criteria に従って、活動性と非活動性の 2 群に分け、マーカー候補分子をこの両群で比較した（表 1、表 2）。活動性 RP とする基準は耳介、鼻、気管の 3 か所のうち、2 か所以上の軟骨炎を認める例および 1 か所とその他 2 つの症状（眼の炎症、関節炎、又は聴覚・前庭症状）を有する例とした。この基準において活動性 RP 患者 8 名、非活動性 RP 患者 7 名となり、この両群の比較を Welch の t 検定によって実施した。

測定した項目は以下の通りである。

IL-1α，IL-1β，IL-2，IL-4，IL-5，IL-6，IL-8，IL-10，IL-12p70，TNF，IFNγ，GM-CSF，CCL2/MCP-1，CCL3/MIP-1α，CCL4/MIP-1β，CCL5/RANTES，CXCL10/IP-10，vascular endothelial growth factor（VEGF），CX3CL1/Fractalkine の測定には Cytometric Bead Array Flex set system（BD Biosciences）を用いた。IL-17, matrix metalloproteinase（MMP）-1，MMP-2，MMP-3，MMP-13，可溶性 TREM-1（sTREM-1），cartilage oligomeric matrix protein（COMP）および抗 typeII コラーゲン抗体は ELISA を用いて、測定を行った。CRP 濃度（CRP および高感度 CRP）は三菱化学メディエンスにおいて N-Latex CRPII キットを用いた nepherometry によって測定された。

（2）血清 sTREM-1 値が RP の疾患活動性を反映し、治療に対して応答するかどうかを調べるために、メトトレキサートによる治療を開始した活動性 RP 患者において、臨床症状、治療内容、sTREM-1 値および CRP 値が経時的に得られた例があり、その結果を解析した。

（3）RP 患者において血清 sTREM-1 値が高値を示す機序を推定するため、健常者 2 名および RP 患者 2 名の末梢血単核球を用いて、5 つの各細胞群における膜型 TREM-1 の発現を解析・比較した。具体的には、フローサイトメトリーを用いて、CD4+T 細胞（CD3+CD4+細胞）、

CD8+T 細胞（CD3+CD8+細胞）、B 細胞（CD3-CD19+細胞）、単球（CD3-CD14+細胞）および NK 細胞（CD3-CD56+細胞）における TREM-1 発現を解析した。

（4）RP 患者の病変部における膜型 TREM-1 発現の有無と局在を調べるために、免疫組織染色を行った。コントロールとして非特異的な炎症による肉芽組織を使用した。

（倫理面への配慮）

臨床検体の収集に際しては、本学の生命倫理委員会で承認された（承認番号：第 1625 号）同意書を用いて、不利益や危険性の排除などに関するインフォームドコンセントを行った。また検体は、提供者を特定できないように個人情報管理者が連結不可能匿名化により番号化し、患者の人権擁護に努めた。

C. 研究結果

（1）28 種類のマーカー候補分子を活動性 RP 患者群と非活動性 RP 患者群で比較すると、RP のマーカーとして報告のある CRP，COMP および抗 typeII コラーゲン抗体は確かに活動性 RP 患者において高値を示すが、非活動性 RP 患者群との比較において有意差を示すことができなかった。それに対して、sTREM-1 は活動性 RP 患者群において有意に高値を示した（$p = 0.0403$）（表 2）。

（2）図 1 に示すように、メトトレキサートによる治療を開始後、嗄声が改善すると同時に、720.5 pg/ml と異常高値を示していた sTREM-1 レベルが 106.6 pg/ml まで低下した。この値は我々が以前、決定した「健常者と RP 患者を判別するカットオフ」である 158 pg/ml を下回るレベルである。また、重要なことにメトトレキサート投与前、CRP 値は 0.41 mg/dl と正常値に近く、疾患活動性が捉えられていない状況においても、sTREM-1 値は異常高値を

示した。

（３）図２に RP 患者１名の解析結果を示す。その結果から単球（CD3-CD14+細胞）においてのみ、膜型 TREM-1 の発現レベルが高いことが判明した。そこで、RP 患者および健常者由来の単球上の膜型 TREM-1 発現レベルを比較したが、両者に発現の差を認めなかった（図３）。

（４）膜型TREM-1はRP患者の病変部にある軟骨細胞上では検出されず、病変部にある炎症性肉芽組織の血管内皮細胞において発現していることが明らかになった（図４）。

D. 考案

昨年度までに健常者と比較して RP 患者血清で有意に高値を示す分子として、sTREM-1、インターフェロンγ、CCL4/MIP-1β、VEGF および MMP-3 を同定していたが、その中で血清 sTREM-1 レベルは活動性RPおよび非活動性RPも区別することが可能であったため、RP の疾患活動性マーカーとして優れていることが明らかとなった。実際、活動性 RP 患者において sTREM-1 値の変動が治療に応答した臨床症状の変化と一致した例を認めた。これは血清 sTREM-1 が RP の疾患活動性マーカーだけでなく、治療応答マーカーである可能性も示唆するものである。

膜型 TREM-1 は免疫グロブリンスーパーファミリーの一員で、主に好中球、単球/マクロファージの細胞膜上に発現していることが知られるが、本研究では少なくとも単球を含む末梢血単核球において、健常者と RP 患者間で膜型 TREM-1 の発現量に差を認めなかった。しかし、RP 病変部の血管内皮細胞ではコントロールでは確認できない膜型 TREM-1 が発現していたことから、活動性の高い RP 患者における末梢血中の sTREM-1 の上昇は血管内皮細胞上の TREM-1 から切断・遊離されてくる可能性が考えられた。

E. 結論

本研究により CRP，COMP および抗 type II コラーゲン抗体よりも優れた RP の疾患活動性マーカーとして血清 sTREM-1 を同定することができた。この血清 sTREM-1 レベルは RP の治療応答性マーカーとしての可能性が示唆された。

F. 研究発表

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RING-finger type E3 ubiquitin ligase
inhibitors as novel candidates for the
treatment of rheumatoid arthritis.
Int. J. Mol. Med, 30:1281-1286, 2012.

