表 1　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) Mean ± SD	Inactive RP (n =7) Mean ± SD	*P**
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　RP患者（n=41）の男女比

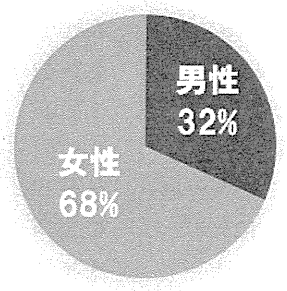


図２　RP患者の発症年齢構成

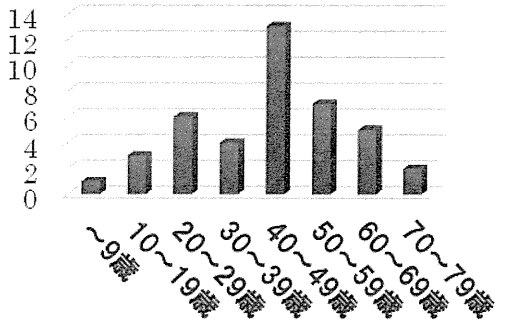
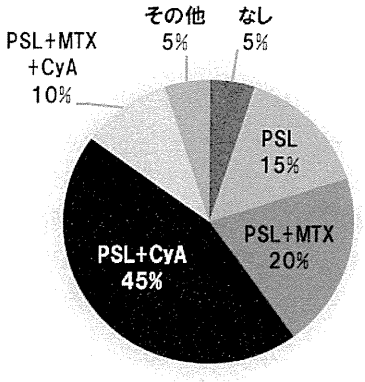
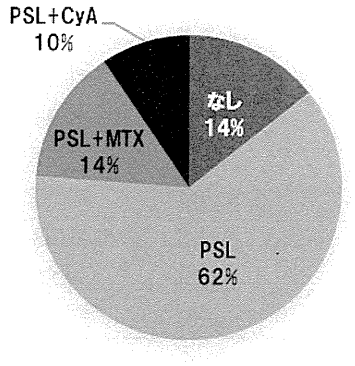


図３　気道病変の有無と現在の治療内容との関連

気道病変のある症例（N=20）



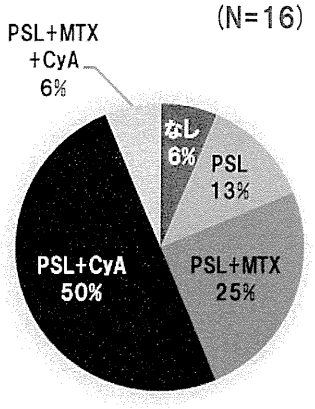
気道病変のない症例（N=21）



PSL=プレドニゾロン、MTX＝メトトレキサート、CyA=シクロスポリン

図４　初発症状としての気道症状の有無と現在の治療内容との関連

気道症状を初発とした症例
（N=16）



気道症状以外を初発とした症例
（N=25）

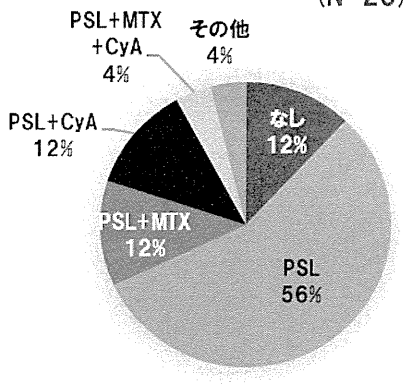
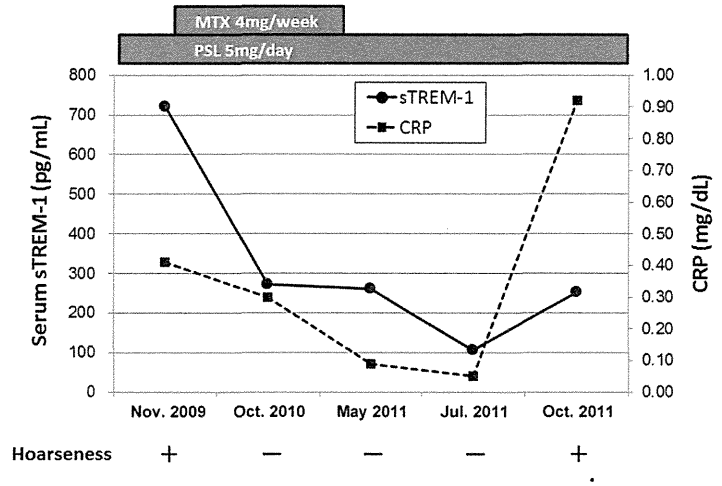


