IgA-class switch recombination [35] and the CD40 ligand and IL-4-induced IgG-class switch recombination, but inhibited the CD40 ligand and IL-4-induced IgE-class switch recombination [31].

Regarding the effects of retinoids on total immunoglobulin production, there are no reports that treatment with ATRA altered serum immunoglobulin level in patients with APL. However, serum IgG2a and IgG2b anti-myosin antibody levels, as well as IgG1, IgG2a, and IgG2b anti-collagen antibody levels, were decreased by Am80 in murine myosin-induced myositis and collagen-induced arthritis, respectively [10, 11].

Dendritic cells

RARα and RXRα are highly expressed in human monocyte-derived DCs, whereas murine splenic DCs express all RAR receptors [36]. ATRA was shown to increase the number of DCs in the spleen and promoted the expression of HLA-DR, CD11c, and CD1c on epidermal DCs [36]. In the presence of inflammation, ATRA also induced DC maturation and upregulated the capacity of antigen presentation through RXR signaling [36], but elicited programmed cell death in DCs in the absence of an inflammatory stimulation [36]. ATRA also suppressed the production of IL-12, but enhanced that of TGF-β and IL-6 from monocytes derived from DCs [36]. These effects may contribute to the regulation of Th differentiation.

On the other hand, ATRA has been shown to increase the expression of matrix metalloproteinases in endothelial cells, which have the potential to boost tumor-specific T cell responses by increasing the migration of tumor-infiltrating DCs to draining lymph nodes [37]. Gut-associated DCs also enhance the differentiation of Treg cells and production of IgA in an ATRA dose-dependent manner in vitro [38, 39]. IgA was decreased in the lamina propria of the small bowel in vitamin A-deficient mice, and the oral administration of an RAR agonist significantly increased serum IgA levels [40]. These findings suggested that gut-associated DCs stimulated with retinoic acid may induce the production of IgA from B cells. Taken together, these findings indicate that retinoic acid has several effects, such as cytokine production, maturation, and B cell stimulation, on DCs.

Monocytes/macrophages

All-*trans* retinoic acid was previously shown to induce the expression of CC chemokine ligand 2 (CCL2) in human monocytes derived from leukemia patients [41]. ATRA also induced the production of IL-10 from monocytes/macrophages, while ATRA suppressed TNF-α and IL-12 from monocytes/macrophages via interactions between RXR and NF-κB [42–44]. ATRA could also attenuate inflammation-induced tissue damage by inducing the production of plasminogen activator inhibitor-2 in peripheral blood mononuclear cells [45]. In addition, ATRA increased the number of T cells, natural killer cells, and macrophages in the lungs and spleen, which attenuated severe infections, such as tuberculosis [46]. RARγ-deficient macrophages exhibited the impaired production of inflammatory cytokines when stimulated with TLR as well as a defective immune response to *Listeria monocytogenes* [47]. Therefore, retinoids play important roles in the activation of monocytes/macrophages with inflammation, including infection.

Neutrophils

Retinoids inhibit the activation of neutrophils by suppressing the production of the superoxide anion and release of protease [48–51]. In addition, we recently reported that Am80 could suppress the production of reactive oxygen species (ROS) and release of elastase from human neutrophils by inhibiting mitogen-activated protein kinase (MAPK) signals in vitro [12]. Am80 could also inhibit the migration speed and chemotaxis directionality of human neutrophils in vitro [12]. Neutrophil extracellular traps (NETs) also play an important role in innate immunity [52]. However, the role of retinoids in the formation of NETs remains unknown.

