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## Association of M2 macrophages, Th2, and B cells with pathomechanism in microscopic polyangiitis complicated by interstitial lung disease

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## ABSTRACT

**Objective.** To address the pathomechanism of microscopic polyangiitis (MPA) complicated by interstitial lung disease (ILD) using serum biomarker profile and pulmonary histopathology.

**Methods.** Serum biomarkers from patients with MPA-ILD (n = 32), MPA without ILD (n = 17), and healthy controls (n = 10) were examined. Based on the biomarker profiles, principal component analysis (PCA) and cluster analysis were performed to classify patients with MPA-ILD into subgroups. Clinical characteristics and prognosis were assessed for each subgroup. Two lung biopsies were examined following hematoxylin-eosin staining and immunostaining.

**Results.** T-cell and macrophage polarization was skewed toward the T helper (Th) 2 cells and M2 macrophages in MPA-ILD group relative to that in MPA without ILD group. The PCA allowed classification of the 19 biomarker profiles into three groups: (1) B cell- and neutrophil-related cytokines, vascular angiogenesis-related factors, extracellular matrix-producing factors, (2) Th1-driven cytokines, M1 macrophage-driven cytokines and Th2-driven cytokines, and (3) M2 macrophage -induced and -driven cytokines. The cluster analysis stratified the patients with MPA-ILD into clinically fibrotic dominant (CFD) and clinically inflammatory dominant (CID) groups.

Notably, severe infections were significantly higher in the CFD group than in the CID group. Immunohistochemical staining demonstrated intense CXCL13 staining in B cells and Th2 cells in the interstitium of MPA-ILD lungs.

**Conclusion.** Activation of M2 macrophages, Th2 cells, and B cells plays a key role in the pathomechanism of MPA-ILD. Classification of MPA-ILD based on serum biomarker profile would be useful in predicting the disease activity and the complication of severe infection in MPA-ILD.

**Key Index Terms:** biomarkers, cytokines, interstitial lung disease, microscopic polyangiitis, pulmonary histopathology.

## INTRODUCTION

Microscopic polyangiitis (MPA) is defined by the Chapel Hill Consensus Conference as necrotizing vasculitis, with a few or no immune deposits, that predominantly affects small vessels [1]. MPA is an anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV). The major target antigens of ANCA are myeloperoxidase (MPO) and proteinase 3 (PR3) [2]. MPA has a higher prevalence in Japan than granulomatosis with polyangiitis (GPA) and MPO-ANCA positivity is more commonly observed [3].

Pulmonary involvement is a major manifestation of AAV, and interstitial lung disease (ILD) is the most frequent pulmonary manifestation reported in Japan. The prognosis of MPA patients with ILD is significantly poorer than that of MPA patients without ILD [4]. Disease activity markers, such as Birmingham Vasculitis Activity Score (BVAS), C-reactive protein (CRP), and ANCA titers, and existing diagnostic markers for ILD, such as Krebs von den Lungen-6 (KL-6) and percent forced vital capacity (FVC), have limited the applicability for MPA-ILD diagnosis and monitoring [5-7]. Hence, new, reliable indicators are warranted for evaluating the severity of MPA-ILD.

ANCA plays a pathogenic role in ANCA-associated vasculitis [8]. The ANCA-cytokine sequence theory was proposed to explain the pathomechanism underlying AAV. The levels of TNF- $\alpha$ , IFN- $\gamma$ , IL-1 $\beta$ , IL-2, IL-6, IL-17, IL-23, IL-8, granulocyte

colony stimulating factor (G-CSF), and B cell activating factor are significantly higher in patients with GPA-dominated AAV than in healthy individuals, indicating that M1 macrophages, T helper 1 (Th1) and Th17 cells, neutrophils, and B cells are activated in AAV [8, 9]. Furthermore, the expression of VEGF, a tissue inhibitor of metalloproteinase (TIMP)-1, matrix metalloproteinase 3, and intercellular adhesion molecule-1, the markers of microvascular damage and repair, is elevated in AAV [9, 10]. However, the complete biomarker profile of patients with MPA has not yet been fully examined. In particular, the immunophenotype and cytokine profile of MPA-ILD remain unknown. Therefore, it was not clear whether these biomarkers are useful indicators for evaluating disease activity and prognosis in MPA-ILD.

We previously reported that the level of serum C-C motif chemokine ligand 2 (CCL2) was significantly higher in MPA patients with ILD than those in MPA patients without ILD. We also demonstrated that CCL2 produced by CD68<sup>+</sup>/CD163<sup>+</sup> macrophages was associated with the pathomechanism of MPA-ILD [11]. However, interaction between cytokines, and inflammatory cells in MPA-ILD has not been revealed. The pathomechanism of MPA-ILD has not been fully elucidated.

To clarify these clinical questions, we comprehensively measured the levels of these serum biomarkers, and classified the patients with MPA-ILD into subgroups based on

the principal component analysis (PCA) and cluster analysis in this study. Furthermore, we explored the potential biomarkers relevant to disease activity in MPA-ILD. Additionally, we evaluated immune cells producing these biomarkers using immunohistochemical analyses of MPA-ILD lungs to reveal the pathomechanism of MPA-ILD.

