

**Supplementary Appendix**

**Title:** Validation of a New Serum Granulocyte/Macrophage Colony-Stimulating Factor Autoantibody Testing Kit

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## Section 1. Supplemental Methods

### *Subjects*

Sera of patients with aPAP used in the training study were collected at 12 hospitals (Hokkaido, Tohoku, Chiba, Niigata, Aichi, Kyoto, and Nagasaki universities, as well as National Center for Global Health and Medicine, Kinki Chuo Chest Medical Center, Kobe City Medical Center General Hospital, and Kurashiki Municipal Hospital). The patients were eligible for the study if they met the following criteria: 1) between 16 and 80 years of age; 2) diagnosed with aPAP based on both high-resolution computed tomography (HRCT) and biopsy and/or BAL cytology findings; and 3) positive serum GM-CSF autoantibody (GMAb) levels ( $>1.0 \mu\text{g/mL}$ ) (1).

For the training control, 30 sera samples were obtained from healthy volunteers at Niigata University, according to the protocol for studies performed in the Niigata University Medical & Dental Hospital. The other 60 sera samples with information regarding gender and age were purchased from Access Biologicals LLC (Vista, CA, USA). The patients and control healthy subjects were matched with regard to age and male-to-female ratio (patients:  $56.4 \pm 14.1$  y.o., healthy subjects:  $58.7 \pm 12.3$  y.o. and patients: 46/32, healthy subjects: 53/37, respectively). A total of 168 training samples were randomly assigned in a blinded manner. A data manager at The Clinical and Translational Research Center (CTRC) in Niigata University managed the correspondence table in secret until key open.

For the validation study, we used the existing, available, and all samples preserved in the Niigata University Medical Dental Hospital, including sera obtained from patients with PAP and other lung diseases with GGO observed on chest HRCT. Regarding PAP, we enrolled the patients whose sera were tested for GMAb from 2010 to 2018, and confirmed via e-mail correspondence with the treating physicians that the diagnosis of PAP had been reached based on the appearance of GGO on HRCT and the pathological findings obtained through BAL or VATS. All sera from patients with aPAP were positive for serum GMAb ( $> 1 \mu\text{g/ml}$ ) measured by the conventional ELISA previously reported (2). We sent an e-mail to the treating physicians to request information regarding the underlying disease of secondary PAP. For five patients with hereditary PAP, we identified abnormal CSF2RA or CSF2RB genes, which have been reported in previous reports (3, 4).

We also enrolled 200 patients with other lung diseases who were diagnosed from January 2012 to May 2018. Of those, 162 patients exhibited GGO on HRCT that was independently confirmed by two pulmonary physicians. PAP was excluded in all 162 patients by BAL or VATS. The diagnoses for these 162 patients had been reached as follows. Pulmonary infection was diagnosed by identification of the pathogens. Drug-induced interstitial lung disease was suspected based on the patient's drug administration history with supplementary findings by BAL. Connective tissue disease (CTD)-associated lung disorders were diagnosed when GGO on HRCT was observed in patients with a CTD that met the diagnostic criteria for that disorder. When we failed to find other causes that may induce

interstitial lung diseases, we diagnosed the patient with idiopathic interstitial pneumonia (IIP). Some of the cases with IIP were regarded as IPF following the ATS/ERS/JRS/ALAT Clinical Practice Guideline (5). In some patients, we classified IIP by surgical lung biopsy according to the ATS/ERS guideline (6) (data not shown).

#### ***Preparation of polyclonal standard GMAb***

A total of 29 ml of sera obtained from 20 patients with aPAP was pooled and equilibrated with 50 ml of Tris Borate Buffer Saline (TBS, pH 7.4), centrifuged at  $10,000 \times g$  for 30 min at 4°C. The supernatant was passed through a 0.45- $\mu$ m filter (Stericup filter unit, Millipore, Burlington, MA, USA), diluted five times with TBS (pH 7.4), mixed with 10 ml of rProtein A Sepharose Fast Flow (GE Health Care, Co. Ltd.), and stirred at 4°C overnight. The resultant rProtein A gel was transferred to an open column, and washed with TBS until the proteins could not be confirmed in the flow through. Subsequently, IgG was eluted with 50 ml of Gentle Elution Buffer, followed by dialysis against 1 L of TBS (pH 7.4) four times. The IgG solution was applied to NHS-activated HiTrap (Amersham Pharmacia Biotech UK Ltd., Little Chalfont, UK) previously coupled with rhGMCSF (Escherichia coli derived; Amoy Top Co Ltd, Amoy, China), washed with TBS (pH 7.4), eluted with 20 ml of 10 mM Glycin HCl pH2.5, and neutralized with 200 mM Tris-HCl pH8.0. The purity of the isolated antibody was confirmed through SDS gel electrophoresis (Supplemental Figure1A).