2. 学会発表

なし

G. 知的財産権の出願・登録状況（予定を含
む)
なし

表1 被験 RP 患者の臨床情報

	RP 患者		
	全例(n = 15)	活動性(n = 8)	非活動性(n = 7)
年齢(年)	47 [10-81]	50.5 [10-74]	44 [27-81]
女性の割合	53.3%	50.5%	57.1%
罹病期間(年)	5 [1–19]	12 [4–19]	4 [1–8]
耳介軟骨炎	46.7%	62.5%	28.6%
鼻軟骨炎	40.0%	62.5%	14.3%
気管軟骨炎	66.7%	87.5%	42.9%
耳症状	53.3%	87.5%	14.3%
関節炎	46.7%	75.0%	14.3%
眼の炎症	33.3%	50.0%	14.3%

表2　活動性RP患者と非活動性RP患者におけるマーカー候補分子の血清濃度の比較

Biomarker candidates[a]	Units	Active RP (n =8)			Inactive RP (n =7)			P*
		Mean	±	SD	Mean	±	SD	
sTREM-1	pg/ml	353.39	±	158.03	200.14	±	95.11	**0.0403**
VEGF	pg/ml	339.19	±	218.10	185.48	±	106.88	0.1066
hs-CRP	ng/ml	0.48	±	0.64	0.10	±	0.08	0.1342
TNF	pg/ml	1.43	±	2.65		N.D.		0.1708
IL-6	pg/ml	2.38	±	4.45		N.D.		0.1752
IL-17A	pg/ml	0.05	±	0.14	0.71	±	1.14	0.2129
MMP-3	ng/ml	334.71	±	400.33	138.44	±	135.59	0.2254
MMP-1	ng/ml	5.35	±	4.35	3.07	±	2.51	0.2658
MMP-13	ng/ml	0.30	±	0.11	0.26	±	0.05	0.3469
IL-1α	pg/ml	1.01	±	2.86		N.D.		0.3506
IL-1β	pg/ml	1.09	±	3.07		N.D.		0.3506
IL-10	pg/ml	1.30	±	3.68		N.D.		0.3506
IL-12p70	pg/ml	0.66	±	1.87		N.D.		0.3506
CX3CL1	pg/ml	12.29	±	34.75		N.D.		0.3506
MMP-2	ng/ml	139.68	±	25.79	125.38	±	31.39	0.3589
COMP	ng/ml	30.26	±	35.31	17.56	±	10.53	0.3598
CXCL10	pg/ml	251.14	±	110.78	204.78	±	121.20	0.4563
IFN-γ	pg/ml	4.54	±	7.29	6.93	±	5.06	0.4703
CXCL8	pg/ml	17.31	±	6.34	15.01	±	8.11	0.5571
CCL2	pg/ml	80.59	±	78.04	62.80	±	30.33	0.5660
CCL4	pg/ml	141.68	±	90.46	124.7	±	33.26	0.6332
IL-4	pg/ml	0.83	±	2.36	0.76	±	2.02	0.9509
CCL5	ng/ml	37.87	±	17.21	37.42	±	15.05	0.9585
αCOLII Ab[b]	U/ml	382.34	±	808.48	162.44	±	311.65	0.5525

RP, relapsing polychondritis; sTREM-1, soluble triggering receptor expressed on myeloid cells-1; VEGF, vascular endothelial growth factor; hs-CRP, high-sensitivity C-reactive protein; TNF, tumor necrosis factor; N.D., not detected; IL, interleukin; MMP, matrix metalloproteinase; CX3CL, chemokine (C-X3-C motif) ligand; COMP, cartilage oligomeric matrix protein; CXCL, chemokine (C-X-C motif) ligand; IFN, interferon; CCL, chemokine (C-C motif) ligand; αCOLII Ab, anti-type II collagen antibody

[a] IL-2, IL-5, GM-CSF, CCL3 の血清レベルはすべての症例において検出限界以下であった.

[b] 検体の不足により、本項目のサンプルサイズは以下の通り (active RP: n = 6, inactive RP: n = 7)..

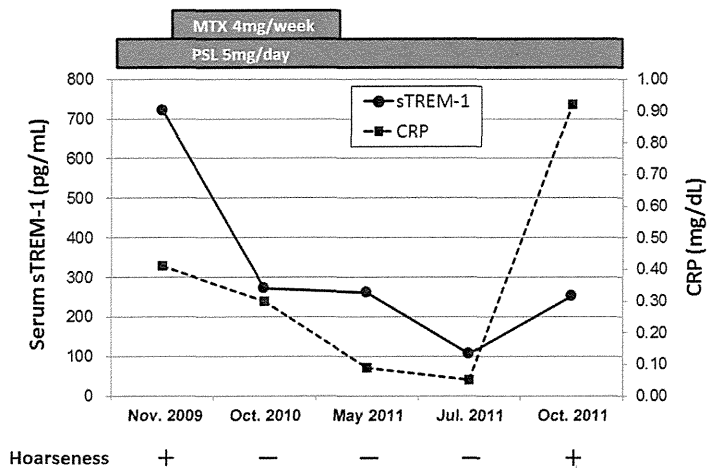*ウェルヒの T 検定による. 0.05 以下の P 値のみ太字で表示した.