図５ 活動性 RP 患者における臨床経過とマーカーの経時的変化の一例



厚生労働科学研究費補助金　（難治性疾患等克服研究事業（難治性疾患克服研究事業））
分担研究報告書

再発性多発軟骨炎の診断と治療体系の確立
－再発性多発軟骨炎における Th1/Th17 細胞機能の検討－

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研究要旨：再発性多発軟骨炎（relapsing polychondritis、以下 RP）は、全身の軟骨に炎症を来たしうる原因不明の難治性疾患である。本邦における患者数は 500 人程度と推察され、疫学・病態研究が端緒についたばかりであり、診断・治療指針は未確立である。

研究代表者らは厚生労働科学研究費補助金難治性疾患克服研究事業「患者支援団体等が主体的に難病研究支援を実施するための体制構築に向けた研究（JPA研究班）」の分担研究を担当し、同班研究がいわゆる「患者主体」レジストリを立ち上げ、それが症状主体の前向き研究を可能にすることを確認した。

そのデータを用いた新規治療方法の開発に向けた、新規パラメーターの検討の一環として昨年度は、血清sTREM-1レベルは活動性RPおよび非活動性RPを区別することが可能であり、RPの疾患活動性マーカーとして優れていることを発見した。本年度はこの自然免疫機能と対をなす、獲得免疫機能を検討した。

方法は、獲得免疫の中心的役割を果たしているT細胞の機能発揮に重要とされるサイトカインの血清濃度を測定した。T細胞機能はTh1、Th17等に細分化され、それぞれに機能発揮に必要なサイトカインが異なり、ヒトの疾患に大きくかかわるとされるためである。その結果はTh1細胞機能亢進が明確になり、そこにTh17細胞が関与している可能性が示唆された。

この獲得免疫の異常は、我々が平成21～23年度厚生労働科学研究費補助金難治性疾患克服研究事業[課題名：再発性多発軟骨炎の診断と治療体系の確立]の中で明らかにした、本疾患におけるメトトレキサートの有効性と密接な関連があるものと考えられる。

今後はこの新規疾患パラメーター検討を、「患者主体」レジストリの綿密な情報とあわせて前向き研究をすることで、本疾患の病態解明および適切な治療方法の確立を目指す。

A. 研究目的

i)研究の背景

再発性多発軟骨炎の疫学調査

再発性多発軟骨炎（relapsing polychondritis、以下 RP）は、原因不明で稀な難治性疾患である。本邦における疫学情報や病態研究は不十分であり、かつ診断・治療のための指針が作成されていない。その為、認知度が低く診断が見過ごされているケースも多く、気道軟骨病変などの臓器病変を伴う患者の予後は極めて不良であり、診断、治療法の確立が急務である。

我々は平成 21 年度厚生労働科学研究費補助金難治性疾患克服研究事業[課題名：再発性多発軟骨炎の診断と治療体系の確立]において、RP に対する患者実態・疫学調査(RP 239 症例)を行ない、本邦の患者実態として、本邦全体の患者数がおおよそ 500 人程度と推察されること、発症年齢は 3 歳より 97 歳まで多年齢層にわたり、平均は 52.7 歳であること、男性と女性の割合がほぼ同じであること、重症例となりやすい気道病変を持つ患者の割合が 50%程度になることを明らかにした。治療においては、気道病変はステロイド単独治療ではその病勢を抑えられないため、免疫抑制剤（メソトレキ