Effects of retinoids on animal models of rheumatic diseases

Several studies demonstrated the efficacy of retinoids in animal models of autoimmune diseases. Treatments with 13-cis-retinoic acid, ATRA, and Am80 attenuated murine and rat collagen-induced arthritis [10, 40, 53, 54]. Am80 inhibited Th17 and enhanced Treg differentiation and decreased anti-collagen antibodies in vivo [10]. ATRA decreased the infiltration of macrophages into the glomeruli, suppressed the expression of CCL2 in the kidney in vivo, and inhibited proteinuria and renal involvement, such as fibrin deposits, necrosis, and crescents in NZB/WF1 mice, which were used as a lupus nephritis model [55]. A treatment with Am80 also ameliorated murine experimental autoimmune myositis [11]. We recently reported that Am80 significantly attenuated *Candida albicans* water-soluble fraction (CAWS)-induced vasculitis, which is characterized by the infiltration of neutrophils into inflamed vessels. Moreover, Am80 inhibited the migration of transferred neutrophils into the site of vasculitis in vivo [12]. Thus, retinoids could be a promising therapeutic target for rheumatic disease.

Current status of retinoid therapy for rheumatic diseases

Retinoids have regulatory effects on immune cells and have been shown to improve rheumatic diseases in animal models. These findings suggest that retinoids may be a new therapy for rheumatic diseases. To date, four clinical trials have been conducted on retinoid therapy for rheumatic diseases, including RA, lupus nephritis, and systemic sclerosis (Table 1).

In the first trial, RA patients were treated with etretinate, a synthetic retinoid, for 24 weeks. One mg/kg/day etretinate was administered to 15 RA patients for the first 4 weeks, and then, the dosage was reduced to 0.5 mg/kg/day. However, 8 of 15 patients discontinued the treatment by week 12 because of severe liver involvement, and arthritis only improved in three patients [7].

The efficacy of 4-HPR (300 mg/day), a synthetic retinoid, was then evaluated in 12 severe and long-standing RA patients for 24 weeks [8]. Six of the 12 patients withdrew before the completion of the study because 2 exhibited toxic effects (visual problems), 2 flare, and 2 gastrointestinal bleeding. Histological changes and metalloproteinase gene expression were evaluated in synovial tissues pre- and post-medication using biopsy samples, and no patient met the predetermined Paulus criteria treatment response. In addition, no improvements were observed in the laboratory parameters, except for a modest decrease in C-reactive protein and no decrease in the mRNAs of metalloproteinases or collagenase in the synovial tissue.

Retinoids, such as etretinate and 4-HRP, were not effective in the treatment of RA patients in these studies. However, Am80, a ligand for RARα and β, but not for RARγ (Table 2 [56]), was used to effectively treat murine CIA [10]. Therefore, the different structures and binding abilities of retinoids to RAR or RXR may have affected the efficacy of these treatments. Am80 also induces fewer side effects than ATRA [12]. Therefore, Am80 may represent a possible retinoid treatment for RA. The effects of Am80 need to be examined in a large number of patients at several clinical stages of RA.

Seven patients with active lupus nephritis were treated with ATRA (10 mg/day) for 6 months in an open clinical trial. Clinical symptoms, proteinuria, and hematuria as well as serum albumin, creatinine, anti-DNA antibody, and CH50 levels were evaluated. Improvements were observed in the clinical symptoms, such as fever and skin rash, and laboratory findings, including proteinuria and anti-DNA antibody levels of four patients. Moreover, they reached the complete remission criteria of nephrotic syndrome. ATRA was not effective in the other three patients and was discontinued after 3 months. No patient had adverse effects to the ATRA therapy [57].

