


**BRIEF REPORT**

# Serum MMP3 Correlated With IL1 $\beta$ Messenger RNA Expressions of Peripheral Blood Mononuclear Cells in Patients With Relapsing Polychondritis With Respiratory Involvement

Jun Shimizu , Sueshige Wakisaka, Tomoko Suzuki, and Noboru Suzuki

**Objective.** Respiratory involvement was intimately associated with poorer prognosis in patients with relapsing polychondritis (RP). We previously reported that high serum matrix metalloproteinase-3 (MMP3) was frequently observed in patients with RP with respiratory involvement. Elevated MMP3 secreted through local inflammation may be associated with the development of airway lesions.

**Methods.** We collected peripheral blood mononuclear cells (PBMCs) and sera from 30 patients with RP and 14 healthy individuals. Interleukin (IL) 1 $\beta$ , IL6, and tumor necrosis factor (TNF)  $\alpha$  messenger RNA (mRNA) expressions were analyzed in freshly isolated and cultured PBMCs with phytohemagglutinin and phorbol myristate acetate stimulation by real-time reverse transcription polymerase chain reaction and serum MMP3 by enzyme-linked immunosorbent assay (ELISA).

**Results.** We confirmed our previous finding that patients with respiratory involvements showed higher serum MMP3 compared with patients lacking respiratory involvement. IL1 $\beta$  mRNA expression was significantly higher in patients with RP than in healthy individuals after mitogenic stimulation. TNF $\alpha$  mRNA expression after stimulation was significantly lower in patients with RP compared with in healthy individuals. We performed correlation analyses between MMP3 and cytokine mRNA expressions in patients with RP. In patients with respiratory involvement, MMP3 correlated with IL1 $\beta$  and IL6 after stimulation. In patients without respiratory involvement, no positive correlations between MMP3 and cytokine mRNA expressions were observed regardless of culture condition. We did not find any positive correlations between MMP3 and TNF $\alpha$  mRNA expression in patients with RP.

**Conclusion.** It is possible that IL1 $\beta$  mRNA expression associates by some means with airway inflammation via the secretion of MMP3 in patients with RP. Involvement of proinflammatory cytokines, including IL1 $\beta$ , was suggested for the pathophysiology of airway lesions in patients with RP.

## INTRODUCTION

Relapsing polychondritis (RP) is characterized by recurrent inflammation and destruction of cartilaginous tissues, including ears, nose, respiratory tract, and joints (1). The inflammation often spreads to other organs such as eyes, inner ears, heart, blood vessels, and kidneys (1). Respiratory involvement is associated intimately with progressive disease course, leading to death in patients with RP (2–5).

The pathogenesis of RP remains largely unknown. Histo-pathological analyses demonstrated that T cells, plasma cells, neutrophils, and macrophages infiltrated into the perichondrium (6). Matrix metalloproteinase-3 (MMP3) was produced by activated chondrocytes in the RP lesions, and the expressing cell numbers positively correlated with apoptotic chondrocyte numbers (6). In an electron microscopic analysis of auricular chondritis, degenerating chondrocytes released matrix vesicles (7). It was suggested that some of the matrix vesicles

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contained proteolytic enzymes and were functionally similar to lysosomes (7). It is conceivable that accelerated immune responses are involved in the chondrocyte degeneration in patients with RP (8).

We showed that serum MMP3 increased significantly in patients with RP with respiratory involvement compared with in healthy individuals; however, this was not associated with disease activity (4). Serum MMP3 was comparable between patients with RP with auricular involvement and healthy individuals (4).

High serum MMP3 was recognized in rheumatoid arthritis (RA), and serum MMP3 was suggested to be released from the synovium (9). In the joint, inflammatory cytokines, interleukin (IL) 1 $\beta$ , IL6, and tumor necrosis factor (TNF)  $\alpha$ , were thought to promote the secretion of MMP3 (9,10,11).