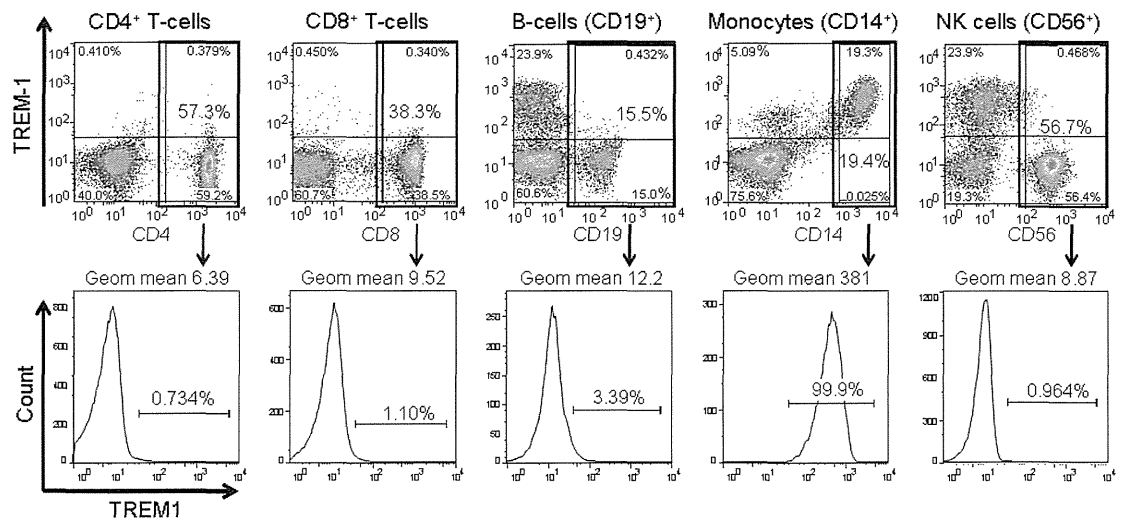図1　活動性 RP 患者における臨床経過とマーカーの経時的変化の一例

図2　末梢血単核球中の TREM-1 の発現

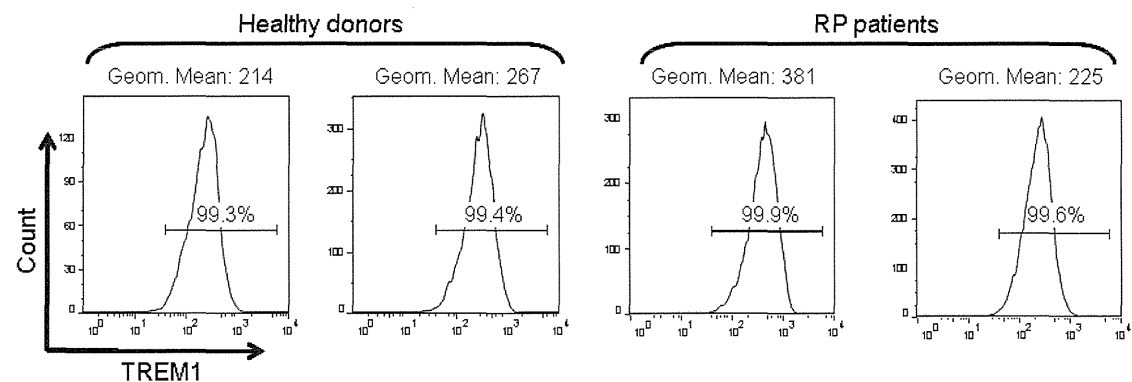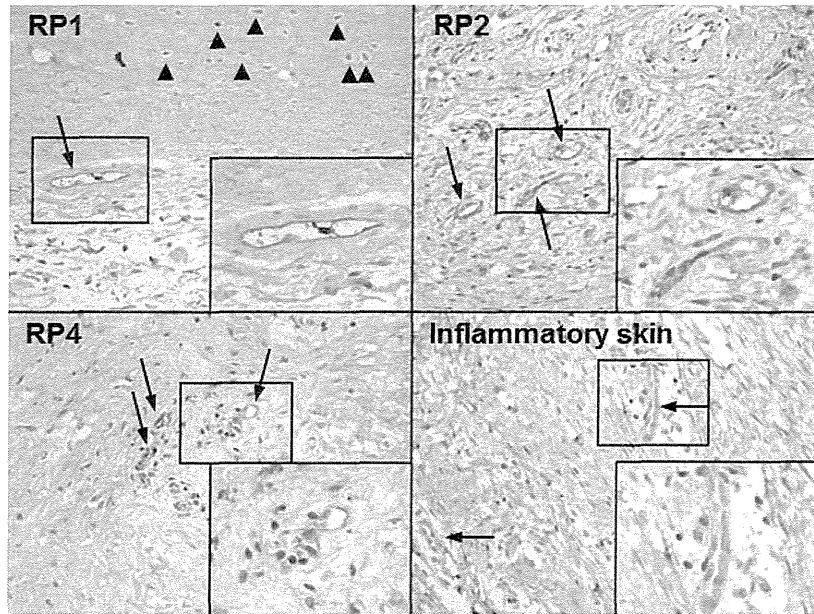図3　健常者および RP 患者における単球上の TREM-1 発現比較

図4　RP 患者の軟骨炎病変部における TREM-1 の局在

Ⅲ．研究成果の発表に関する一覧表

雑誌

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Murata M, Yudoh K, Shimizu H, Beppu M, Nakamura H, Kato T, Masuko K.	Layilin, a talin-binding hyaluronan receptor, is expressed in human articular chondrocytes and synoviocytes and is down-regulated by interleukin-1β.	Mod Rhematol.	6	online	2012
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Ⅳ．研究成果の刊行物・別刷

RING-finger type E3 ubiquitin ligase inhibitors as novel candidates for the treatment of rheumatoid arthritis

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Abstract. Rheumatoid arthritis (RA) significantly affects quality of life. We recently cloned synoviolin, a RING-type E3 ubiquitin ligase implicated in the endoplasmic reticulum-associated degradation (ERAD) pathway. Synoviolin is highly expressed in rheumatoid synovial cells and may be involved in the pathogenesis of RA. Inhibition of synoviolin activity is a potentially useful therapeutic approach for the treatment of RA. We conducted a high-throughput screen of small molecules to find inhibitors of synoviolin autoubiquitination activity. We identified two classes of small molecules, named LS-101 and LS-102, which inhibited synoviolin activity. LS-102 selectively inhibited synoviolin enzymatic activity, while LS-101 inhibited a broad array of RING-type E3 ligases. Moreover, these inhibitors suppressed the proliferation of rheumatoid synovial cells, and significantly reduced the severity of disease in a mouse model of RA. Our results suggest that inhibition of synoviolin is a potentially useful approach in the treatment of RA.

Introduction

Rheumatoid arthritis (RA) is the most common chronic inflammatory joint disease, affecting ~0.5-1% of people in the industrialized world (1). Clinically, the disorder is characterized by joint pain, stiffness, and swelling due to synovial inflammation and effusion. The clinical features of RA are based on several pathological processes including chronic inflammation, overgrowth of synovial cells, bone and joint destruction, and fibrosis. Currently, the goal of RA treatment is the control of underlying inflammatory process to prevent joint damage using non-steroidal anti-inflammatory drugs, glucocorticoids, and disease-modifying anti-rheumatic drugs (DMARD). The most widely used small molecule DMARD is methotrexate, which shows the highest retention rate compared with other agents (2). In recent years, biological agents such as inhibitors of tumor necrosis factor (TNF) signaling have become available for clinical use; however, this therapy is prohibitively expensive, and although TNF inhibitors are clinically as effective as methotrexate, the frequency and extent of response are more restricted. In fact, many patients can lose the clinical response to TNF inhibition, highlighting the need for other treatment modalities to further improve the outcome of RA (3,4).