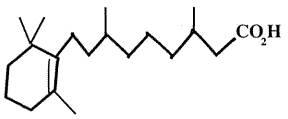
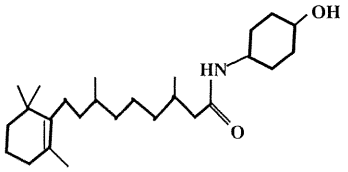
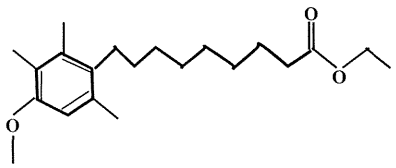
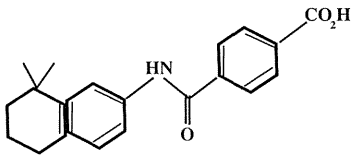
Thirty-one patients with systemic sclerosis (7 were treated with etretinate monotherapy, 5 with etretinate plus immunosuppressive therapies, 13 with immunosuppressive therapy only, and 6 with no treatment) were evaluated using the modified Rodnan total skin thickness score [58]. A significant improvement was defined as a 75 % reduction in the score. The skin thickness scores in 6 of the 7 patients treated with etretinate monotherapy, 3 of 5 with etretinate plus immunosuppressive therapy, 1 of 13 with immunosuppressive therapy only, and 0 of 6 with none therapy significantly improved. These findings suggested that etretinate may be a useful treatment for skin involvement associated with systemic sclerosis [58].

Retinoid trials for other rheumatic diseases, including vasculitis and myositis, have not yet been conducted. Am80 was effective for the treatment of myositis and vasculitis in animal models [11, 12]. Retinoids may also be used to treat these diseases.

Table 1 Clinical reports of the efficacy of retinoids in the treatment of rheumatic diseases

Retinoid	Disease (number of patients)	Duration	Results
Etretinate	RA ($n = 15$)	24 weeks	Clinical improvement ($n = 3$)
			No change ($n = 4$)
			Withdraw ($n = 8$)
4-HRP	RA ($n = 12$)	24 weeks	No change ($n = 6$)
			Withdraw ($n = 6$)
ATRA	Lupus nephritis ($n = 7$)	6 months	Clinical improvement ($n = 4$)
			Withdraw ($n = 3$)
Etretinate	Systemic sclerosis ($n = 31$)	20–70 months	Clinical improvement
	Etretinate alone ($n = 7$)		Etretinate alone ($n = 6$)
	Etretinate plus immunosuppressive therapies ($n = 5$)		Etretinate plus immunosuppressive therapies ($n = 3$)
	Immunosuppressive therapy alone ($n = 13$)		
	Non treatment ($n = 6$)		

Table 2 Synthetic retinoids

Retinoid	Activity/specificity	Structure
ATRA	Pan-RAR agonist	
4-HPR	RAR agonist	
Etretinate	Pan-RAR and Pan-RXR agonist	
Am80	RARα and β agonist	

There are currently no ongoing clinical trials on retinoids for rheumatic diseases. However, further clinical trials are expected for rheumatic diseases.

Conclusion

Retinoids have immunoregulatory functions, and treatments with retinoids were shown to be effective for arthritis, nephritis, myositis, and vasculitis in experimental animal models. Some clinical studies confirmed the efficacy of retinoids for lupus nephritis and systemic sclerosis. Therefore, retinoids may be a new therapy for rheumatic diseases; however, evidence for the positive impact of retinoids on rheumatic patients is scarce. Further clinical trials are needed to elucidate the efficacy of retinoids for the treatment of rheumatic diseases.

Conflict of interest Dr. Nanki reports grants and personal fees from Chugai Pharmaceutical Co., LTD., grants and personal fees from Eisai Co., LTD., grants from Ono Pharmaceutical Co., LTD., grants and personal fees from Mitsubishi Tanabe Pharma Corporation., grants and personal fees from Takeda Pharmaceutical Co., LTD, grants and personal fees from AbbVie Inc., personal fees from UCB Japan Co. Ltd., personal fees from Astellas Pharma Inc., grants from Pfizer Japan Inc., personal fees from Bristol-Myers Squibb, personal fees from Santen Pharmaceutical Co., Ltd., personal fees from Taisho Toyama Pharmaceutical Co., Ltd., and grants from Asahi Kasei Pharma Corporation outside the submitted work.