セート）が必要となることを発見した（文献 1）。

そこで現在免疫抑制剤を用いた臨床試験を計画しており、そのため新たな患者登録・追跡システムが必要となった。その際、適切でかつ正確な臨床検査データの収集と、疾患機序解明のための新規疾患パラメーターの開発が不可欠である。

ii) 本年度研究の目的＝新規疾患パラメーターの開発

これまで RP 患者の疾患活動性は CRP や抗 type II コラーゲン抗体によって評価されており、急性期には多くの RP 患者で高値を認める。しかしながら、CRP が正常範囲内にある症例でも軟骨の破壊・線維化が進む例も多く、CRP では疾患活動性の評価が困難な面がある。また抗 type II コラーゲン抗体も疾患活動性との相関が報告されているが、陽性者は RP 患者の 30～50%にすぎず、感度・特異度もあまり高くないという報告もある。そこで、昨年度までに RP を検出する感度のよいマーカーを同定する目的で 28 種類のマーカー候補分子の中から、健常者と比較して RP 患者血清で有意に高値を示す分子を探索し、可溶性 TREM-1(sTREM-1)、インターフェロンγ、CCL4/MIP-1β、VEGF および MMP-3 を同定した。その中でも血清 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り、RP の疾患活動性マーカーとして優れていることを発見した。

ヒトの免疫機能は、自然免疫と獲得免疫にてなりたっており、(1)(2)の自然免疫に加えて獲得免疫の異常を研究することは治療効果を上昇させるために重要なことと考える。近年、様々なヒト免疫疾患において Th1 細胞および Th17 細胞という獲得免疫の主要な細胞に異常がみられることが報告されている（後述）。本年度はこの細胞群の維持に必須であるサイトカインの血中濃度を測定する。

iii) 期待される研究成果

①患者登録・追跡における IT 技術の積極利用による、高効率化。

②情報収集の多元化による、患者訴えの綿密な収集。

③JPA 研究班を通じて、国レジストリ作成への意見反映の試み。

④RP の病態・病勢を的確に反映する、簡便な検査法の確立。

⑤RP に有効性が高いと考えられるメソトレキセート治療の前向き研究における評価方法の確立。

B. 平成 25 年度研究結果

i) Th1 細胞、Th17 細胞

獲得免疫の要である T 細胞は、生体の局所にあわせた機能の発揮のため、網内系においていくつかのサブセットに分化する。Th1 細胞と Th17 細胞は、代表的な炎症惹起性のサブセットのひとつであり、前者は細胞内寄生菌、後者は細胞外寄生菌および真菌の排除にあたるとされている。また、その分化には Th1 細胞は IL-12、Th17 細胞は TGFβおよび IL-6 というサイトカインが必要であり、さらに Th17 細胞はその増殖・維持に IL-23 を要する。

ii) ヒト免疫疾患と Th1 細胞、Th17 細胞

双方のサブセットともに関節リウマチに代表されるヒト免疫疾患の原因の一つであることが証明されており、抗体療法が臨床的に使用され始めている。

我々は厚生労働科学研究難治性疾患等克服研究事業（難治性疾患克服研究事業）ベーチェット病に関する調査研究の分担研究において、ベーチェット病にて①末梢血における Th17 細胞の増加および Th1 細胞の減少、②末梢血ナイーブ CD4+T 細胞が、IL-12 の存在下にて Th1 細胞様、IL-23 の存在下にて Th17 細胞様の過反応を示すこと等を発見し、双方の

細胞ともに病態に密接に関連している可能性を提示した。(文献 2〜5)

iii) RP 血清中 Th1/Th17 細胞関連サイトカイン濃度の検討

本年度は疾患および健常者血清中のIFNγ、IL-12、IL-17 および IL-23 濃度を観察し、生体内での T 細胞分化誘導環境を検討した。

対象は RP6 例、健常者 8 例、疾患コントロールとしてベーチェット病 4 例。それぞれのキットを用い測定した。

RP において Th1 細胞が産生する IFNγの上昇、Th1 細胞の維持に重要な IL-12 の低下がみられ、Th1 細胞の過剰な活性化とそのネガティブフィードバックが考察された(図 1)。