To address this need, we have been investigating the mechanism of outgrowth in rheumatoid synovial cells (RSCs). First, we demonstrated the crucial role of Fas antigen-induced apoptosis in synovial cell hyperplasia (5). Then, while studying cellular functions of RSCs, we cloned synoviolin from these cells (6). Synoviolin, a mammalian homolog of Hrd1p/Der3p (7-9), is an endoplasmic reticulum (ER)-resident E3 ubiquitin ligase with a RING motif that is involved in ER-associated degradation (ERAD) pathway. Synoviolin is also highly expressed in synoviocytes of patients with RA (6,10-12). Overexpression of synoviolin in transgenic mice leads to advanced arthropathy caused by reduced apoptosis of synoviocytes (6). We postulated that hyperactivation of the ERAD pathway by overexpression of synoviolin prevents ER-stress-induced apoptosis, leading to synovial hyperplasia (13). Synoviolin^{+/-} knockout mice showed resistance to the development of collagen-induced arthritis (CIA) due to enhanced apoptosis of synovial cells (6). Consistent with our hypothesis, cells from these mice show impaired ERAD due to the lack of synoviolin. In addition, synoviolin ubiquitinates and sequesters the tumor suppressor p53 in the cytoplasm, thereby negatively regulating its biological functions in transcription, cell cycle regulation, and apoptosis by targeting it instead for proteasomal degradation (14). Therefore, synoviolin regulates apoptosis in response to ER stress (through ERAD) as well as p53-dependent apoptosis.

Together, these studies implicated synoviolin as a candidate pathogenic factor in arthropathy, and suggested that the gene dosage of this protein correlates with the onset of arthropathy. Furthermore, elevated synoviolin levels were identified in

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circulating monocytes in association with resistance to treatment with infliximab (a monoclonal antibody against TNF) (10). Therefore, blocking the function of synoviolin could be clinically beneficial in RA patients. This study attempted to identify an inhibitor of synoviolin that acts by blocking its enzymatic activity.

Materials and methods

Screening of synoviolin inhibitor. Purified glutathione S-transferase (GST)-synoviolin Δ transmembrane domain (TM) was mixed with glutathione-SPA beads (Amersham Pharmacia Biotech) in buffer (50 mM Tris-HCl, pH 7.4, Protease inhibitor cocktail, 14 mM β-mercaptoethanol, 0.5 μl cell lysate/well, 0.2 mg SPA bead/well) and incubated for 30 min at room temperature. Glutathione-SPA beads were washed twice, and then mixed with the candidate synoviolin inhibitor compounds in buffer (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 2 mM NaF, and 10 nM okadaic acid) in the presence of ATP (2 mM), ^{33}P-labeled ubiquitin (0.38 μg/well), E1 (25 ng/well) (Affiniti Research), and E2 (0.3 μg/well) (UbcH5c). After incubation for 90 min at room temperature, buffer comprising 0.2 M boric acid, pH 8.5, 2 mM ethylenediaminetetraacetic acid (EDTA), and 2% Triton-X100 was added to stop the reaction. The beads were allowed to settle and the amount of ^{33}P-ubiquitin incorporated into the GST-synoviolin beads was determined using a Microbeta Scintillation counter.

The primary screen was conducted with multiple compounds per well (10-20 compounds per well) at an estimated screening concentration of 2-10 μM. Compound mixtures showing potential activity in the primary screen were then rescreened at one compound per well to determine the active compound within the mixture. Three equivalents of a single compound per well follow-up screening were evaluated. Reconfirmed active compounds were resynthesized and tested in a dose-response experiment to determine potency.

In vitro ubiquitination assay. The *in vitro* ubiquitination assay used in this study was described previously (15). Briefly, 40 ng of E1 (Affiniti Research), 0.3 μg of E2 (UbcH5c), 0.75 μg of ^{32}P-labeled ubiquitin (a gift from T. Ohta), and 1 μg of recombinant E3 ubiquitin ligases were incubated for 30 min at 37°C. Samples were analyzed as described above.

Cells. HeLa cells were obtained from ATCC. Synovial cells were isolated from synovial tissue obtained patients with rheumatoid arthritis (RA) who met the American College of Rheumatology criteria for RA at the time of orthopedic surgery. These cells were cultured in Dulbecco's modified Eagle's medium (Sigma).

Proliferation assay. The proliferation of rheumatoid synovial cells (RSCs) was evaluated using Alamar blue (BioSource International) according to the manufacturer's instructions.

Induction of CIA. CIA was induced as described previously (6). Briefly, bovine type II collagen (Collagen Research Center) was dissolved overnight in 0.05 M acetic acid at 4°C, and then emulsified in complete Freund's adjuvant (Difco) to a final concentration 1 mg/ml. DBA/1 male mice (7-week-old) were

immunized by subcutaneous injections containing 100 μg of collagen emulsion. After 3 weeks, mice were boosted with 200 μg collagen emulsion in Freund's complete adjuvant. Then, the mice were treated daily for 4 weeks with the inhibitor compounds at 1.3, 4.0, and 12.0 mg/kg/day in olive oil, vehicle control intraperitoneally, or oral administration of 0.25 mg/kg/day dexamethasone in methylcellulose as a positive control.

The mice were monitored daily for signs of arthritis using an established scoring system (16): 0, no swelling or redness; 1, swelling, redness of paw or 1 joint; 2, two joints involved; 3, more than two joints involved; 4, severe arthritis of entire paws and joints. All paws were evaluated in each animal and the maximum score per animal was 16.

Histological studies. The knee and elbow joints were fixed in 4% paraformaldehyde. After decalcification with EDTA, the joints were embedded in paraffin, and 4-μm sections were prepared for staining with hematoxylin and eosin. The extent of arthritis in the joints was assessed according to the method reported by Tomita *et al* (17): 0, normal synovium; 1, synovial membrane hypertrophy and cell infiltration; 2, pannus and cartilage erosion; 3, major erosion of cartilage and subchondral bone; 4, loss of joint integrity and ankylosis.

Statistical analysis. All data are expressed as mean ± SEM. Differences between groups were examined for statistical significance using Student's t-test. A P-value <0.05 denoted the presence of a statistically significant difference.

Ethical considerations. The ethics committee for Animal Experiments of St. Marianna University School of Medicine approved the mice experiments described in this study. Furthermore, all the experimental protocols described in this study were approved by the Ethics Review Committee of St. Marianna University School of Medicine (Approval number 01008), and the written informed consent was obtained from all patients.