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SHORT COMMUNICATION

The risk of serious infection in patients with rheumatoid arthritis treated with tumor necrosis factor inhibitors decreased over time: a report from the registry of Japanese rheumatoid arthritis patients on biologics for long-term safety (REAL) database

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Abstract To investigate changes in the risk for serious infections (SIs) over time in Japanese rheumatoid arthritis (RA) patients treated with tumor necrosis factor inhibitors (TNFIs). This prospective cohort study included Japanese RA patients who began treatment with a TNFI from 2005 to 2007 (2005 group, $n = 716$, 634.2 patient years [PY]) and from 2008 to 2011 (2008 group, $n = 352$, 270.1 PY) at the time or after their enrollment in the registry of Japanese RA patients on biologics for long-term safety (REAL) database. Patients were observed for 12 months or until discontinuation of their initial TNFI in the REAL database. Drug discontinuation reasons and retention rates were analyzed. Incidence rates of serious adverse events (SAEs) were calculated with 95 % confidence intervals (CIs). The Cox proportional hazard model was applied to estimate the risk for SIs. The retention rate in the 2008 group was significantly lower than the 2005 group ($p < 0.001$). Discontinuation rates due to lack of efficacy or good control for the 2008 group were significantly higher than the 2005 group ($p < 0.001$). The crude incidence rate ratios comparing the 2008 group with the 2005 group for SAEs were 0.93 (95 % CI 0.65–1.34) and for SIs were 0.50 (0.24–1.03). The 2008 group had significantly lower risk for SIs than the 2005 group after adjusting for covariates (hazard ratio: 0.43 [0.20–0.93]). These results indicate significant decrease of the risk for SIs with TNFI treatment over time; this may be explained by evidence-based risk management of RA patients given TNFIs.

Keywords Rheumatoid arthritis · Epidemiology · Tumor necrosis factor inhibitor · Infection · Risk

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For the REAL study group. The REAL study group is given in "Appendix".

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Introduction

Tumor necrosis factor inhibitor (TNFI), the first approved biological disease-modifying antirheumatic drug (biological DMARD), has been widely used to treat patients with rheumatoid arthritis (RA) [1–3]. In 2003, infliximab (IFX) was the first approved biological DMARD for treatment of RA in Japan, followed by etanercept (ETN) in 2005, adalimumab (ADA) in 2008, golimumab in 2012, and certolizumab pegol in 2013 [4]. The data from postmarketing surveillance programs (PMS) implemented for these TNFIs by pharmaceutical companies [5–7], and those from prospective cohort studies for RA patients given TNFIs [8–10] have provided indispensable evidence for clinical practice.

The effectiveness and safety of a drug are strongly influenced by the selection of patients for whom it is prescribed. The launch of a new drug with indications similar to those of an older drug creates a situation where patients with a most suitable profile are "channeled" into the new therapy, thus creating differences in baseline clinical profiles from patients who were treated with the original drug. Such differences cause potential bias in estimation of drug effectiveness and safety [11]. The emergence of new clinical evidence leads to changes in the prescription practice of physicians, which may also over time affect treatment response or drug safety. It has been reported that treatment responses to TNFIs were significantly improved by changing patterns in prescriptions of TNFIs [12]. However, changes in the safety profile of TNFIs have not been described.

In this study, we hypothesized that safety profiles of treatment with TNFIs have improved over time. Thus, we compared risk for SAEs, including serious infections (SIs) between patients who started TNFIs from 2005 to 2007, shortly after the approval of the first TNFI in Japan, and from 2008 to 2011.

Patients and methods

Database

The registry of Japanese RA patients on biologics for long-term safety (REAL) is a prospective cohort established to investigate the long-term safety of biologicals in RA patients. Details of the REAL have been previously described [8]. Briefly, the criteria for enrollment in the REAL include patients meeting the 1987 American College of Rheumatology (ACR) criteria for RA, and starting or switching to treatment with biologicals or starting, adding or switching to non-biological DMARDs at the time of enrollment in the database, which was started in June 2005 and closed in January 2012.