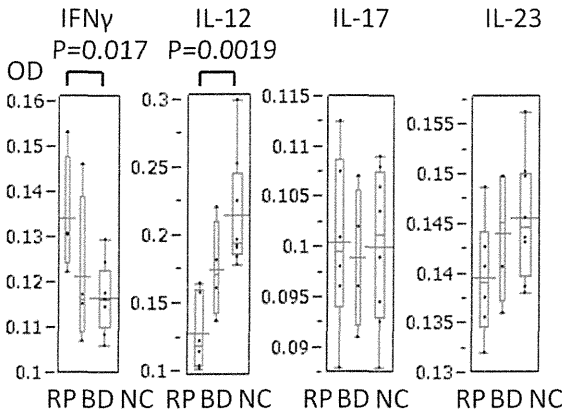

図1 RP、ベーチェット病(BD;疾患コントロール)、健常者(NC)の血中Th1/Th17細胞関連サイトカイン濃度

さらにそのIFNγ濃度はIL-23濃度と正相関(P=0.028)を示した (図2)。IL-23がその活性に重要に関与するTh17細胞もTh1細胞機能を介して、二次的にRPの病態形成に関わることが示唆される。

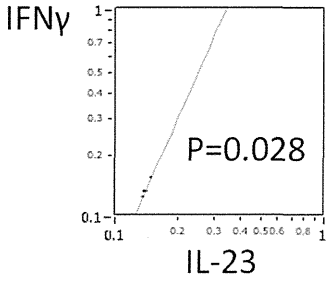

図2 RPにおける血中サイトカイン濃度の相関検討

C. 結語

結果のii)項で記載したように、我々はTh1/Th17細胞機能とその相互関係を鋭敏に反映するリンパ球機能検査を確立しており、今後RPでの詳細な解析を行う。前述のJPA研究班による新患者レジストリ方法と本検査法を有機的に結合させることで、迅速な治療指針の確立を図ることを今後の目的とする。

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Ⅲ．研究成果の発表に関する一覧表

研究成果の刊行に関する一覧表

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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-