#### ***Measuring the IgG concentration of the affinity purified polyclonal GMAb***

The IgG concentration of the affinity purified polyclonal GMAb solution was estimated via UV absorption (Nanodrop, Thermo Fisher, Waltham, MA, USA), and determined through IgG ELISA using a capture antibody (anti-human IgG goat antibody, MBL) The HRP-conjugated anti-human IgG goat antibody (MBL) was detected, followed by color development using a chromogenic peroxidase substrate solution (3, 3', 5, 5'-tetramethylbenzidine [TMB], Moss, Pasadena, MD, USA). Optical absorbance at wavelength 450 nm (A450) was measured using a SUNRIZE plate reader (TECAN, Männedorf, Switzerland). Human IgG (Wako Pure Chemical Industries, Osaka, Japan) was used as the reference standard. The IgG concentration of the stock affinity purified polyclonal GMAb was determined to be 200  $\mu$ g/mL by averaging the values from six replicate measurements.

#### ***Humanized monoclonal GMAb standard***

The murine monoclonal anti-GM-CSF antibody 33-8F was established as previously reported (7). For use as a standard in the GMAb measuring kit, 33-8F was chimerized with human IgG through genetic engineering technologies. Briefly, the human CK and CH1-3 regions were fused to the VL and VH regions of the 33-8F coding cDNA sequence, ligated to a mammalian expression vector, and transfected to CHO cells. The secreted GMAb chimeric mAb 33-8F-H was purified from the culture supernatant with nProtein A sepharose (GE Healthcare, Chicago, IL, USA) according to the manufacturer's instruction and dialyzed to PBS. The purity was confirmed by SDS-PAGE (Supplemental Figure 1B).

Glycerol was added to the antibody solution at a final concentration of 50% (v/v) and stored at  $-30^{\circ}\text{C}$ .

### ***Preparation of recombinant human GM-CSF antigen***

Full-length human GM-CSF ORF with C-terminal 6x His tag, followed by a single stop codon sequence, was synthesized and cloned into pET28a (+) (Merk Millipore, Burlington, MA, USA) with NcoI/XhoI sites, expressed and purified. The resultant construct contained the additional amino acids HHHHHH, C-terminal to the human GM-CSF sequence, and was termed pET28a (+)-GM-CSF-His.

pET28a (+)-GM-CSF-His was transformed to BL21-codonPlus (DE3)-RIL *E. coli* (Agilent, Santa Clara, CA, USA), and recombinant GM-CSF-His was expressed as previously described (8). Briefly, passaged *E. coli* was cultured at  $37^{\circ}\text{C}$ , and protein expression was induced until the OD 600 nm reached 0.6 with 1 mM (final concentration) isopropyl b-D-1-thiogalactopyranoside (IPTG), followed by subsequent additional culture for 5 h at  $37^{\circ}\text{C}$ . The cells were centrifuged, washed once with 30 mL PBS, and the cell pellet was stored at  $-80^{\circ}\text{C}$ .