We hypothesized that local inflammation with excessive secretion of IL1 $\beta$ , IL6, and TNF $\alpha$  induced MMP3 production by chondrocytes and led to destruction of airway cartilaginous tissues in patients with RP. We here investigated IL1 $\beta$ , IL6, and TNF $\alpha$  messenger RNA (mRNA) expressions in peripheral

blood mononuclear cells (PBMCs) and compared those with serum MMP3 in patients with RP.

## PATIENTS AND METHODS

**Patients.** We obtained PBMCs and sera of 30 patients diagnosed with RP based on Damiani criteria (12) and 14 age- and sex-matched healthy individuals. Demographic and clinical data of RP patients and healthy individuals are summarized in Table 1. All patients who participated in this study were clinically stable at the time of sample collection, with a mean RP Disease Activity Index (RPDAI) (13) score of 23.1. All 30 patients with RP were negative for myeloperoxidase- and proteinase 3-antineutrophil cytoplasmic antibodies and had no comorbid autoimmune diseases. Two patients underwent tracheostomy during the disease courses. This study was approved by the institutional review board of St. Marianna University School of Medicine and was registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN000018937). We conducted our research according to the principles expressed in the Declaration of Helsinki. We

**Table 1.** Demographic and clinical data of patients with RP and healthy individuals

	Patients With RP			Healthy Individuals
	Total	Resp	Non-Resp	
Patients, n	30	16	14	14
Demographic data				
Age, years	53.2 $\pm$ 2.7	52.5 $\pm$ 3.2	53.8 $\pm$ 4.5	46.2 $\pm$ 3.8
Age at onset, years	42.7 $\pm$ 3.3	42.4 $\pm$ 4.3	42.9 $\pm$ 5.1	
Disease duration, years	10.3 $\pm$ 1.6	9.7 $\pm$ 2.1	10.9 $\pm$ 2.4	
Male:female ratio	10:20	7:9	3:11	8:6
RPDAI score	23.1 $\pm$ 3.3	28.8 $\pm$ 5.2	16.6 $\pm$ 3.4	
Cumulative incidence of clinical complications, %				
Auricular	70.0	62.5	78.6	0
Nasal	53.3	62.5	42.9	0
Inner ear	20.0	25.0	14.3	0
Joint	33.3	43.8	21.4	0
Eye	63.3	68.8	57.1	0
Airway	53.3	100 <sup>a</sup>	0	0
Skin	0.67	12.5	0	0
Cardiovascular	13.3	18.8	7.1	0
Neurological	0.33	0	7.1	0
Renal	0.67	6.3	7.1	0
Medication utilization rates, %				
Steroids	86.7	100 <sup>a</sup>	71.4	0
Immunosuppressants	60.0	62.5	57.1	0
Methotrexate	30.0	37.5	21.4	0
Cyclosporine	20.0	25.0	14.3	0
Tacrolimus	13.3	12.5	14.3	0
Mizoribine	6.7	0	14.3	0
Azathioprine	3.3	6.3	0	0
Cyclophosphamide	0	0	0	0
Biologic agents	26.7	25.0	28.6	0
Infliximab	13.3	6.3	21.4	0
Tocilizumab	10.0	12.5	7.1	0
Adalimumab	3.3	6.3	0	0
Etanercept	0	0	0	0

Abbreviation: Non-Resp, patients without respiratory involvement; Resp, patients with respiratory involvement; RP, relapsing polychondritis; RPDAI, Relapsing polychondritis disease activity index.

<sup>a</sup> Significant differences between Resp and Non-Resp ( $P < 0.05$ ).