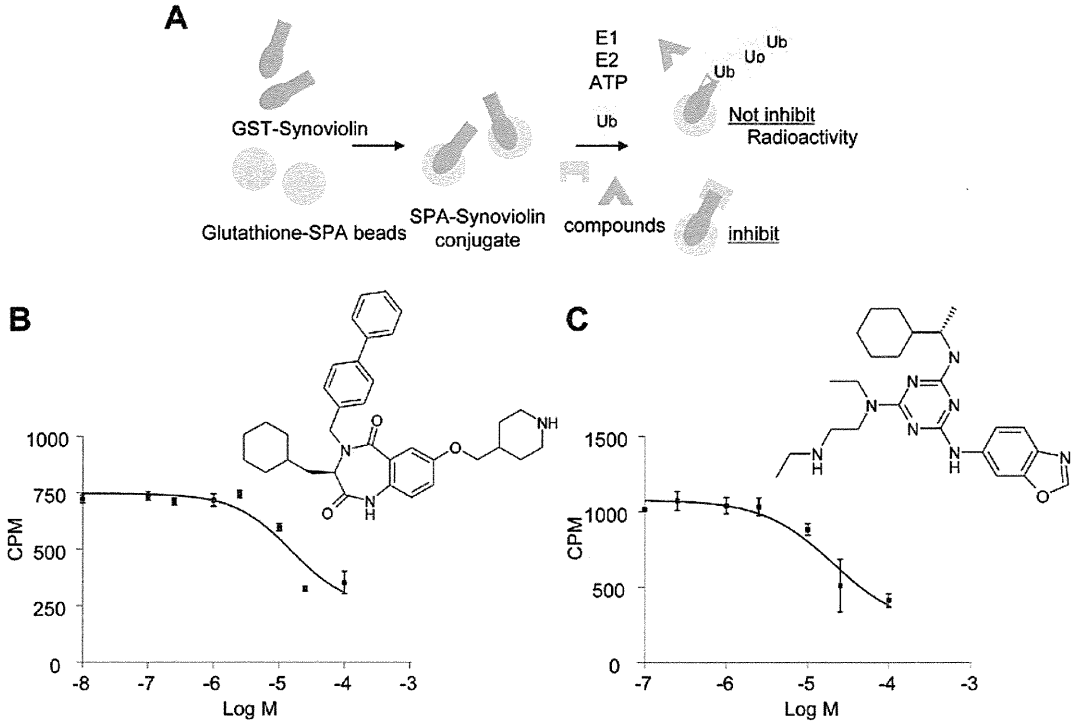

Figure 1. Screening for synoviolin inhibitors. (A) Scheme of high-throughput screening of synoviolin-induced ubiquitination assay. (B) Inhibition of synoviolin ^{33}P-polyubiquitination by LS-101 and chemical structure of LS-101. (C) Inhibition of synoviolin ^{33}P-polyubiquitination by LS-102 and chemical structure of LS-102.

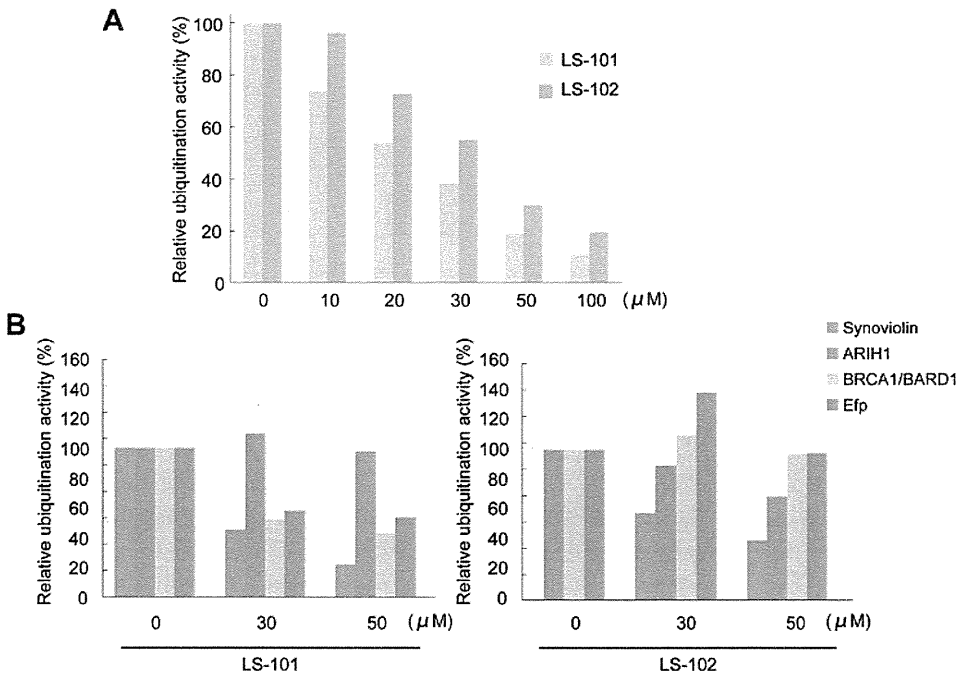

Figure 2. Effects of LS-101 and LS-102 on *in vitro* ubiquitination. (A) Both LS-101 and LS-102 inhibited the autoubiquitination of synoviolin in a dose-dependent manner. The IC_{50} of LS-101 was 20 μM and that of LS-102 was 35 μM. (B) Selectivity of LS-101 (left) and LS-102 (right) against other E3 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