Results

High-throughput compound screening for inhibitors of synoviolin. To identify small molecule inhibitors of synoviolin autoubiquitination, we screened the Lead Discovery Service program of Pharmacopeia, which includes more than four million compounds from Pharmacopeia's Compound Collection (18). Herein we monitored ^{33}P-autoubiquitinated synoviolin in cell lysates containing GST-synoviolinΔTM in the presence of ATP, E1, E2, and ^{33}P-labeled ubiquitin (Fig. 1A). The primary screen was conducted with multiple compounds per well (10-20 compounds per well) at an estimated screening concentration of 2-10 μM. Mixtures of compounds showing potential activity in the primary screen were then rescreened individually. Compounds demonstrating activity in this reconfirmation assay were resynthesized and retested. Two unique compounds, termed LS-101 and LS-102, inhibited the autoubiquitination of synoviolin with a 50% inhibitory concentration value (IC$_{50}$) of ~15 μM (Fig. 1B) and 20 μM (Fig. 1C), respectively.

LS-101 and LS-102 inhibit the autoubiquitination of synoviolin. Further evaluation of LS-101 and LS-102 in an *in vitro* ubiqui-
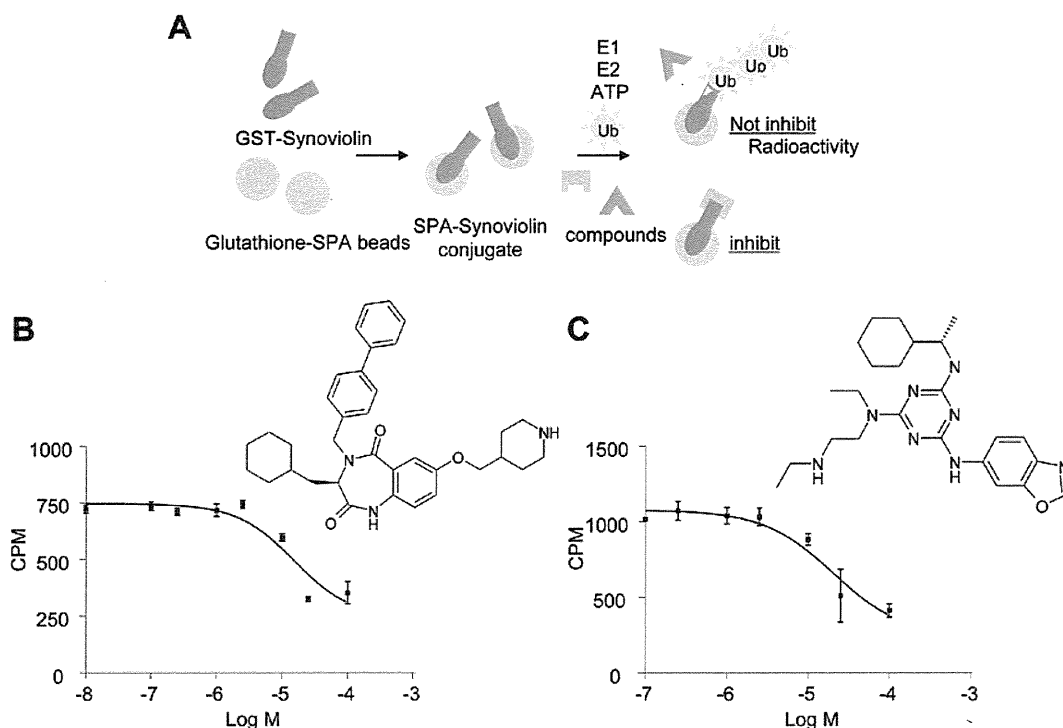
Figure 1. Screening for synoviolin inhibitors. (A) Scheme of high-throughput screening of synoviolin-induced ubiquitination assay. (B) Inhibition of synoviolin ^{33}P-polyubiquitination by LS-101 and chemical structure of LS-101. (C) Inhibition of synoviolin ^{33}P-polyubiquitination by LS-102 and chemical structure of LS-102.
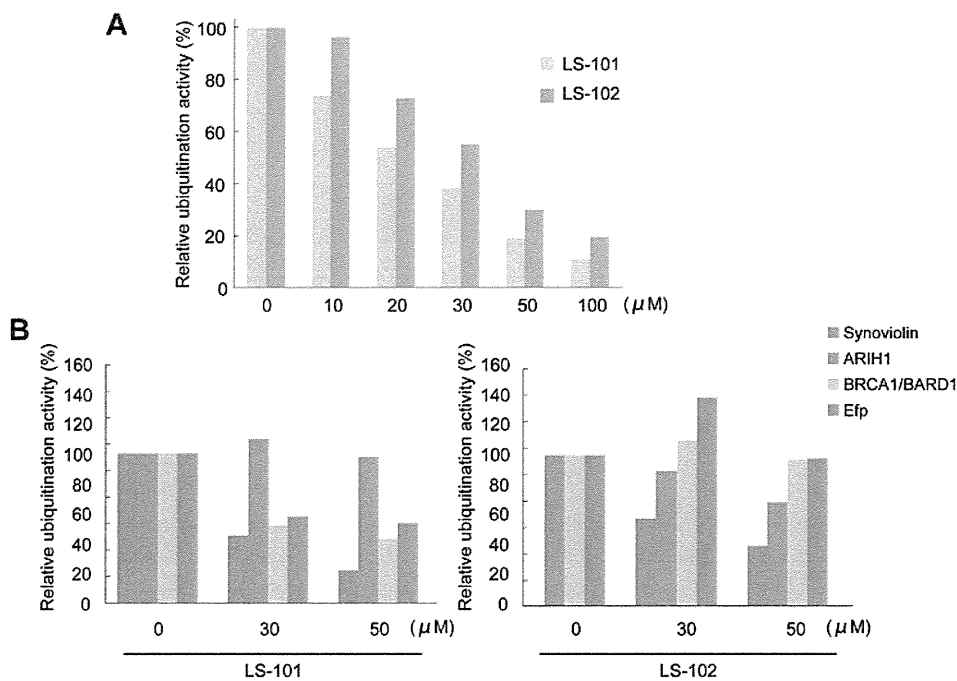

Figure 2. Effects of LS-101 and LS-102 on *in vitro* ubiquitination. (A) Both LS-101 and LS-102 inhibited the autoubiquitination of synoviolin in a dose-dependent manner. The IC_{50} of LS-101 was 20 μM and that of LS-102 was 35 μM. (B) Selectivity of LS-101 (left) and LS-102 (right) against other E3 ubiquitin ligases. LS-102 inhibited synoviolin selectively compared with LS-101. Data are mean ± SEM of 3 experiments.