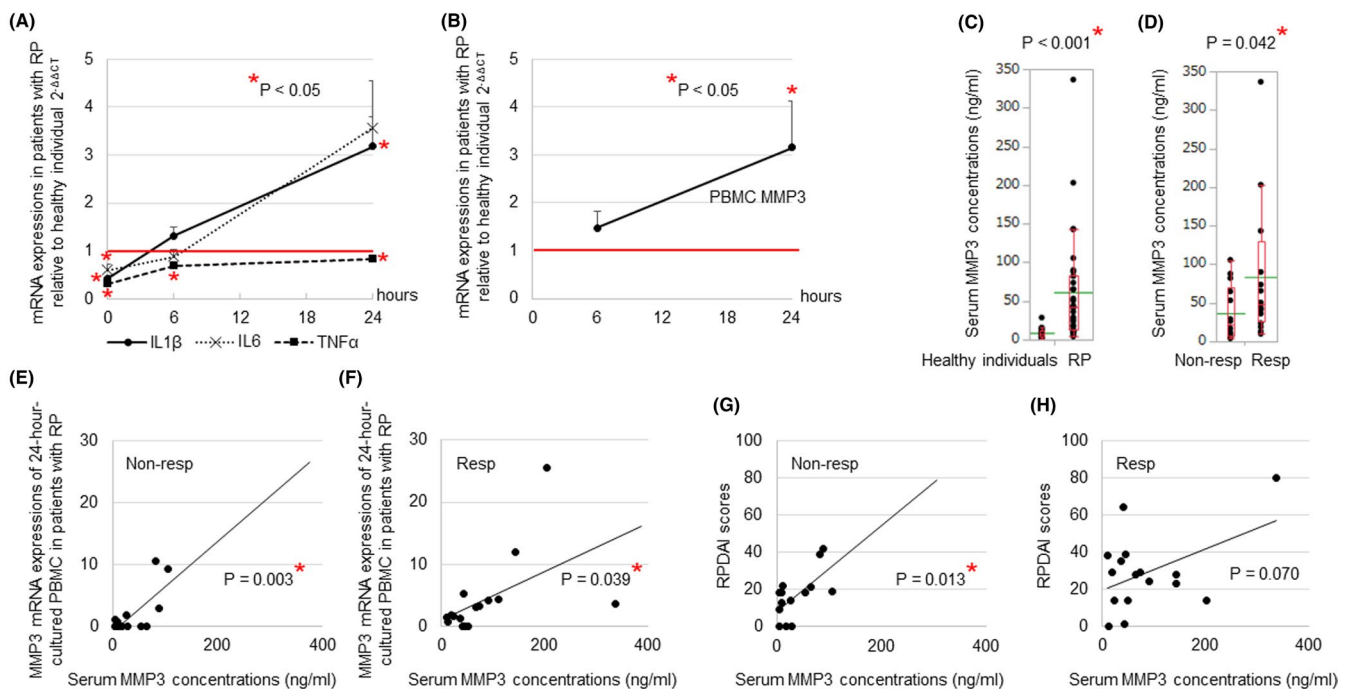
obtained written informed consent from each individual prior to enrollment in the study.

**Cell culture, mRNA expression analyses, and an ELISA assay.** PBMCs were cultured for up to 24 hours with 1  $\mu\text{g/ml}$  phytohemagglutinin (Sigma-Aldrich) and 4 ng/ml phorbol myristate acetate (Sigma-Aldrich). We extracted total RNA from freshly isolated, 6-hour-cultured, and 24-hour-cultured PBMCs. We studied four combinations of TaqMan primers and probes from Life Technologies Japan of IL1 $\beta$ , IL6, TNF $\alpha$ , and MMP3. Eukaryotic 18S ribosomal RNA was used as an endogenous control.

Relative gene expressions of patients with RP and healthy individuals were estimated by the  $\Delta\Delta\text{Ct}$  method. The changes of the cytokine mRNA expressions of PBMCs in patients with RP were calculated by adjusting to healthy individuals ( $2^{-\Delta\Delta\text{Ct}}$  of healthy individuals = 1, indicated by a red line in Figure 1A).

We measured serum concentrations of MMP3 using an ELISA kit (R&D Systems).