## **METHODS**

### **Patient information**

This study was performed according to the Declaration of Helsinki and its amendments and was approved by the Ethics Committee of Osaka Medical and Pharmaceutical University (approval no. 1529). Written informed consent was obtained from all study subjects. Forty-nine patients at the Osaka Medical and Pharmaceutical University Hospital, who fulfilled the Chapel Hill Consensus definition of MPA, were recruited into this study between December 2011 – April 2019 [1]. All patients were ANCA positive and/or had their biopsies evaluated for confirmed diagnosis of small-vessel necrotizing vasculitis. All patients were admitted to our hospital for the first remission induction therapy. All patients received immunosuppressive treatments based on the discretion of the physician. All clinical and laboratory findings, treatment, and

outcomes were extracted from medical records. More details of the methods, such as exclusion criteria of MPA patients, recruitment of healthy controls (HCs), measurement of laboratory parameters, pulmonary function testing, evaluation of high-resolution computed tomography scoring, and evaluation of disease severity and outcome, are provided as Supplementary Methods.

### **Measurement of serum cytokines and biomarkers**

Serum samples of the study subjects were collected before initiating immunosuppressive therapy and stored at -70°C until measurement. All patients were in active phases, and they were not treated with glucocorticoids and immunosuppressive therapy before sampling. Serum levels of biomarkers [IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, IL-17A, IL-23 (heterodimers), CCL2, M-CSF, CXCL13, TNF- $\alpha$ , IFN- $\gamma$ , G-CSF, and granulocyte-macrophage (GM)-CSF] were measured using Human Magnetic Luminex Screening Assay (LXSAHM, R&D Systems, Minneapolis, MN, USA). Serum levels of TIMP-1, PDGF, VEGF, TGF- $\beta$ , and leucine-rich alpha 2 glycoprotein (LRG) were measured by ELISA kits (TIMP-1, PDGF, and TGF- $\beta$ : R&D Systems, Minnesota, USA; VEGF: Abcam, Cambridge, UK; and LRG: IBL, Fujioka, Japan). The detection limits for all potential biomarkers are shown in Supplementary Methods. The range of standard



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curves for each cytokine, and the number of samples whose serum cytokine levels were over the detection limits in patients with MPA and HCs are shown in Supplementary Table 1.

### **PCA of serum biomarkers and Cluster analyses in MPA-ILD patients**

We performed PCA to elucidate the relationship among serum biomarkers. Out of 21 biomarkers, IL-17A and IL-23 were excluded from the PCA as their levels were below the detection limit (Supplementary Table 1). Also, cluster analyses were performed by the Ward method using the biomarker values of MPA-ILD patients [12]. More details of PCA and Cluster analyses are provided as Supplementary Methods (Supplementary Figure 1, 2).

### **Immunohistochemical analysis**

Lung tissue specimens were obtained from two patients with MPA-ILD during surgical lung biopsy or bronchoscopy. An autopsy lung sample of a patient with hypopharyngeal cancer was used as a negative control. We performed immunohistochemical staining for CCR4, which is a chemokine receptor of Th2 cells [13], IL-4, CXCL13, and CD20 in MPA-ILD lungs. Details of immunohistochemical analysis are shown in Supplementary

Methods.

### **Statistical analysis**

Data are presented as the median (interquartile range). Fisher's exact test was performed when appropriate, and Mann-Whitney *U*-test was performed to compare the median values. Correlations were evaluated using Spearman's correlation coefficients. Relative risks and 95% confidence interval (CI) were calculated using a log-linear Poisson regression model of person-years. The Kaplan-Meier method was used to evaluate severe infection-free survival curves and the log-rank test to assess the significance of differences between groups. Univariate and multivariate Cox proportional hazards regression analyses were used to evaluate the risk factor for severe respiratory infections. Results with  $P < 0.05$  were considered statistically significant. The data were analyzed using JMP® 14 (SAS Institute Inc., Cary, NC, USA) and GraphPad Prism (version 8.0; GraphPad Software, La Jolla, CA, USA).

## **RESULTS**

### **Baseline characteristics of MPA patients**

Out of the 49 enrolled patients diagnosed with MPA, 46 patients were MPO-ANCA+,

one patient was PR3-ANCA+, whereas two patients were MPO+ and PR3-ANCA+.

The clinical characteristics of patients are listed in Table 1. The mean age of the patients was 73 years and 49% of the patients were females. The median initial White blood cell (WBC) count, serum level of albumin, CRP, and MPO-ANCA titer were 12480/mm<sup>3</sup>, 2.4 g/dL, 10.1 mg/dL, and 96.5 U/mL, respectively. The median total BVAS was 17 and the proportion of patients with five-factor score (FFS)  $\leq 1$ , 2, and  $\geq 3$  was 24.5%, 55.1%, and 20.4%, respectively. Thirty-two patients presented with ILD on admission. A comparison of clinical characteristics and disease severity classification between MPA patients with and without ILD was presented in Supplementary Table 2.

### **Comparison of biomarker levels observed in patients with MPA with and without ILD and HCs**

Firstly, we evaluated 21 biomarker levels in patients with MPA (n = 49) and in HCs (n = 10). The levels of Th1-, Th2-, M1/M2 macrophage-, B cell-, and neutrophil-related cytokines; pro-fibrotic biomarkers; and vascular angiogenesis-related biomarkers were significantly higher in patients with MPA than in the HCs (Supplementary Table 3).