### ***Measurement of the concentration of GMAb using the new ELISA***

The standard GMAb ELISA used in this study was based on previous reports (2,9-11) with some modifications. The concentration of GMAb in the serum samples was measured using the anti-GM-CSF autoantibody measuring kit (the new kit, MBL2490023, MBL, Co. Ltd., Ina, Japan) according to the manufacturer's instruction. The kit consists of solutions of diluted standard antibody ready to use, dry plate that was previously coated and blocked with a blocking reagent, the diluted peroxidase labeled second antibody, and sample dilution buffer (Supplemental Figure 2). Briefly, serum samples were diluted (1/201) in reaction buffer or the standard monoclonal GM-CSF antibody 33-8F-H (0, 1.6, 6.7, 46, 186, and 312 U/ml) and applied to each well of 96-well plates coated with 100  $\mu\text{L}$  of 4.0  $\mu\text{g}/\text{ml}$  *E. coli*-derived recombinant human GM-CSF. This was followed by addition of a blocking reagent, incubation for 1 h at room temperature, and washing (four times) with ELISA wash buffer. Horseradish peroxidase-conjugated anti-human IgG goat polyclonal antibody (MBL) was added into each well, and plates were incubated for 1 h at room temperature. Color development was performed using a chromogenic peroxidase substrate solution (3, 3', 5, 5'-tetramethylbenzidine, Moss, Pasadena, MD, USA). The reaction was terminated by adding 100  $\mu\text{L}$  0.5 N sulfuric acid. Optical absorbance at wavelength 450 nm (A<sub>450</sub>) was measured using a SUNRIZE plate reader (TECAN, Männedorf, Switzerland). The concentration (U/mL) of GMAb was determined as follows. The optical density of six point standards with known GMAb concentrations in the same plate was used to produce a four-parametric logistic curve with Microsoft Excel (Microsoft Corp, Seattle, WA, USA).

***Measurement of the concentration of GMAb using the conventional ELISA*** with minor modifications

For the comparison of performance, conventional ELISA was also used with minor modifications. Firstly, the 33-8F-H monoclonal antibody, instead of J152-4C, was used as the standard antibody. Secondly, sample dilution was performed as described earlier in this section (1/201 dilution in the reaction buffer) with conventional dilution buffer (PBS, 1%BSA, 0.1%Tween 20). Stabilicoat was used as a blocking reagent, as previously described (2). Autoantibodies to aminoacyl-tRNA synthetases (ARSs) and melanoma differentiation-associated gene 5 (MDA-5) were measured using ELISA as previously described (Ran Nakashima et al. PLOS ONE, 9, 2014; Sato S, Murakami A, Kuwajima A, Takehara K, Mimori T, Kawakami A, et al. (2016) Clinical Utility of an Enzyme-Linked Immunosorbent Assay for Detecting Anti-Melanoma Differentiation-Associated Gene 5 Autoantibodies. PLoS ONE 11(4): e0154285. doi:10.1371/journal.pone.0154285)

### ***Statistics***

After estimating cut-off values for prediction, we conducted an external validation study. The external validation study evaluated the performance of the model (e.g., sensitivity and positive predictive value, as described later) in data other than those used for estimating the cut-off values. External validation is essential to support generalizability of the prediction using the cut-off value for patients other than those in the training data.

## Section 2. Supplemental Results

### ***Determination of the concentration of monoclonal GMAb using the polyclonal GMAb standard***

The concentration of the mouse-human chimeric monoclonal antibody 33-8F-H (secondary standard) was determined with the anti-GM-CSF autoantibody measuring kit using affinity purified polyclonal GMAb standards as the primary standard (Supplemental Figure 1A and B). One unit per ml of GMAb (1 U/ml) was defined as the concentration of 1.0 µg/ml affinity purified polyclonal GMAb. Thus, the polyclonal GMAb solution was used for the primary standard and preserved at -80°C until calibration of the monoclonal secondary standard. To calibrate a wide range of GMAb concentrations, a standard curve for a lower titer (< 12.5 U/mL) was determined via linear approximation (Supplemental Figure 1C), while a standard curve for higher concentrations (> 12.5 U/mL) was determined via cubic approximation (Supplemental Figure 1D). When the mouse-human chimeric monoclonal antibody 33-8F-H (secondary standard) in the kit was calibrated against the aforementioned primary polyclonal GMAb standards, 0.6 µg/ml of the monoclonal antibody accounted for 1 U/mL of the primary standard. The mouse-human chimeric monoclonal antibody 33-8F-H (secondary standard) was used for all subsequent measurements of GMAb concentration in clinical samples.