**Statistical analysis.** We compared demographical data of patients with those of normal individuals by using a Wilcoxon rank-sum test or Fisher's exact test with statistical software JMP 13.0.0 (SAS Institute Japan). We compared relative gene expression



**Figure 1.** Evaluation of proinflammatory cytokine and matrix metalloproteinase-3 (MMP3) messenger RNA (mRNA) expressions and serum MMP3 concentrations in patients with relapsing polychondritis (RP). Asterisks indicate significant differences between each group of patients with RP and healthy individuals ( $P < 0.05$ ). **A**, Interleukin (IL) 1 $\beta$ , IL6, and tumor necrosis factor (TNF)  $\alpha$  mRNA expressions in freshly isolated, 6-hour-cultured, and 24-hour-cultured peripheral blood mononuclear cells (PBMCs) with phytohemagglutinin (PHA) and phorbol myristate acetate (PMA) stimulation were estimated by the  $\Delta\Delta\text{Ct}$  method. The changes of the cytokine mRNA expressions of PBMCs in patients with RP were calculated by adjusting to healthy individuals ( $2^{-\Delta\Delta\text{Ct}}$  of healthy individuals = 1, indicated by a red line). IL1 $\beta$ , IL6, and TNF $\alpha$  mRNA expressions decreased significantly in freshly isolated PBMCs of patients with RP compared with in those of healthy individuals. IL1 $\beta$  mRNA expression was significantly higher in patients with RP than in healthy individuals 24 hours after mitogenic stimulation. TNF $\alpha$  mRNA expression 24 hours after stimulation was significantly lower in patients with RP compared with healthy individuals. **B**, We measured BMP3 mRNA expressions in freshly isolated, 6-hour-cultured, and 24-hour-cultured PBMCs with PHA and PMA stimulation. We did not detect BMP3 mRNA expressions in freshly isolated PBMCs of either patients with RP or healthy individuals. BMP3 mRNA expression was significantly higher in patients with RP than in healthy individuals 24 hours after mitogenic stimulation. **C**, Comparison of serum BMP3 between patients with RP and healthy individuals. Serum BMP3 increased significantly in patients with RP compared with in healthy individuals. **D**, Comparison of serum BMP3 between a subgroup of patients with respiratory involvement (Resp Subgroup) and those without respiratory involvement (Non-Resp Subgroup). Serum BMP3 increased significantly in the Resp Subgroup compared with that in the Non-Resp Subgroup. These biological parameters of patients with RP and healthy individuals are displayed with dot plots in **C** and **D**. A box plot and a mean level (green line) of each group of patients with RP and healthy individuals are indicated. **E**, A significant positive correlation was observed between 24-hour-cultured PBMC BMP3 mRNA and serum BMP3 in the Non-Resp Subgroup. **F**, A significant positive correlation was observed between 24-hour-cultured PBMC BMP3 mRNA and serum BMP3 in the Resp Subgroup. **G**, A significant positive correlation was observed between the RP Disease Activity Index (RPDAI) score and serum BMP3 in the Non-Resp Subgroup. **H**, We did not observe a significant positive correlation between the RPDAl score and serum BMP3 in the Resp Subgroup.

levels and ELISA titers by using a Wilcoxon rank-sum test. Several parameter titers were expressed as mean  $\pm$  standard error of the mean. A *P* value of less than 0.05 was considered significant.

## RESULTS

**Proinflammatory cytokine and MMP3 mRNA expressions of PBMCs in patients with RP.** We first assessed IL1 $\beta$ , IL6, TNF $\alpha$ , and MMP3 mRNA expressions in PBMCs and compared those between patients with RP and healthy individuals.