Next, we evaluated 21 biomarker levels in the MPA patients with ILD (n = 32) and MPA without ILD (n = 17) (Figure 1, Supplementary Table 4). The initial serum levels

of GM-CSF and IL-2 were lower in the MPA-ILD group compared with those in the MPA without ILD group ( $P = 0.02$  and  $0.04$ , respectively). In contrast, the initial serum CCL2 levels were significantly higher in MPA-ILD group compared with those in the MPA without ILD group ( $P = 0.002$ ) (Figure 1A).

We also evaluated cytokine ratios between the MPA-ILD group, MPA without ILD group and HCs (Figure 1B). The ratio of M-CSF/GM-CSF, which steers the balance between M1 and M2 macrophages [14], was significantly higher in the MPA-ILD group than in the HC group ( $P = 0.01$ ). The ratio of IL-10/IL-2, which represents the balance between Th1 and Th2 [15], was significantly higher in the MPA-ILD group than in the MPA without ILD group ( $P = 0.03$ ).

These findings suggested that the T-cell and macrophage polarization was skewed toward Th2 and M2 macrophages in the patients with MPA-ILD relative to the patients with MPA without ILD.

### **PCA based on biomarkers observed in MPA-ILD**

For visualization of the biomarkers in patients with MPA-ILD ( $n = 32$ ), we performed two-dimensional PCA (Figure 2A). PCA retained three components that explained 57% of all variance in the data. The PCA coordinates for the first three varimax-rotated PCs

are presented in Supplementary Table 5. The first component was composed of IL-6, CXCL13, IL-8, G-CSF, TIMP-1, TGF- $\beta$ , LRG, VEGF, and PDGF. The second component consisted of GM-CSF, IL-2, IL-4, IL-13, IL-1 $\beta$ , and TNF $\alpha$ , and the third component comprised of CCL-2, IL-10, M-CSF, and IFN- $\gamma$ . The diversity in the MPA-ILD biomarkers was driven by three groups: (1) B cell- (IL-6 and CXCL13) and neutrophil-related cytokines (IL-8 and G-CSF), vascular angiogenesis-related factors (PDGF and VEGF), and extracellular matrix-producing factors (TGF- $\beta$ , LRG, and TIMP-1), (2) Th1-driven cytokines (GM-CSF and IL-2), M1 macrophage-driven cytokines (IL-1 $\beta$  and TNF $\alpha$ ), Th2-driven cytokines (IL-4 and IL-13), and (3) M2 macrophage-induced and -driven cytokines (IL-10, M-CSF, and CCL-2).

### **Correlation between initial serum biomarkers and disease activity indicators of MPA-ILD**

The correlation between the serum biomarker levels and disease activity indicators of MPA-ILD are presented in Supplementary Table 6. The inflammatory markers, such as WBC and CRP, correlated significantly with the PCA-defined group 1 biomarkers. The total GGO scores correlated significantly with the initial levels of CXCL13 ( $R = 0.53$ ), and the total fibrosis scores correlated significantly with the initial levels of CXCL13

and IL-13 ( $R = 0.38$  and  $0.35$ , respectively).

### **Statistical cluster analysis based on biomarkers in patients with MPA-ILD**

We used cluster analysis to identify subgroups among the patients with MPA-ILD based on their serum biomarker levels (Figure 2B). Individual patients were divided in two groups as shown in Figure 2C. The PCA score plot confirmed that the patients with MPA-ILD were localized according to the PC1-PC2 axes. Comparison of the biomarker levels between Cluster 1 and Cluster 2 has been listed in Supplementary Table 7. The biomarker levels in group 1 were significantly higher in Cluster 2 than in Cluster 1. The initial serum level of IL-4 was significantly higher in Cluster 1 than in Cluster 2 ( $P = 0.012$ ).

### **Comparison of the clinical characteristics and prognosis between Cluster 1 and Cluster 2 in patients with MPA-ILD**

We compared the clinical characteristics of patients with MPA-ILD in Cluster 1 and 2 (Table 2). The initial WBC count and serum CRP levels were significantly higher in Cluster 2 than in Cluster 1 ( $P = 0.0001$  and  $< 0.0001$ , respectively). The initial serum albumin, and KL-6 and Surfactant protein D levels were significantly higher in Cluster

1 than those in Cluster 2 ( $P = 0.0003$ ,  $0.049$ , and  $0.033$ , respectively). Additional details on the frequency of systemic symptoms are provided in Supplementary Table 8. These results suggested that MPA-ILD patients were classified into two clinically different subgroups based on their biomarker profiles: Cluster 1 was the clinically fibrotic-dominant (CFD) group and Cluster 2 was the clinically inflammatory-dominant (CID) group.

The incidence rates of severe infection and severe respiratory infection were evaluated at the median follow-up period of 25.1 months. The incidence of severe infection was significantly higher in patients belong to the CFD group (52.9 %) than those in CID group (13.3%;  $P = 0.028$ ). Patients in the CFD group had a higher relative risk of severe infection than those in the CID group (relative risk, 5.9; 95 % CI, 1.5 – 38.9). Kaplan–Meier survival curves were plotted to estimate the probability of severe infection-free survival between the CFD and CID groups. The 4-year severe infection-free survival rate was significantly lower in the CFD group (15.8%) than in the CID group (82.1%) (log-rank test,  $p=0.02$ ) (Supplementary Figure 3). Out of the nine patients, seven were hospitalized due to respiratory infections in the CFD group.