Data were retrieved from the REAL database on March 5, 2012, for this study. The REAL study was approved by the ethics committees of the participating 27 institutions. The procedures followed were in accordance with the

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Japanese guidelines for epidemiological studies and with the Helsinki Declaration of 1975, as revised in 1983. All patients in the REAL signed an informed consent form at enrollment in the REAL.

Data collection

Each patient's recorded baseline data included demography, disease activity, physical disability, comorbidities, treatments, and laboratory data at the beginning of the observation period. A follow-up form was submitted to the REAL data center every six months by site investigators to report occurrence of SAEs, current RA disease activity, treatments, and laboratory data [8].

Patients

By March 2012, 1,945 RA patients were registered in the REAL, of these 1,069 patients started administration of IFX, ETN, or ADA at the time of enrollment or after enrollment in the REAL. Our analysis included 716 patients who started IFX or ETN in 2005–2007 (2005 group) and 353 patients who started IFX, ETN, or ADA in 2008–2011 (2008 group).

Follow-up

The start date of the observation was the date an initial TNFI was administered to a patient. Observation was terminated: (1) 12 months after the start of the observation period, (2) on the date of death or loss to follow-up, (3) on enrollment in a clinical trial, (4) on the date of the last administration of TNFI, if therapy with the initial TNFI in the REAL was discontinued for more than 90 days, (5) on the date when the initial TNFI in the REAL was changed to another biologic, or (6) on March 5, 2012, whichever came first.

Definition of serious adverse events (SAEs)

Our definition of SAEs, including SIs, was in accordance with the International Conference on Harmonization [13]. Bacterial infections requiring intravenous administration of antibiotics and opportunistic infections were also regarded as SIs [14].

Statistical analysis

Drug retention rates were calculated by the Kaplan–Meier method and compared using the log-rank test between the two groups. Risk factors for SIs during continuous treatment with the TNFI for up to 1 year were identified using the Cox regression hazard model with the forced entry method. These statistical analyses were conducted using SPSS (version 20.0, SPSS Inc., Chicago, IL USA). All p values were two-tailed, and $p < 0.05$ was considered statistically significant.

Results

Baseline characteristics of patients

Baseline data for the two groups are shown in Table 1. Compared with the 2005 group, the 2008 group had shorter disease duration ($p = 0.001$) and lower disease activity ($p = 0.001$) and was treated with higher doses of methotrexate ($p = 0.010$) and lower dosage of oral corticosteroids ($p < 0.001$). The rate of previous use of three or more non-biological DMARDs was lower in the 2008 group ($p = 0.001$) (Table 1). The median duration of follow-up (interquartile [IQR]) was 1.00 (IQR 0.51, 1.00) year in the 2005 group and 1.00 (IQR 1.00, 1.00) year in the 2008 group.

Types and occurrence of SAEs

During the observation period, 103 SAEs and 42 SIs in the 2005 group and 41 SAEs and 9 SIs in the 2008 group were observed. The crude incidence rate ratio (IRR) comparing the 2005 group with the 2008 group for all SAEs was 0.93 [95 % confidence interval (95 % CI) 0.65–1.34] and for SIs was 0.50 (95 % CI 0.24–1.03) (Table 2).

Drug discontinuation reasons and retention rates

There were significant differences in the reasons for discontinuation between the two groups ($p = 0.049$ by χ^2 test). The adjusted residuals indicate that a significantly higher percentage of patients in the 2008 group discontinued TNFI due to good control (Supplementary Table 1). The discontinuation rate for the 2008 group due to good control ($p < 0.001$, log-rank test) or to lack of efficacy ($p < 0.001$, log-rank test) was significantly higher than that for the 2005 group (Supplementary Figure 1).