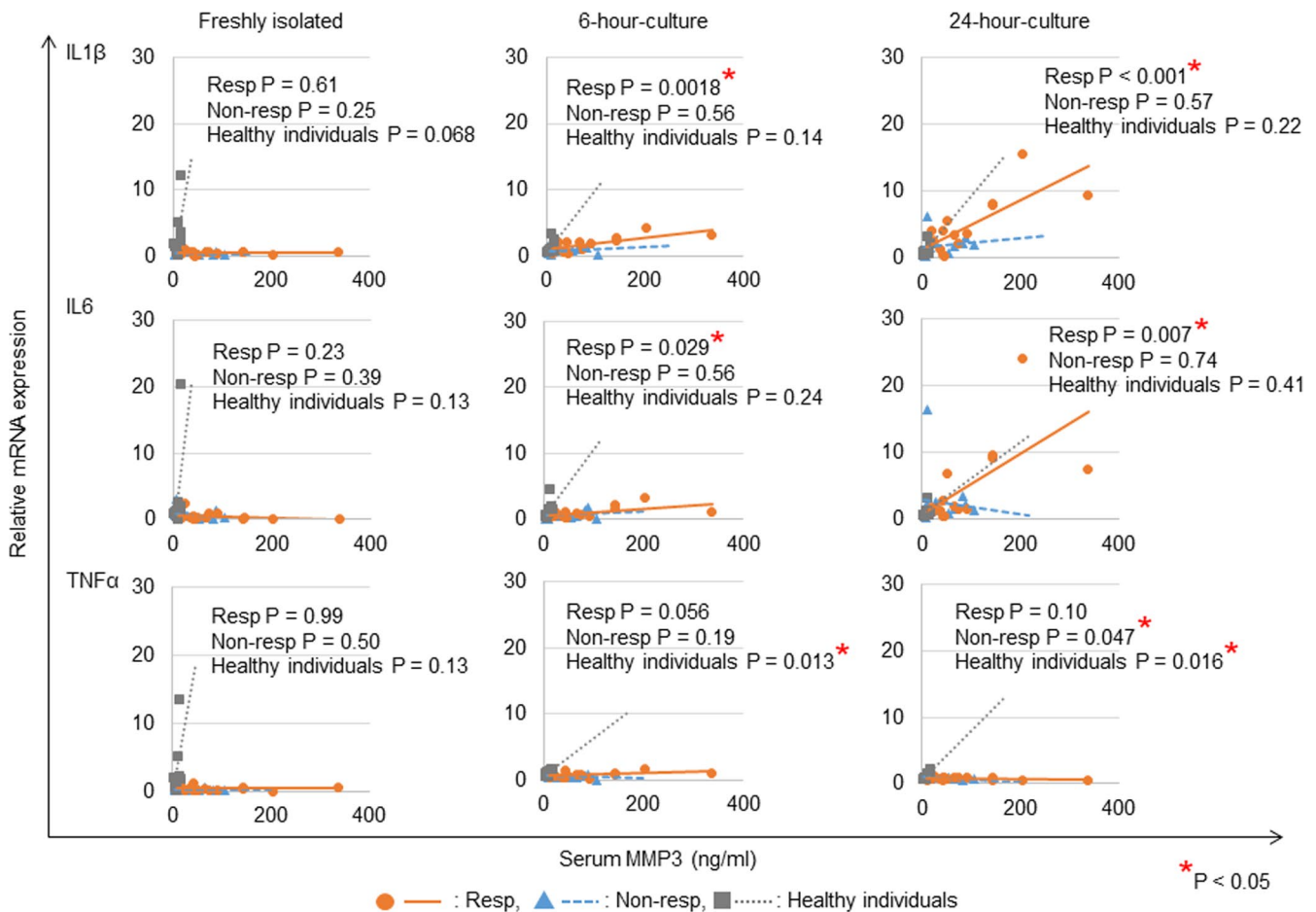
IL1 $\beta$ , IL6, and TNF $\alpha$  mRNA expressions of freshly isolated PBMCs decreased significantly in patients with RP compared with healthy individuals (Figures 1A and 2). IL1 $\beta$  increased significantly in patients with RP compared with healthy individuals 24 hours after stimulation (Figure 1A). In the same culture condition, the increase in IL6 did not reach the statistical significance in patients with RP compared with healthy individuals (*P* = 0.06, not

significant; Figure 1A). TNF $\alpha$  kept significantly decreased levels in patients with RP compared with healthy individuals throughout the culture period (Figure 1A).