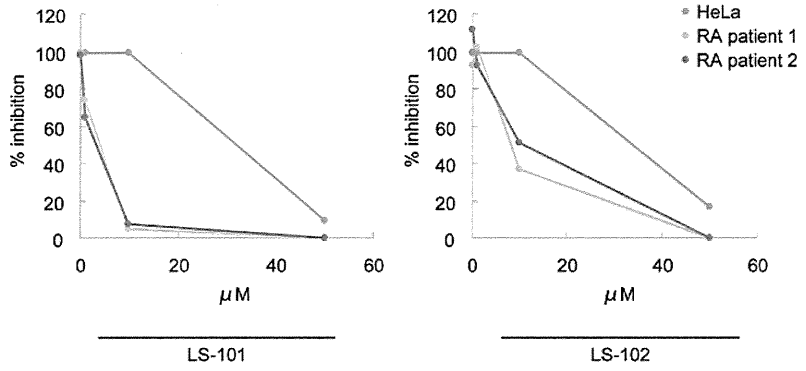

Figure 3. Effects of LS-101 and LS-102 on cell growth of RSCs. HeLa cells and RSCs derived from two RA patients were treated with synoviolin inhibitors for 12 h at the indicated concentrations. LS-101 and LS-102 repressed the proliferation of each RSC population tested. Data are expressed as the mean percentage of inhibition of the vehicle-treated control group ± SEM; (n=3).

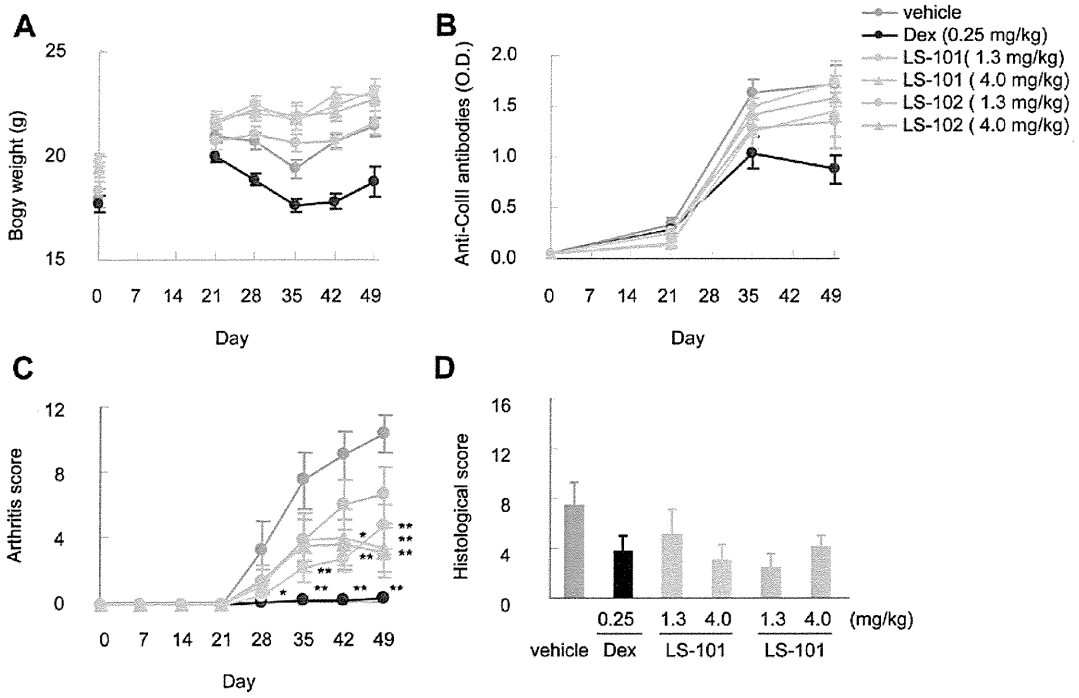

Figure 4. Effects of LS-101 and LS-102 in mouse CIA. DBA/1 mice immunized on day 0 and boosted on day 21 with type II collagen were treated with the vehicle alone, 0.25 mg/kg dexamethasone (Dex), or with 1.3, 4.0 mg/kg LS-101 or LS-102 from day 21 to 49. (A) Change in body weight. (B) The level of anti-type 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, polyglutamine 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 inhibitors 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 inflammation, 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 inflammation, because suppressing synoviolin blocks synovial cell outgrowth directly due to sequential events following immunization 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.

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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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