#### **Evaluation of factors associated with severe respiratory infection in patients with**

## MPA-ILD

Among the severe infection, 81% (9 out of 11) cases were related to severe respiratory infections. Next, we evaluated factors associated with severe respiratory infection using Cox proportional hazards regression analysis (Supplementary Table 9). In the univariate analysis, higher KL-6 levels and lower %FVC were significantly associated with an increased severe respiratory infection rate ( $P=0.01$  and  $0.04$ , respectively). After adjusting for age and sex, the multivariate analysis revealed that higher KL-6 levels were independently associated with an increased severe respiratory infection rate ( $P=0.001$ ).

## Immunohistochemical analyses of lungs of patients with MPA-ILD

We previously observed that T-cell and macrophage polarization was skewed toward Th2 and M2 macrophages in the MPA-ILD group relative to the MPA without ILD group. The serum IL-4 and IL-13 levels were associated with ILD activity, whereas the serum CXCL13 levels significantly correlated with the GGO and fibrosis score. CXCL13 promotes B cell migration to lymphoid follicles [16], whereas IL-4 and IL-13 are produced by Th2 cells [17]. Based on these findings, we hypothesized that B- and Th2-cells were associated with the pathomechanism underlying MPA-ILD. Hence, to



evaluate pulmonary fibrosis in patients with MPA-ILD, we performed an immunohistochemistry analysis to evaluate the expression of CXCL13, CD20, and CCR4 in the lung biopsy specimens obtained from two patients prior to initiating immunosuppressive therapy (Figure 3). The detailed clinical characteristics are presented in Supplementary Table 10. Hematoxylin and eosin staining revealed a cystic fibrotic airspace lined by bronchiolar epithelium and filled with inflammatory cells, including neutrophils and macrophages. Interstitial infiltration of lymphocyte, lymphoid follicles (LFs), and bronchiolitis was observed. Strong CXCL13 immunostaining was confined to B-cells in LFs, whereas CCR4-positive cells were located in the interstitium of MPA-ILD lungs. Strong IL-4 immunostaining was confined to these CCR4-positive cells, confirming CCR4-positive cells as CCR4-positive Th2 cells (Supplementary Figure 4). The number of CXCL13-producing B cells and CCR4-positive Th2 cells was higher in the lungs of both MPA-ILD patients than in control group. The median number of CXCL13-positive B cells in each case was 26, 46, and 4, respectively; the median number of Th2 cells for each case was 81, 54, and 6, respectively. Thus, these results suggested that CXCL13 producing B cells and Th2 cells played a key role in the pathomechanism of MPA-ILD.

## DISCUSSION

The pathomechanism underlying MPA-ILD has not been elucidated. In this study, we validated the findings of our previous study [11], demonstrating that CCL2-producing alveolar M2 macrophages are associated with the pathomechanism in MPA-ILD. The crosstalk between Th2 and M2 macrophages is associated with the pathomechanism of idiopathic pulmonary fibrosis (IPF) [18]. The results of the present study suggest that the immunophenotype of MPA-ILD cases is polarized into Th2 and M2 macrophages, which may be similar to that of IPF patients.

Our hypothesis on the pathomechanism of MPA-ILD based on biomarker profiles is presented in Figure 4A. First, secretion of IL-2 and GM-CSF by Th1 cells results in M1 macrophage differentiation [19]. M1-macrophages produce IL-1b and TNF- $\alpha$ , thereby priming neutrophils [20]. In addition, IL-4 and IL-13 secreted by Th2 cells activate the B cells [17]. Second, IL-6 induces differentiation of B cells into plasma cells [21], and CXCL13 acts as a chemoattractant to promote the infiltration of B cells into inflammatory lesions [22]. This process triggers ANCA production and neutrophil activation [23]; IL-8 and G-CSF induce neutrophil activation [24, 25]. PDGF and VEGF are released by injured vascular endothelial cells to induce angiogenesis, whereas TIMP-1, TGF- $\beta$ 1, and LRG are produced during the repair process [26-31]. Third, IL-4

and IL-13, produced by Th2 cells and M-CSF, induce the differentiation of M2 macrophages that produce IL-10 and CCL2 [11, 20].

In patients with IPF, the serum CXCL13 level was significantly higher than the control group [16]. CXCL13 facilitates B cell homing to the inflammatory pulmonary lesions via CXCR5 and is associated with the pulmonary fibrosis [16]. Furthermore, IL-13 production by alveolar macrophages is increased in IPF, and promotes fibrosis by regulating expression of TGF- $\beta$ 1 [32]. In the present study, the serum CXCL13 level positively correlated with the extent of pulmonary inflammation, and serum CXCL13 and IL-13 levels positively correlated with the extent of pulmonary fibrosis. Hence, these cytokines may be useful biomarkers for predicting the activity and severity of MPA-ILD.