Starting years of TNFI associated with risk for serious infection

We initially performed univariate analyses to compare patients who did and did not develop SIs (data not shown) and selected the following variables for multivariate analysis with consideration of medical significance: age, gender, presence of comorbidities, patient group (2008 vs. 2005), type of TNFI (monoclonal antibody vs. soluble receptor), and the use of oral corticosteroids at baseline. Cox

Table 1 Patient characteristics at the start of the observation period

	2005 group ($n = 716$)	2008 group ($n = 352$)	p value
Age (years)	56.1 ± 13.3	57.9 ± 14.8	0.021
Gender [female (%)]	81.8	81.2	0.814
Disease duration (years)[a]	7.0 (2.9, 14.0)	4.9 (1.8, 12.6)	0.001
DAS28(3/CRP) (number)	4.6 ± 1.2 ($n = 702$)	4.3 ± 1.3 ($n = 313$)	0.001
Steinbrocker's stage III or IV (%)[b]	53.6	37.5	<0.001
Steinbrocker's class 3 or 4 (%)[b]	29.5	21.0	0.003
Previous biologicals use (%)	11.2	17.3	0.005
Number of previous non-biological DMARDs ≥3 (%)	51.0	35.5	<0.001
MTX use (%)	68.6	80.7	<0.001
MTX dosage (mg/week)	7.5 ± 2.1	8.0 ± 2.4	0.010
Oral corticosteroid use (%)	71.2	53.7	<0.001
Corticosteroid (mg/day)[c]	5.8 ± 2.8	5.1 ± 2.5	<0.001
IFX use (%)	45.3	38.9	<0.001
ETN use (%)	54.7	26.7	
ADA use (%)	0	34.4	
Any comorbidities (%)	32.1	33.0	0.785
Chronic pulmonary diseases (%)[d]	21.2	21.9	0.809
Diabetes mellitus (%)	11.2	10.5	0.745
Liver diseases (%)	4.9	4.5	0.805
Kidney diseases (%)	3.6	1.1	0.020
TMP-SMX use (%)	2.4	19.0	<0.001

TNFI tumor necrosis factor inhibitor, *DAS28* disease activity score including 28-joint count, *CRP* C-reactive protein, *DMARDs* disease-modifying antirheumatic drugs, *MTX* methotrexate, *IFX* infliximab, *ADA* adalimumab, *ETN* etanercept, *TMP-SMX* trimethoprim-sulfamethoxazole

Values are mean ± SD unless otherwise indicated. For univariate analysis, the chi-square test for categorical variables and Mann–Whitney test were used to compare continuous variables between the two groups

[a] Values are median (interquartile)

[b] Steinbrocker classification was used to define RA disease stages and classes

[c] The oral corticosteroid dose was converted to the equivalent prednisolone dosage

[d] Pulmonary diseases include interstitial pneumonia, chronic obstructive pulmonary disease, bronchial asthma, prior pulmonary tuberculosis, and bronchiectasis

regression models reveal that the 2008 group had significantly lower risk for SIs than the 2005 group (hazard ratio: 0.43 [95 % CI 0.20–0.93], $p = 0.032$) after adjusting for the covariates (Table 3).

Comparison of disease activities between the groups

In patients with DAS28 (3/CRP) data at baseline and year 1 ($n = 540$ for 2005 group, $n = 178$ for 2008 group), the 2005 group had significantly higher DAS28 (3/CRP) scores than the 2008 group at both times (mean ± standard deviation in 2005 vs. 2008; 4.59 ± 1.23 vs. 4.32 ± 1.25 at baseline, $p = 0.011$; 2.80 ± 1.08 vs. 2.50 ± 0.97 at year 1, $p = 0.001$). A significantly higher percentage of patients in the 2008 group achieved low disease activity (DAS28 [3/CRP] <3.2) at year 1 compared with the 2005 group (80.9 % in the 2008 group, 68.7 % in the 2005 group, $p = 0.002$).