We did not detect MMP3 mRNA expressions in freshly isolated PBMCs of either patients with RP or healthy individuals. MMP3 mRNA expression was significantly higher in patients with RP than in healthy individuals 24 hours after mitogenic stimulation (Figure 1B).

**Elevated serum MMP3 concentrations in patients with RP, especially in those with respiratory involvement.** We then measured serum MMP3 and compared the titers between patients with RP and healthy individuals. Serum MMP3 increased significantly in patients with RP compared with in healthy individuals (Figure 1C).

We reported that serum MMP3 increased significantly in patients with RP with respiratory involvement compared with



**Figure 2.** Correlation analyses between serum matrix metalloproteinase-3 (MMP3) and proinflammatory cytokine messenger (mRNA) expressions of freshly isolated, 6-hour-cultured, and 24-hour-cultured peripheral blood mononuclear cells in patients with relapsing polychondritis (RP) with respiratory involvement (Resp Subgroup; *n* = 16), patients with RP without respiratory involvement (Non-Resp Subgroup; *n* = 14), and healthy individuals (*n* = 14). In the Resp Subgroup, MMP3 correlated with interleukin (IL) 1 $\beta$  and IL6 after stimulation. In the Non-Resp Subgroup, positive correlation between MMP3 and cytokine mRNA expressions was not observed regardless of the culture condition. Asterisks indicate significant correlation (*P* < 0.05).

healthy individuals, whereas serum MMP3 levels were comparable between patients with RP without respiratory involvement and healthy individuals (4).

Here, we divided patients of this study into the following two subgroups: a subgroup of patients with respiratory involvement (Resp Subgroup) and a subgroup of patients without respiratory involvement (Non-Resp Subgroup). Serum MMP3 increased significantly in patients in the Resp Subgroup compared with those in the Non-Resp Subgroup (Figure 1D). Serum MMP3 showed positive linear correlations with PBMC MMP3 mRNA expressions in both the Resp and Non-Resp Subgroups (Figure 1E and F).

We did not find any statistical differences in the demographic data, cumulative incidence of clinical complications, and medication utilization rates between the Resp and Non-Resp Subgroups, except for incidence of airway involvement and steroid use rates (Table 1). We did not observe any significant differences in serum MMP3 levels between patients with RP who received biologic agents (that is, anti-TNF $\alpha$  and/or anti-IL6 agents) and those who did not (Supplemental Figure 1).

**Correlation analyses of RPDAl and serum concentrations of MMP3 in patients with RP.** We performed correlation analyses to evaluate the relations between RPDAl and serum MMP3 in each subgroup. We observed a significant positive correlation between RPDAl scores and serum MMP3 concentrations in the Non-Resp Subgroup but not in the Resp Subgroup (Figure 1G and H).

**Correlation analyses of proinflammatory cytokine mRNA expressions and serum concentrations of MMP3 in patients with RP.** We performed correlation analyses to evaluate relationships between the cytokine mRNA expressions and serum MMP3 concentrations in each subgroup (Figure 2). Spontaneous expressions of cytokine mRNA were very low both in the Resp Subgroup and in the Non-Resp Subgroup (freshly isolated in Figure 2). In the Resp Subgroup, serum MMP3 correlated with IL1 $\beta$  and IL6 at both 6 and 24 hours after mitogenic stimulation. In the Non-Resp Subgroup, no positive correlations between serum MMP3 and cytokine mRNA expressions were observed regardless of the culture condition. Indeed, we did not find any positive correlations between serum MMP3 and TNF $\alpha$  in patients with RP.

## DISCUSSION

In this study, serum MMP3 concentrations correlated with IL1 $\beta$  mRNA expressions and with IL6 mRNA expressions after stimulation of PBMCs in patients with RP with respiratory involvement (Figures 1A and 2). To our surprise, we did not find any positive correlations between serum MMP3 and TNF $\alpha$  mRNA expression in our RP patient cohort.