Matsumoto *et al.* reported that patients with AAV can be divided into three subgroups based on immunophenotyping; 1) antibody production-related group, 2) cytotoxic activity-related group, and 3) neutrocytosis/lymphocytopenia-related group [33]. Similarly, Watanabe *et al.* also classified AAV patients into four subgroups according to their clinical characteristics; 1) patients with MPO-ANCA negative group, 2) patients with an elevated serum Cr and high CRP levels, 3) patients without serum Cr elevation, and 4) patients with elevated serum Cr, but without high CRP levels [34].

However, none of the studies have classified the patients with MPA-ILD into subgroups based on their clinical characteristics and biomarkers. This is the first study to report two immunophenotypes in patients with MPA-ILD based on their biomarker profile; the CFD and the CID groups.

In this study, there were no significant differences in the total BVAS score and revised FFS between the CFD and CID groups. Suzuki et al. reported that the total BVAS score cannot predict the severity of MPA-ILD because ILD was not included in BVAS [6]. Furthermore, Comarmond et al. reported that revised FFS was not associated with mortality in MPA-dominated AAV with ILD [35]. These previous reports support our findings, and the total BVAS score and revised FFS may not predict the severity and prognosis of MPA-ILD.

The CFD group presented a higher incidence of severe infections than the CID group after immunosuppressive therapy because the disease activity of ILD was higher in the CFD group than in the CID group, leading to the high complication of severe infections in the CFD group. [36, 37] As a result, severe infections may be more complicated in the CFD group than in the CID group even though the contexts of treatment were not different between the groups. Based on these findings, the cluster classification based on serum biomarkers in the present study has clinical significance in MPA-ILD.

The typical pathological findings of MPO-ANCA<sup>+</sup> ILD include extensive interstitial fibrosis, lymphoid hyperplasia, and bronchiolitis [7, 38]. In the present study, the histopathological features of the patient samples were consistent with those reported earlier. However, little was known regarding the localization of immune cells in MPA-ILD, and this is the first study to reveal that CXCL13-producing B cells in the LF and Th2 cells in the interstitium are associated with the pathomechanism of MPA-ILD.

We propose the following pathomechanism focusing on MPA-ILD (Figure 4B). In patients with MPA-ILD, B cells in the LF of lungs potentially produce CXCL13 and recruit B cells into the pulmonary inflammatory lesion sites. Th2 cells, located in the interstitium of ILD, produce IL-4 and IL-13 and induce the differentiation of alveolar resident macrophages into M2 macrophages [18], facilitating the production of CCL2 and IL-10. Thus, cytokine producing Th2 cells and M2 macrophages promote fibroblast migration into the inflammatory lesions, transforming them into myofibroblasts [39]. During this process, extracellular matrix is produced and culminates in the formation of lung fibrosis.

However, this study had several limitations. First, only Japanese patients were enrolled in this study, and MPO-ANCA<sup>+</sup> MPA was the dominant disease type. Hence, it remains to be seen whether these findings would be applicable to other ethnicities.

Second, the treatment strategy was determined at the physician's discretion, and may have caused an indication bias. Third, immunohistochemical analyses of MPA-ILD lungs were performed in only two cases, and we cannot conclude the immunopathological characteristics of MPA-ILD. More number of MPA-ILD specimens is needed to elucidate the pathomechanism of MPA-ILD. Finally, the data in this study may have been affected by tertiary care bias as 23 out of 32 patients with MPA-ILD had ILD prior to the MPA diagnosis. Hence, further investigations are warranted to clarify the immunophenotypes associated with and pathomechanism underlying MPA-ILD based on a larger, prospective study.

In conclusion, this is the first report to reveal the pathomechanism underlying MPA-ILD based on serum biomarkers and pulmonary histopathology. Classification of MPA-ILD based on serum biomarker profile may be useful in predicting the disease activity and prognosis of MPA-ILD. Specific therapeutic strategies for the classified groups may be useful in the future. Biomarkers and receptors related to Th2 cells, M2 macrophages, and B cells should be evaluated in future studies to identify novel targets for MPA-ILD treatment.

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## FIGURE LEGENDS

### **Figure 1. Comparison of biomarker levels and cytokine ratios among MPA-ILD, MPA without ILD, and HC**

(A) Serum Th1 and M2 macrophage related cytokine levels in patients with MPA-ILD and MPA without ILD before their treatment. (B) M1/M2 macrophage-related cytokine ratios and Th1/Th2 cytokine ratios in the patients with MPA-ILD, MPA without ILD, and healthy control subjects. The level of the serum biomarkers are presented as scatter diagrams. Each black bar represents the median value. The Mann-Whitney *U* test was performed to compare the median values.  $P < 0.05$  denotes statistical significant. \* $P < 0.05$ , \*\* $P < 0.01$ . MPA-ILD: microscopic polyangiitis with interstitial lung disease; HC: healthy controls; NS: not significance.

### **Figure 2. PCA and statistical cluster analysis based on biomarker levels in patients with MPA-ILD**

(A) Visualization of biomarkers in two dimensions. Dimensions 1 and 2 are the first two PCs. (B) Hierarchical statistical clustering of biomarker levels in patients with MPA-ILD. Subgroups are indicated in red and green. (C) PC1 and PC2 values of individual patients in the two subgroups. PCA: principal component analysis; MPA-ILD: microscopic

polyangiitis with interstitial lung disease.