Discussion

In this study, the IR of SIs in the 2005 group was consistent with previous reports [9, 14], while the 2008 group showed a 50 % reduction in the IR of SIs, without statistical significance. Patients in the 2005 group appeared to be more susceptible to SIs than those in the 2008 group because of higher dosage of oral corticosteroids, higher disease activity, more advanced disease, and poorer physical function at baseline, all of which were identified as risk factors for SIs [8, 15]. After adjusting for these baseline characteristics, patients in the 2008 group had significantly lower risk for SIs (Table 3) than those in the 2005 group.

Several factors can be considered as determinants of the decreased risk for SIs. The first contributing factor is the safety results from PMS studies. The PMS studies of TNFIs in Japan revealed the types, incidence rates, and risk factors for infections [5–7]. Risk factors, such as older age,

Table 2 Number and incidence rates of serious adverse events in rheumatoid arthritis patients

	TNFI 2005 634.2 PY IR (/100PY)	TNFI 2008 270.1PY IR (/100PY)	TNFI 2008 vs. TNFI 2005, Crude IRR[a] (95 % CI)
ALL SAEs			
Number of events	103	41	0.93 (0.65–1.34)
IR/100 PY (95 % CI)	16.2 (13.3–19.6)	15.2 (11.1–20.4)	
Serious infections (SIs)			
Number of events	42	9	0.50 (0.24–1.03)
IR/100 PY (95 % CI)	6.62 (4.84–8.86)	3.33 (1.65–6.08)	
Serious respiratory tract infections			
Number of events	27	5	0.43 (0.17–1.13)
IR/100 PY (95 % CI)	4.26 (2.87–6.10)	1.85 (0.70–4.06)	
Other infections			
Number of events	15	4	0.63 (0.21–1.89)
IR/100 PY (95 % CI)	2.37 (1.38–3.80)	1.48 (0.50–3.52)	
Pulmonary diseases except for infection			
Number of events	11	6	1.28 (0.47–3.46)
IR/100 PY (95 % CI)	1.73 (0.92–3.00)	2.22 (0.92–4.58)	
Malignancies			
Number of events	3	5	3.91 (0.93–16.38)
IR/100 PY (95 % CI)	0.47 (0.13–1.26)	1.85 (0.70–4.06)	
Others			
Number of events	47	21	1.05 (0.63–1.75)
IR/100 PY (95 % CI)	7.41 (5.51–9.76)	7.77 (4.96–11.66)	

TNFI tumor necrosis factor inhibitor, *PY* patient year, *IR* incidence rate, *IRR* incidence rate ratio, *CI* confidence interval, *SAE* serious adverse event

[a] Crude incidence rate per 100 PY and crude incidence rate ratio with their 95 % CI were calculated for each category of serious adverse events occurring from the first to the last dose of infliximab, etanercept, or adalimumab

Table 3 Multivariate analysis of independent risk factors for serious infections in rheumatoid arthritis patients

All values at baseline	Hazard ratio (95 %CI)	*p* value
Age by decade	1.76 (1.31–2.39)	<0.001
Gender (male)	0.45 (0.18–1.16)	0.099
Steinbrocker's class 3 or 4 (vs. 1 or 2)	1.26 (0.68–2.32)	0.460
Comorbidities yes (vs. no)[a]	2.23 (1.18–4.22)	0.014
Concomitant use of corticosteroid	1.79 (0.85–3.75)	0.126
2008 group (vs. 2005)	0.43 (0.20–0.93)	0.032
IFX or ADA (vs. ETN)	1.63 (0.88–3.03)	0.124