In RA, serum MMP3 was thought to be derived from synovium and to correlate with disease activity (9). Associations between serum MMP3 and IL1 $\beta$  mRNA expression in patients with RP in the Resp Subgroup, but not in those in the Non-Resp Subgroup, may indicate that IL1 $\beta$  induces MMP3 effectively in somewhere in the respiratory tract (9). It is also possible that TNF $\alpha$  locally produced in the lesions may lack the ability to induce MMP3 by the bronchial chondrocytes in patients with RP with respiratory involvement. The positive correlation between serum MMP3 and cytokine mRNA expressions in the Resp Subgroup may be associated with efficient immune responses of adaptive immunity in patients with RP using a tracheal lesion-associated autoantigen, matrilin-1 (8).

Th1-skewed responses in the peripheral blood were observed in patients with RP (2,8,10). Several studies demonstrated that T cell-related cytokines associated with TNF $\alpha$  mRNA destabilization, presumably through the regulation of RNA-binding proteins in the macrophages (14–16). It may be reasonable to assume that RNA modification plays a role in the decreased expression of PBMC TNF $\alpha$  mRNA in patients with RP, but additional work will be needed to test the hypothesis.

We found that PBMC MMP3 mRNA expressions correlated with serum MMP3 in both the Resp and Non-Resp Subgroups (Figure 1E and F). Pathologically, RP immunocompetent cells migrated into perichondrial tissues, and MMP3 production was recognized in both the cartilage and perichondrial tissues (6,8). We suggest that activated PBMCs with upregulated mRNA expressions of inflammatory cytokines and MMP3 have a potential to induce the release of MMP3 from chondrocytes into peripheral circulation, partially similar to the synovitis of RA (9).

We observed a significant positive correlation between RPDAl scores and serum MMP3 in the Non-Resp Subgroup but not in the Resp Subgroup (Figure 1G and H). Indeed, in the Non-Resp Subgroup of this study, three patients had eye involvement, two patients had arthritis, two patients had vestibular dysfunction, one patient had encephalitis, and one patient had angina and renal failure. The organ involvement may associate with their serum levels of MMP3.

RP as an inflammatory disease with recurrent attacks and RA as a major systemic autoimmune disease may differ from each other in the inflammatory processes of the local lesions involving MMP3. It is possible that the combination of IL1 $\beta$ , IL6 and TNF $\alpha$  and that of TNF $\alpha$  and IL6 may play a role in the pathophysiology of RP and RA, respectively.

It was reported that patients with familial Mediterranean fever, a major autoinflammatory disease caused by the abnormal secretion of IL1 $\beta$ , were complicated by the development of RP in five cases (17). A recent study revealed that somatic mutations of a major ubiquitylation enzyme associated with adult-onset autoinflammatory diseases, including RP, through overactivation of mutated hematopoietic stem cells and peripheral blood myeloid

cells (18). These data suggest that the immune responses observed here may have certain effects on the bronchial inflammation in RP.

In human inflammatory diseases, *ex vivo* PBMC hyporesponsiveness against *in vitro* stimulation was frequently observed, and the low sensitivity was considered to associate with disease activities and therapeutic responses (19,20).

It is interesting to clarify whether the reduction of MMP3 production by some means, including anti-IL1 agents, may bring about remission of airway destruction in patients with RP with progressive disease.

In conclusion, we suggested that IL1 $\beta$  and IL6 mRNA expressions in PBMCs after stimulation associated with serum MMP3 in patients with RP with respiratory involvement. Some patients with RP were treated with biologic agents to inhibit IL1 $\beta$ , IL6, and TNF $\alpha$  (21), with substantial success. Functional relationships between each anticytokine therapy and cytokine expression profiles of the patients require further investigations.

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## AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Noboru Suzuki had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Study conception and design.** Shimizu, Noboru Suzuki.

**Acquisition of data.** Shimizu, Wakisaka, Tomoko Suzuki, Noboru Suzuki.

**Analysis and interpretation of data.** Shimizu, Noboru Suzuki.

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