**Figure 3. Immunohistochemical staining in lung biopsy sections from patients with MPA-ILD**

Representative images of sections stained with hematoxylin and eosin, anti-CXCL13 antibody, anti-CD20 antibody, and anti-CCR4 antibody in the lung biopsy samples of control subjects and patients with MPA-ILD. Accumulation of CD20/CXCL13<sup>+</sup> B cells (white arrow) and CCR4<sup>+</sup> Th2 cells (black arrow) is shown. A, F, K: Magnification (40×), scale bar 100 μm. B, G, L: Magnification (100×), scale bar 100 μm. C, D, E, H, I, J, M, N, O: Magnification (400×), scale bar 100 μm. MPA-ILD: microscopic polyangiitis with interstitial lung disease; HE: hematoxylin-eosin; CXCL: C-X-C motif chemokine ligand; CD: cluster of Differentiation; CCR: CC chemokine receptor.

**Figure 4. Pathomechanism of MPA-ILD suggested by PCA based on serum biomarkers and immunohistochemical staining of MPA-ILD.**

(A) Systemic pathomechanism of MPA-ILD. M1 macrophages arise in response to IL-2 and GM-CSF, which was produced by T helper (Th) 1 cells. M1 macrophages producing IL-1b and TNF-a lead to priming of neutrophils. Also, Th2 producing IL-4



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and IL-13 activates B cells (shown in blue). IL-6 and CXCL13 induced accumulation of B cells and differentiation to plasma cells, leading to the production of ANCA. ANCA and cytokines, such as IL-8 and G-CSF, activate neutrophil and cause vascular injury. PDGF and VEGF are released by injured vascular endothelial cells, while TIMP-1, TGF- $\beta$ 1, and LRG are produced during the repair process (shown in red). To suppress the immune response, M2 macrophages arise in response to M-CSF and produce CCL2 and IL-10 (shown in green).

(B) In MPA-ILD, B cells in lymphoid follicle of lungs produce CXCL13 and recruit B cells into the pulmonary inflammatory lesion sites. Th2 cells release IL-4 and IL-13, and these cytokines induce the differentiation of alveolar resident macrophages into M2 macrophages. M2 macrophages produce CCL2 and IL-10. Th2- and M2 macrophage producing cytokines promote fibroblasts to differentiate into myofibroblasts and to produce extracellular matrix in wound healing repair process. MPA-ILD: microscopic polyangiitis with interstitial lung disease.

**Table 1.** Clinical characteristics, and disease severity classification of patients in MPA

Characteristics	MPA (n= 49)
Age, years	73 (70-79)
Female, n (%)	24 (49.0)
Laboratory findings	
WBC, /mm <sup>3</sup>	12480 (7895-14870)
Alb, g/dl	2.4 (2-3.1)
Cr, mg/dl	1.3 (0.74-1.9)
CRP, mg/dl	10.1 (2.9-13.6)
Positive, anti-MPO-ANCA, n (%)	48 (98.0)
Positive, anti-PR3-ANCA, n (%)	3 (6.1)
MPO-ANCA titer, U/mL	96.5 (62.8-260) <sup>a</sup>
BVAS at onset	17 (8.5-21.5)
Five factor score 2009	
≤ 1	12 (24.5)
2	27 (55.1)
≥ 3	10 (20.4)
EUVAS-defined disease activity	
Localized	3 (6.1)
Early systemic	9 (18.4)
Systemic	30 (61.2)
Severe	7 (14.3)

The laboratory markers are presented as the median (interquartile range). MPA: microscopic polyangiitis; WBC: white blood cell; Alb: albumin; Cr: creatinine; CRP: C-reactive protein; MPO-ANCA: myeloperoxidase-anti-neutrophil cytoplasmic autoantibody; PR3-ANCA: proteinase 3-anti-neutrophil cytoplasmic antibody; BVAS: Birmingham Vasculitis Activity Score; EUVAS: European Vasculitis Study Group. <sup>a</sup>Number of subjects, n= 48