tination assay showed that the inhibition of synoviolin activity by both LS-101 and LS-102 was dose-dependent (LS-101; IC_{50}=20 μM, LS-102; IC_{50}=35 μM) (Fig. 2A). To assess the selectivity of the compounds for other E3 ubiquitin ligases, we determined the effects of LS-101 and LS-102 on the enzymatic

activity of the following RING-finger type E3 ubiquitin ligases: ariadne, *Drosophila*, homolog of, 1 (ARIH1) (19), breast cancer 1 gene (BRCA1)/BRCA1-associated RING domain 1 (BARD1) (20), and estrogen-responsive RING-finger protein (Efp) (21). LS-101 inhibited the activity of BRCA1/BARD1 and Efp
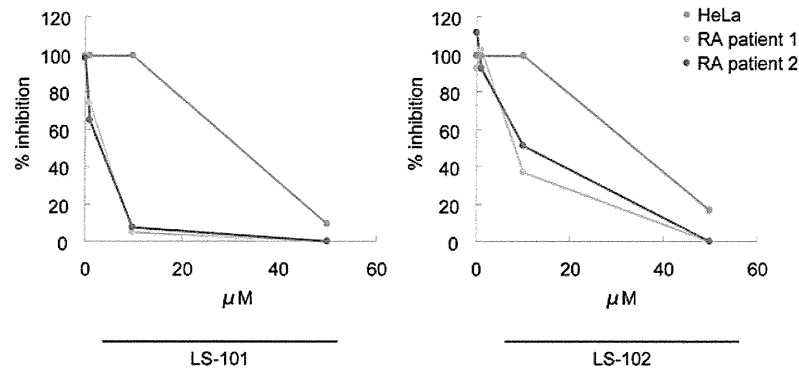
Figure 3. Effects of LS-101 and LS-102 on cell growth of RSCs. HeLa cells and RSCs derived from two RA patients were treated with synoviolin inhibitors for 12 h at the indicated concentrations. LS-101 and LS-102 repressed the proliferation of each RSC population tested. Data are expressed as the mean percentage of inhibition of the vehicle-treated control group ± SEM; (n=3).
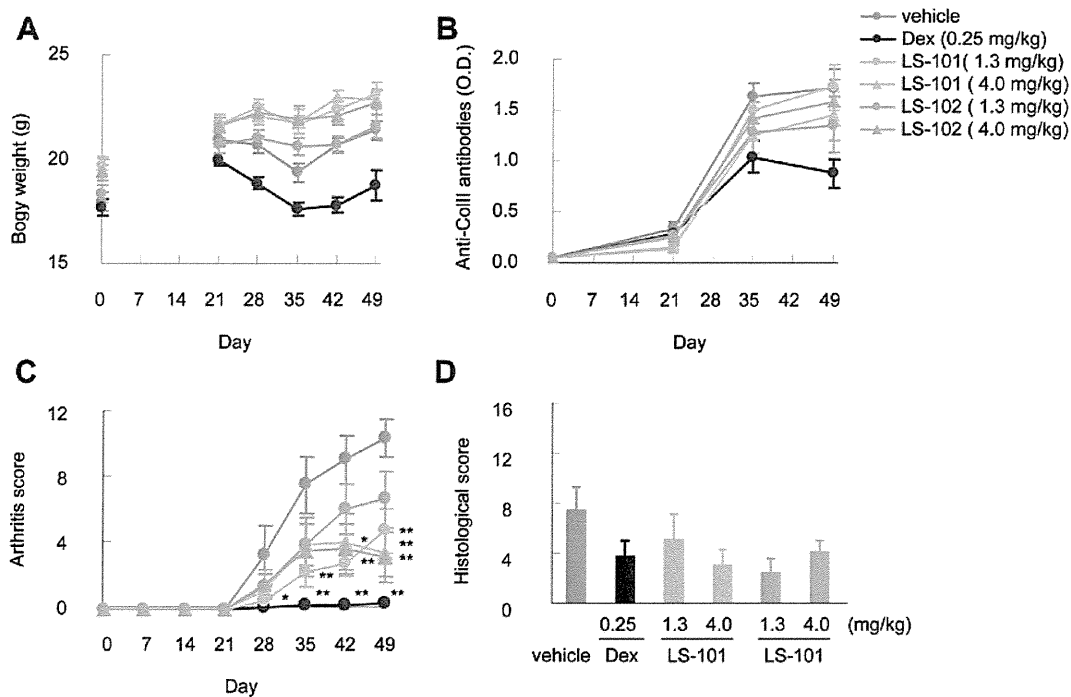

Figure 4. Effects of LS-101 and LS-102 in mouse CIA. DBA/1 mice immunized on day 0 and boosted on day 21 with type II collagen were treated with the vehicle alone, 0.25 mg/kg dexamethasone (Dex), or with 1.3, 4.0 mg/kg LS-101 or LS-102 from day 21 to 49. (A) Change in body weight. (B) The level of anti-type II collagen antibodies. (C) Total arthritis score. (D) Histological arthritis score. Data are mean ± SEM (initial n=12; final n=7). $^*P<0.05$, $^{**}P<0.01$.

(Fig. 2B), although this effect was weaker than that observed with synoviolin (Fig. 2B). Moreover, LS-101 had no effect against the enzymatic activity of ARIH1 (Fig. 2B). On the other hand, LS-102 did not inhibit the activity of other E3 ubiquitin ligases, only affecting synoviolin (Fig. 2B). These results suggested that LS-102 is a more selective synoviolin inhibitor than LS-101.

LS-101 and LS-102 inhibit proliferation of RSCs. We next tested LS-101 and LS-102 for their effects on the proliferation of RSCs, using HeLa cells as a control. LS-101 and LS-102 inhibited HeLa cell growth only at very high concentrations (LS-101; IC_{50}=31.3 μM, LS-102; IC_{50}=32.7 μM). However, treatment of RSCs with these compounds suppressed synovial cell growth dose-dependently and with much greater potency than that observed in HeLa cells (Fig. 3). A similar effect was also observed in another line of RSCs (Fig. 3). In addition, LS-101

inhibited synovial cell proliferation more potently than LS-102 (LS-101; IC_{50}=4.2 μM, LS-102; IC_{50}=5.4 μM). These results demonstrated that blockade of synoviolin function reduced the proliferation of RSCs, and that RSCs are more susceptible to this effect than HeLa cells. Consistent with these findings, higher expression levels of synoviolin were observed in RSCs than in HeLa cells (6).

