observed on stimulation of peripheral blood mononuclear cells of the patient with GM-CSF, while expression of phosphorylated STAT5 was observed on stimulation of those of the control with GM-CSF as well as on stimulation of those of both with IL-2 (Fig. 3 in Ref. 1).

Next, we investigated the expression of the α and β chains of the GM-CSF receptor on the PBMCs, by western blotting. The expression of GM-CSF-R β and IL-3R α on patient monocytes was comparable with that on the controls; however, expression of GM-CSF-R α was not detected in the former (Fig. 4 in Ref. 1). The result was further confirmed by RT-PCR (Fig. 5 in Ref. 1). Together, these results indicated defects in

the molecules that transmit GM-CSF signals to STAT5.

cDNAs of *CSF2RB*, reverse-transcribed from the mRNA of the patient PBMCs, harbored no mutations or deletions (data not shown). PCR amplicons for each exon of *CSF2RA* from the patient PBMCs were screened for their nucleotide sequences, revealing a homozygous single-base mutation C>T at nt 508 in exon 7 (Fig. 6 in Ref. 1). This mutation was a nonsense mutation that gave rise to a stop codon (Q170X). Further evaluation by aCGH demonstrated a 22.8-Mb region of loss of heterozygosity (LOH) in Xp22.33p22.11, encompassing the *CSF2RA* gene."

Methods for analyses of GM-CSF-stimulated phosphorylation of STAT5 in PBMC, GM-CSFR α expression, genetic screening, and SNPs were previously described.¹

Clinical course after WLL

We decided to use WLL as a treatment for hereditary PAP caused by the *CSF2RA* deletion. We first performed unilateral WLL of the right lung. Right unilateral WLL increased her PaO₂ and decreased KL-6 and lung opacity (Supplementary Table 3 and Fig. 2). As for lung opacity, right unilateral WLL improved the opacity of not only the right lung but also left lung that was not lavaged directly at that time (Supplementary

Fig. 2). We then performed unilateral WLL of the left lung to complete WLL. Left unilateral WLL further increased PaO₂ and decreased KL-6 and lung opacity (Supplementary Table 3 and Fig. 2). Left unilateral WLL also improved the opacity of not only the left lung but also the right lung that was not lavaged directly (Supplementary Fig. 2). Concentrations of IL-3 and IL-5 after WLL were 73.3 pg/ml and 4.1 pg/ml, respectively.¹

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Elderly-onset hereditary pulmonary alveolar proteinosis and its cytokine profile.
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Supplementary Table 1. Laboratory findings on admission

Complete blood count			Blood biochemistry			Arterial blood gas		
WBC	7560	/μL	AST	42	IU/L	pH	7.460	
Neut	67.8	%	ALT	11	IU/L	PaO ₂	74.0	Torr
Lymp	23.1	%	LDH	338	IU/L	PaCO ₂	38.0	Torr
Mono	8.7	%	BUN	19	mg/dL	HCO ₃ ⁻	26.7	mEq/L
Eos	0.3	%	Cr	0.74	mg/dL			
			CRP	0.10	mg/dL	Lung function test		
RBC	442×10 ⁴	/μL				VC	1.48	L
Hb	13.1	%	KL-6	4314	U/mL	%VC	75.1	%
PLT	24.1×10 ⁴	/μL	SP-D	400	ng/mL	FEV ₁	1.19	L
			CEA	13.2	ng/mL	FEV ₁ %	80.5	%
			IgE	148	IU/mL	DLco	6.4	mL/min/mmHg
						%DLco	49.4	%

Supplementary Table 2. BALF findings on admission

BALF		
Total cells	1.3×10 ⁵	/ml
M	64	%
Lymp	26	%
Neut	9	%
Eos	1	%
Baso	0	%
CD4/8	1.24	