<b>Table 2.</b> Comparison of clinical characteristics, disease severity classification, outcome, and treatments of MPA-ILD patients according to cluster analysis			
Characteristics	Cluster1 (n= 17)	Cluster 2 (n=15)	<i>P</i> value
Age, years	77 (72-85)	74 (70-80)	0.39
Female, n (%)	7 (41.2)	8 (53.3)	0.72
Disease duration of ILD (month)	20 (0-48)	1 (0-24)	0.38
Laboratory findings			
WBC, /mm <sup>3</sup>	7870 (5860-10045)	14190 (12480-16520)	0.0001 ***
Alb, g/dl	3.3 (2.6-3.7)	2.1 (1.9-2.5)	0.0003 ***
Cr, mg/dl	1.26 (0.82-1.7)	0.79 (0.65-1.39)	0.10
CRP, mg/dl	2.5 (0.43-6.0)	11.9 (10.1-14.1)	<0.0001 ***
MPO-ANCA titer (IU/mL)	101 (62.3-274.5)	95.0 (57.3-166)	0.65
KL-6, U/ml	571 (240-733)	289 (236-403)	0.049*
SP-D	140 (49-312) <sup>a</sup>	48.3 (21.6-95.3) <sup>b</sup>	0.033*
PFT findings			
%FVC	87.5 (71.5-92.7) <sup>c</sup>	92.8 (76.3-102.6) <sup>d</sup>	0.12
%DLco, ml/min/mmHg	44.2 (26.5-52.1) <sup>e</sup>	46.4 (37.6-52.2) <sup>f</sup>	0.61
Chest HRCT score			
Total GGO score	7.32 (3.66-9.16)	5.99 (3.66-7.64)	0.56
Total Fibrosis score	5.65 (3.16-8.65)	4.32 (3.31-6.32)	0.40
BVAS at onset	14 (9.5-21)	16 (7-21)	0.94
Five factor score 2009			
≤1, n (%)	2 (11.8)	5 (33.3)	0.21
2, n (%)	13 (76.5)	7 (46.7)	0.14
≥3, n (%)	2 (11.8)	3 (20)	0.65
EUVAS-defined disease activity			
Localized, n (%)	2 (11.8)	0 (0)	0.49
Early systemic, n (%)	3 (17.7)	3 (20)	1.00
Systemic, n (%)	9 (52.9)	11 (73.3)	0.29
Severe, n (%)	3 (17.7)	1 (6.7)	0.60
<b>Outcome</b>			
Severe infection	9 (52.9)	2 (13.3)	0.028*
Respiratory-related severe infection	7 (41.2)	2 (13.3)	0.12
<b>Initial treatment</b>			

PDN, mg/day	45 (30-52.5)	45 (37.5-65)	0.28
MPDN pulse, n (%)	0 (0)	3 (20.0)	0.09
Immunosuppressants			
IVCY, n (%)	8 (47.1)	4 (26.7)	0.29
Total IVCY dose (g)	1.6 (1.1-2.3)	1.3 (0.8-2.5)	0.67
RTX, n (%)	1 (5.9)	1 (6.7)	1.000
IVIG, n (%)	0 (0)	0 (0)	NS
AZA/MMF/TAC/MZB, n (%)	15 (88.2)/0 (0)/1 (5.9)/0 (0)	11 (73.3)/1 (6.7)/0 (0)/1 (6.7)	0.38/0.47/1.00/0.45
Apheresis			
Plasma exchange, n (%)	1 (5.9)	0 (0)	1.000

The laboratory markers are presented as the median (interquartile range). The P-values were estimated using Fisher's exact test or Wilcoxon rank sum test. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001. MPA: microscopic polyangiitis; ILD: interstitial lung disease; Disease duration of ILD: the period from appearance of respiratory symptoms to start of treatment; WBC: white blood cell; Alb: albumin; Cr: creatinine; CRP: C-reactive protein; MPO-ANCA: myeloperoxidase-anti-neutrophil cytoplasmic autoantibody; KL-6: Krebs von den Lungen-6; Sp-D: Surfactant protein D; PFT: pulmonary function test; FVC: forced vital capacity; Dlco: diffusing capacity of the lung for carbon monoxide; HRCT: high resolution computed tomography; GGO: ground-glass opacity; BVAS: Birmingham Vasculitis Activity Score; EUVAS: European Vasculitis Study Group; PDN: prednisolone; MPDN: methylprednisolone; IVCY: intravenous cyclophosphamide; RTX: rituximab; IVIG: intravenous immunoglobulin; AZA: azathioprine; MMF: mycophenolate mofetil; TAC: tacrolimus; MZB: mizoribine; NS: not significance. <sup>a</sup>Number of subjects, n= 11. <sup>b</sup>Number of subjects, n= 11. <sup>c</sup>Number of subjects, n= 15. <sup>d</sup>Number of subjects, n= 14. <sup>e</sup>Number of subjects, n= 13. <sup>f</sup>Number of subjects, n= 14.