LS-101 and LS-102 reduce clinical severity scores in a CIA model. To evaluate the *in vivo* efficacy of synoviolin inhibitors, we tested LS-101 and LS-102 in a mouse model of arthritis over a period of 28 days. No reduction of body weight was observed during the administration of these compounds (Fig. 4A). Moreover, the production of anti-type II collagen antibodies resulting from type II collagen immunization in both the LS-101 and LS-102 group was comparable to that

observed in the vehicle control group (Fig. 4B). Intraperitoneal treatment with LS-101 or LS-102 starting on day 21 reduced the clinical severity scores compared to vehicle controls (Fig. 4C). The efficacy was observed at both 1.3 mg/kg and 4.0 mg/kg doses in this experiment, although the protective effect of LS-101 at 1.3 mg/kg against CIA was stronger than the same dose of LS-102. At 4.0 mg/kg, there was no difference in the effects between LS-101 and LS-102. Finally, histological analysis showed lower histological arthritis scores in mice treated with the synoviolin inhibitors compared with wild-type mice (Fig. 4D).

Discussion

The selective degradation of proteins in eukaryotic cells is carried out by the ubiquitin proteasome system (UPS), whereby proteins are targeted for degradation by covalent ligation to small polypeptide ubiquitin (22,23). This reaction requires the sequential actions of three enzymes: E1, E2, and E3 ligases (22,23). E3 ligases are responsible for conferring selectivity to ubiquitination by recognizing specific substrates. Bioinformatic analysis has identified over 600 E3 ligases, with RING-type E3 ligases constituting the largest subfamily within this group (24). Accordingly, RING E3 ligases have been linked to the control of multiple cellular processes and to many human diseases such as diabetes mellitus, polygluta-mine disease, and Parkinson's diseases (24-26). In the UPS, the proteasome inhibitory agent bortezomib (Velcade) was recently approved for the treatment of multiple myeloma and mantle cell lymphoma (27). Bortezomib induces apoptosis of a wide variety of cancer cells, and is the first proteasome inhibitor to gain FDA approval (28-30). However, widespread clinical use of bortezomib continues to be hampered by the appearance of dose-limiting toxicities, drug-resistance, and interference by some natural compounds (31). Thus, despite the efficacy of bortezomib for treating lethal diseases such as cancer, the associated toxicities prevent its use for the treatment of chronic diseases such as RA. Thus, it is important to develop inhibi-tors of the ubiquitin-proteasome enzymatic cascade upstream from the proteasome to impact fewer cell processes and reduce toxicity. E3 ligases are attractive such targets given their large number and substrate specificity. We recently cloned the E3 ubiquitin ligase synoviolin, which localizes to the ER lumen and has enzymatic activity. We have also demonstrated that this protein plays crucial roles in the pathological processes of RA (6), and could therefore be a candidate novel therapeutic target of RA (32).

In this study, we identified two potent small compounds as inhibitors of synoviolin enzymatic activity using high-throughput screening (Fig. 1). Moreover, *in vivo* studies showed no serious toxicity associated with these compounds in terms of survival and weight loss during treatment (Fig. 4A). Biochemical characterization of the two compounds, LS-101 and LS-102, demonstrated that they both inhibit the autoubiquitination activity of synoviolin *in vitro* (Fig. 2), with LS-101 showing stronger efficacy (IC_{50}=20 μM) than LS-102 (IC_{50}=35 μM), but less selectivity (Fig. 2). It was unclear from this study why LS-101 showed a weak inhibitory effect on BRCA1/BARD1 and Efp activity, and further study is needed to understand the molecular basis for this observation. LS-101 and LS102 inhibited

the proliferation of RSCs and to a much lesser extent, HeLa cells (Fig. 3). The difference in cell sensitivities to these compounds could be, at least in part, due to the expression level of synoviolin, namely, high levels of synoviolin in RSCs would contribute to the cell overgrowth and therefore, inhibition of synoviolin in these cells would in turn suppress proliferation. These cells may also have different requirements for synoviolin, such that repressing synoviolin activity in RSCs would lead to growth suppression. Prophylactic administration of either LS-101 or LS-102 also significantly reduced the severity of murine CIA (Fig. 4C). Since LS-101, a nonselective inhibitor, reduced clinical severity scores in CIA similarly to LS-102, blocking synoviolin enzymatic activity seems crucial in the pathological process of CIA. These findings suggest that the suppression level of synovial cell growth and incidence of arthritis reflect the efficacy of these compounds rather than their selectivity, and that in RA, synoviolin might have an indispensable role among E3 ligases.

RA comprises multiple processes such as chronic inflamma-tion, overgrowth of synovial cells, joint destruction, and fibrosis. During the course of inflammation, synovial cells, macrophages, T cells, and B cells all contribute to the production of cytokines such as interleukin (IL)-1, IL-6, IL-10, TNF, and transforming growth factor β (TGF-β) (33,34). These cytokines, in turn, stimulate the overgrowth of synovial cells to form a mass of synovial tissue, called pannus, which invades and destroys the bone and cartilage through osteoclast activation and protease production (33-37). This chronic inflammation state ultimately leads to fibrosis. Our study proved that synoviolin is, at least in part, involved in the overgrowth of synovial cells (6) and fibrosis (38) among these processes. The IL-17 induction of synoviolin may also contribute to RA chronicity (39), and synoviolin has been shown to target misfolded MHC class I heavy chains (40). In this study, antibody titers were elevated in synoviolin inhibitor-treated mice to levels comparable to those in vehicle controls (Fig. 4B). Thus, as with the study of synoviolin$^{+/-}$ knockout mice in CIA, it is difficult to clarify the function of synoviolin with respect to the chronicity of inflam-mation, because suppressing synoviolin blocks synovial cell outgrowth directly due to sequential events following immuni-zation of type II collagen (6). Our results confirm that further studies of the association between chronic inflammation and synoviolin are clearly warranted.