### ***Reproducibility and stability of ELISA***

The reproducibility of ELISA was evaluated by repeatedly measuring (five times) the concentration of GMAb in sera obtained from 78 patients with aPAP. The mean value, 95% upper CI, and 95% lower CI of cumulative data appeared to be stable after three repeated measurements, indicating the high reproducibility of ELISA (Supplemental Figure 3A). Subsequently, five positive samples were frozen at -80°C and thawed at 25°C five times. The concentration of GMAb was measured in the samples obtained after each freeze-thaw cycle. The concentrations of GMAb in these patient samples were within a 15% change (Supplemental Figure 3B).

When rhGM-CSF-coated plates were stored at 37°C, the concentration of GMAb in the three positive samples was stable for 4 weeks (Supplemental Figure 3C). The reference standard in the kit (mouse-human chimeric monoclonal antibody 33-8F-H) was also stable at 37°C for ≥ 4 weeks (Supplemental Figure 3D).



### Section 3. Supplemental figure legends

#### **Supplemental Figure 1. Characteristics of the GMAb reference standards.**

A: Purity of the polyclonal GMAb (primary standard) and B: the mouse-human chimeric monoclonal antibody 33-8F-H (secondary standard) were confirmed using SDS-PAGE followed by staining with Coomassie Brilliant Blue (CBB). C: Calibration curve of optical densities at 450 nm for the narrow range of concentrations (0 – 12.5 µg/ml) of polyclonal GMAb and (D) for the wide range of polyclonal GMAb (12.5 – 400 µg/ml).

#### **Supplemental Figure 2.**

Appearance of the newly developed anti-GM-CSF autoantibody measuring kit (MBL2490023). The kit consists of 1) dilution buffer, 2) 3, 3', 5, 5'-tetramethylbenzidine solution, 3) 10x concentrated wash buffer, 4) peroxidase labeled secondary anti-IgG antibody ready to use, 5) Stop buffer, 6) standard anti-GM-CSF monoclonal antibody ready to use, 7) negative control, 8) positive control, 9) dry plate that was previously coated with recombinant GM-CSF and blocked with a blocking reagent.

#### **Supplemental Figure 3. Reproducibility and storability of the ELISA kit.**

A: Reproducibility of the anti-GM-CSF autoantibody measuring kit (MBL2490023) was assessed through five independent measurements of the concentration of GMAb in 78 patients with aPAP. The mean concentration (-◆-) and 95 % upper (-□-) or lower (-△-) confidence interval are shown for measurements 1–3, 1–4, and 1–5.

B: Influence of the number of freeze-thaw cycles on the measurement values. Sera from five patients were frozen at –80°C and thawed at 25°C one to five times. At each cycle, the concentration of GMAb was measured. The absolute concentration of GMAb at each cycle is shown for five patients.

C: Influence of the storage of 96-well assay plates coated with recombinant GM-CSF at 37°C on the measurement values.

D: Stability of the mouse-human chimeric monoclonal antibody 33-8F-H. The antibody was preserved at 37°C or –80°C for 1, 2, 3, and 4 months, and the percentage of binding with the assay plate at 37°C to that at –80°C is shown.

#### **Supplemental Figure 4. Distribution of the concentration of GMAb in the sera of patients with aPAP in the training and validation studies.**

Distribution of the concentration of GMAb in sera obtained from patients with aPAP in the training (A) and validation (B) studies. The vertical axis indicates the number of cases, whereas the horizontal axis shows the concentration of GMAb.

#### **Supplemental Figure 5. HRCT images of five cases with serum GMAb >1.65 U/ml.**

Case 1: Diffuse homogeneous GGO pattern on HRCT in a patient with secondary PAP, pathologically confirmed by VATS.

Case 2: A case with unclassifiable IIP, whose HRCT showed GGO with tractionbronchioloectasis distributing from the subpleural and bronco-vascular bundle.

Case 3: A patient with CTD (PM/DM), whose HRCT showed GGO mainly around broncho-vascular bundle with traction bronchiectasis.

Case 4: A patient with CTD (UCTD), whose chest HRCT revealed diffuse GGO with reticulation,traction bronchioloectasis, and mild emphysema.

Case 5: A patient with unclassifiable IIP with GGO distributed in subpleural, and bronco-vascular bundle regions. Honeycombing or traction bronchiectasis was not observed in the GGO.

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