Supplementary Table 3. Change in laboratory findings after treatment with WLL

	On admission	Before WLL	After right unilateral WLL	After WLL
KL-6	4314	—	1962	507
SP-D	400	—	239	244
CEA	13.2	—	5.0	1.9
PaO ₂	74.0	47.0	57.0	73.0
PaCO ₂	38.0	42.0	42.0	40.5
VC	1.48	—	1.46	1.62
FEV ₁	1.19	—	1.02	1.06
%DLco	49.4	—	53.4	82.9

FIGURE LEGENDS

Supplementary Fig. 1. CT and BALF findings. (A) Chest radiogram of the patient. (B)

High-resolution CT of the patient. (C) BALF presented a milky appearance with

lymphocytosis (26%). (D) Foamy macrophages and amorphous materials in BALF of

the patient. ("Elderly-onset hereditary pulmonary alveolar proteinosis and its cytokine

profile" © Ito M, et al. 2017 (Licensed under CC BY 4.0.);

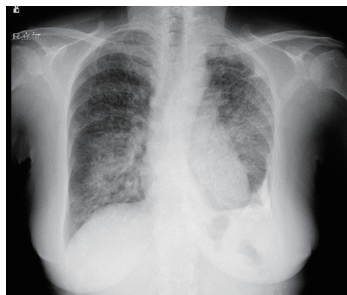
<https://bmcpulmmed.biomedcentral.com/articles/10.1186/s12890-017-0382-x>).

Supplementary Fig. 2. Change in chest radiogram findings after treatment with WLL.

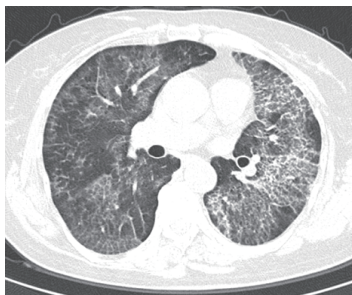
(A) Chest radiogram findings on admission. (B) Chest radiogram findings after

unilateral WLL of the right lung. (C) Chest radiogram findings after WLL.

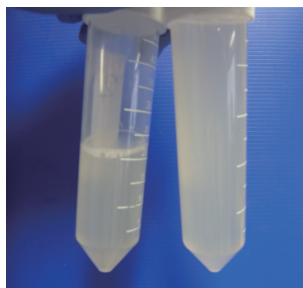
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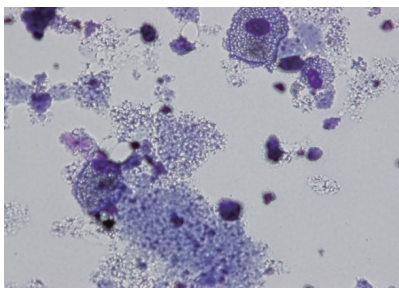
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C)

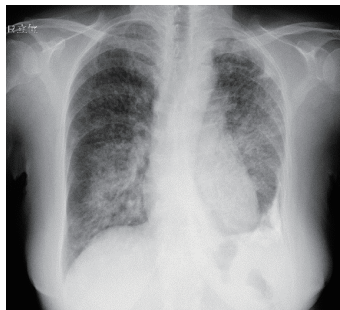


D)

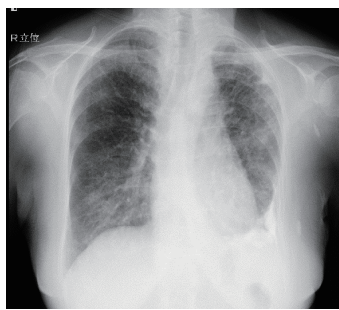


Supplementary figure1

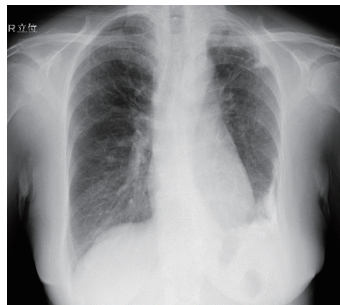
A)



B)



C)



Supplementary figure2