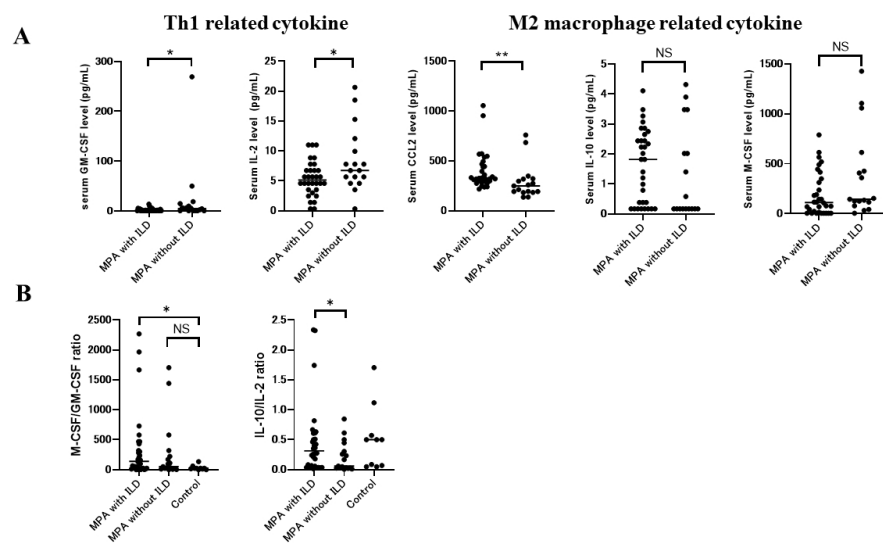


Figure 1

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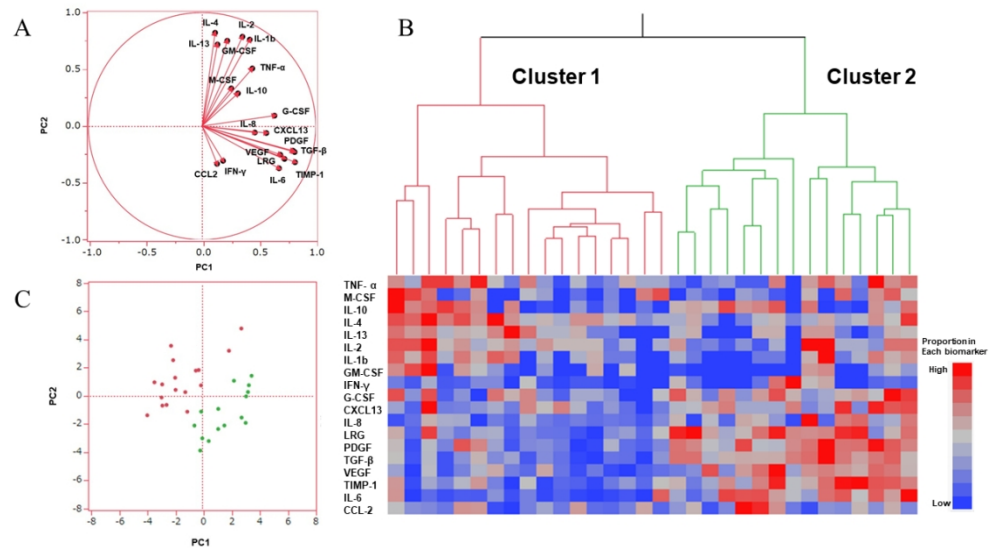


Figure 2

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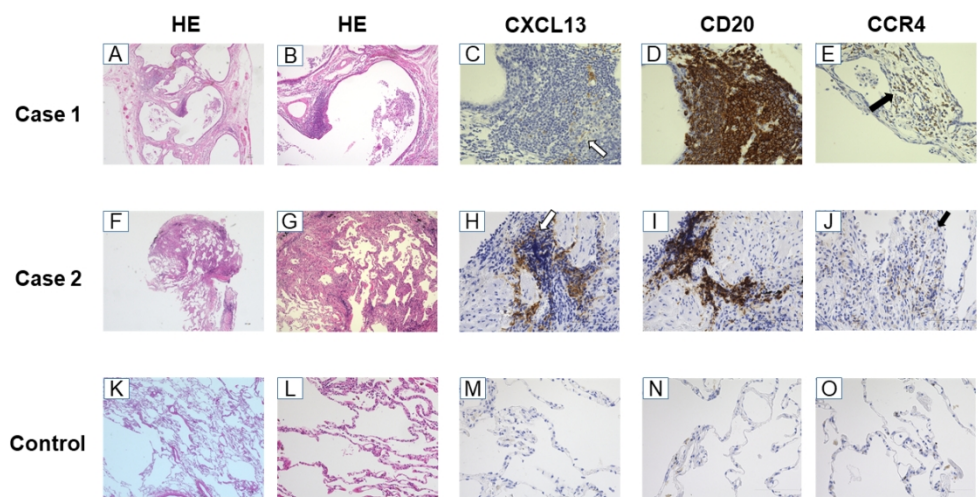


Figure 3

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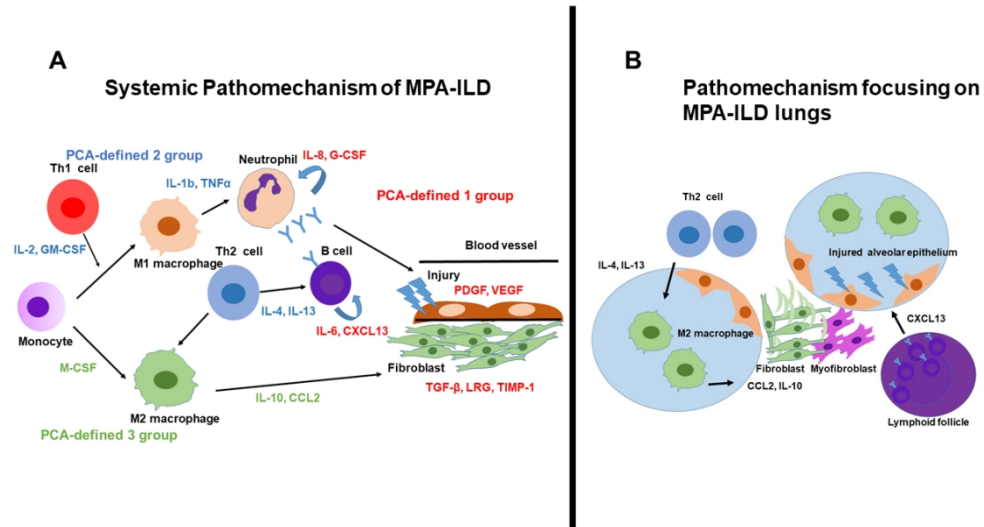


Figure 4

54x30mm (600 x 600 DPI)