Eight biological agents are currently approved for clinical use in treatment of RA, and these drugs have dramatically changed the outcome of RA during the past decade (3,4). However, some patients still fail to respond to the biological treatment or develop adverse effects such as an increased risk of infection. Moreover, these agents are associated with high costs and discomfort arising from the subcutaneous or intravenous administration. Thus, there is a clear need for the development of cheaper, orally administered therapies with fewer side effects. In this regard, spleen tyrosine kinase (Syk) inhibitor, an orally administered drug, has been developed for the treatment of RA (41,42). Dual blockade of TNF and IL-17 was also reported recently as a strategy for halting RA disease from progression to the extent seen when only one cytokine is blocked (43). The involvement of synoviolin in both the TNF and IL-17 pathways further implicates inhibitors of this enzyme as potential candidate drugs for treatment of RA.

In conclusion, we identified two strong synoviolin inhibitors, and confirmed that synoviolin is an ideal molecular target for RA for disease modification and treatment. We are now proceeding with the optimization of LS-101 and LS-102, and hope our research will lead to the development of a new therapy for RA.

Acknowledgements

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ORIGINAL ARTICLE

Serum level of soluble triggering receptor expressed on myeloid cells-1 as a biomarker of disease activity in relapsing polychondritis

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Abstract

Objectives We aimed to identify a serum biomarker for evaluating the disease activity of relapsing polychondritis (RP).

Methods We measured and compared serum levels of 28 biomarkers potentially associated with this disease, including soluble triggering receptor expressed on myeloid cells-1 (sTREM-1), high-sensitivity C-reactive protein (hs-CRP), and cartilage oligomeric matrix protein (COMP), in 15 RP patients and 16 healthy donors (HDs). We divided the 15 RP patients into active RP ($n = 8$) and inactive RP ($n = 7$) groups, depending on the extent of the disease, and compared candidate markers between groups. The localization of membrane-bound TREM-1 in the affected tissue was examined by immunohistochemistry.

Results Serum levels of sTREM-1, interferon-γ, chemokine (C–C motif) ligand 4, vascular endothelial growth factor, and matrix metalloproteinases-3 were significantly higher in RP patients than HDs. Among these markers, sTREM-1 had the highest sensitivity and specificity (86.7 and 86.7 %, respectively). Furthermore, the serum level of sTREM-1 was significantly higher in active RP patients than inactive RP patients ($p = 0.0403$), but this was not true for hs-CRP or COMP. TREM-1 was expressed on endothelial cells in RP lesions.

Conclusions The serum level of sTREM-1 may be a useful marker of disease activity in RP.

Keywords Relapsing polychondritis · Serum marker · Soluble triggering receptor expressed on myeloid cells-1

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Introduction

Relapsing polychondritis (RP) is a rare inflammatory disorder of unknown etiology; it is characterized by recurrent, widespread chondritis of systemic cartilages, specifically those in the ear, eye, nose, large airways, and joints [1–3]. RP is occasionally life-threatening, as its progression leads to fatal dyspnea due to cartilage destruction in large airways. To detect such disease progression, the accurate assessment of disease activity is important. Today, this assessment is performed by analyzing a combination of clinical manifestations, laboratory findings, and imaging results.

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However, it is still difficult to conduct proper evaluations. This is partly because there are no established biomarkers for evaluating the disease activity of RP, although several potential biomarkers—such as CRP, antibody to type II collagen, and cartilage oligomeric matrix protein (COMP)—have been reported previously [3–7]. For example, CRP is the most commonly used marker of inflammation, and its serum level is frequently used to assess RP disease activity [3, 4]. However, RP patients with normal CRP levels are often observed to experience advanced fibrosis of the airways, suggesting insidious chronic inflammation in those tissues, which is difficult to detect by CRP [8]. It has also been reported that antibodies to type II collagen reflect RP disease activity [6]. However, these antibodies were only detected in 30–50 % of RP patients [6, 9]. Furthermore, it has been reported that this measure lacks sensitivity and specificity [10]. Therefore, in the current study, we aimed to identify more sensitive biomarkers that would be able to detect those small differences that cannot be detected by antibodies to type II collagen or CRP.

To do so, this study excluded highly active RP patients. We measured 28 candidate markers that had been previously shown to be involved in RP, inflammation, or cartilage destruction. The levels of these markers were compared not only between RP patients and healthy donors (HDs) but also between active RP and inactive RP patients. Our results showed that the serum level of soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) is most suitable as a disease-activity marker in RP.

TREM-1 is a type I transmembrane receptor of the immunoglobulin superfamily. The soluble form of TREM-1 (sTREM-1) is thought to be released from TREM-1-expressing cells by proteolytic cleavage of membrane-bound TREM-1 [11]. The serum level of sTREM-1 has been found to be elevated in patients with sepsis and has therefore been considered as a marker of microbial infection [12].

Materials and methods

Patients and samples

Fifteen patients (8 women and 7 men) diagnosed with RP according to Damiani's criteria [13, 14] and 16 healthy donors (HD) serving as age-matched and sex-matched controls (Table 1) were recruited from St. Marianna University Hospital, Kanagawa, Japan. They were enrolled between November and December 2009. In this study, we used the patient information (disease condition, disease duration, medication, etc.) obtained at the time of enrollment (Table 1). None of the patients had any other inflammatory disorders, such as overt infections or collagen diseases. To detect small differences that cannot be detected by CRP, this study enrolled RP patients in the chronic phase—not the acute phase—and further excluded patients who had highly active RP, such as those with acute respiratory failure. From among them, we divided the 15 RP patients into two groups (active RP and inactive RP) according to the definition by Lekpa et al. [7]. Briefly,

Table 1 Demographics, clinical characteristics, and medication of subjects

	HD	RP		
	(n = 16)	Total (n = 15)	Active (n = 8)	Inactive (n = 7)
Demographics				
Age (years)[a]	40.5 [27–67]	47 [10–81]	50.5 [10–74]	44 [27–81]
Female sex	50.0 %	53.3 %	50.5 %	57.1 %
Clinical characteristics				
Disease duration (years)[a]		5 [1–19]	12 [4–19]	4 [1–8]
Auricular chondritis		46.7 %	62.5 %	28.6 %
Nasal chondritis		40.0 %	62.5 %	14.3 %
Laryngotracheal chondritis		66.7 %	87.5 %	42.9 %
Ear symptoms		53.3 %	87.5 %	14.3 %
Arthritis		46.7 %	75.0 %	14.3 %
Ocular inflammation		33.3 %	50.0 %	14.3 %
Medication				
Prednisolone		86.7 %	87.5 %	85.7 %
Methotrexate		33.3 %	50.0 %	28.6 %
Azathioprine		20.0 %	25.0 %	14.3 %

HD healthy donor, *RP* relapsing polychondritis

[a] Data are expressed as median [range]

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