Cox hazard model analysis, adjusted for the variables included in the table

CI confidence interval, *IFX* infliximab, *ADA* adalimumab, *ETN* etanercept

[a] Comorbidities include pulmonary, liver, kidney diseases, and diabetes mellitus

presence of diabetes mellitus, or pulmonary diseases, were incorporated into the Japanese guidelines for treatment with TNFIs [3] and updated periodically thereafter. Japanese guidelines for treatments with TNFIs state that administration of TNFIs to patients with any of the above risk factors should be carefully considered. The guidelines have enabled Japanese rheumatologists to select appropriate patients for TNFI therapy. The second factor is the improved risk management of RA patients given these drugs. Bacterial pneumonia has been identified as the most frequent infection in Japanese RA patients given TNFIs, and Japanese RA patients have relatively higher incidence of tuberculosis and *Pneumocystis jirovecii* pneumonia than RA patients in other countries [5–7]. Hence, pneumococcal vaccination and chemoprophylaxis with isoniazid or trimethoprim-sulfamethoxazole (TMP-SMX) for high-risk patients have been recommended in the Japanese guidelines for treatment with TNFIs since 2007 [3]. In the patient population of this study, a significantly higher percentage of patients received TMP-SMX in the 2008 group compared with the 2005 group (Table 1). The third factor is the approval of alternative treatments, such as tocilizumab and abatacept. In this population, the discontinuation rate for the 2008 group due to lack of efficacy was significantly higher than that for the 2005 group (Supplementary Figure 1). In the 2008 group, some patients whose disease activities could not be sufficiently controlled by TNFIs were switched to other classes of biological DMARDs and excluded from this analysis.

Recent changes in treatment for RA are possible unadjusted confounders of the lower risk for SIs seen in the 2008 group. The ACR 2008 recommendations for the use

of non-biological and biological DMARDs in RA [16], the European League Against Rheumatism (EULAR) 2010 recommendations for the management of RA [17], and the updated guideline for TNFIs by the Japan College of Rheumatology (JCR) in 2012 [18] have enabled rheumatologists to begin treatment with a TNFI at an earlier stage. Although patients in the 2008 group in this study were not influenced by the updated JCR guideline, because they started TNFIs between 2008 and 2011, the ACR and EULAR recommendations may have influenced the use of TNFIs in that group. Disease duration in the 2008 group was significantly shorter than that in the 2005 group (Table 1), indicating that the rheumatologists in the participating institutions started TNFIs for their RA patients earlier in the course of disease. Generally, patients with shorter disease durations tend to have lower prevalence of comorbidities, earlier stages of RA, better physical function, and lower rates and dosages of concomitant corticosteroids than those with longer disease duration [19]. However, we had already incorporated these factors as covariates in the multivariate analysis.

There are limitations to this study. First, the number of patients in the 2008 group was smaller than in the 2005 group, which could affect the sensitivity of the analysis. Second, we could not adjust for control of disease activity in the multivariate analysis because data were lacking for year 1 in some patients. Because it has been reported that higher disease activity was associated with the development of SIs [8, 20], better control of disease activity in the 2008 group may have led to reduced risk for SIs. Third, we could not use the history of previous infections and health assessment questionnaire scores as covariates in the multivariate analysis because the REAL database lacks these data.

In conclusion, the adjusted risk for SIs in Japanese RA patients receiving TNFIs decreased significantly over time. This observation may partly be explained by the progress in evidence-based risk management during treatment with TNFI and indicates that continuing pharmacovigilance activity is a requisite for proper use of TNFIs in clinical practice.

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Conflict of interest Tokyo Medical and Dental University (TMDU) receives unrestricted research grants for Departments of Pharmacovigilance with which TMDU pays salary for ***RS***. ***TN*** has received grants from Eisai Co Ltd. and has received speaking fees from Mitsubishi-Tanabe Pharma Co. Tokyo Medical and Dental University (TMDU) receives unrestricted research grants for Departments of Pharmacovigilance with which TMDU pays salary for ***TN***. Tokyo Medical and Dental University (TMDU) receives unrestricted research grants for Departments of Pharmacovigilance with which TMDU pays salary for ***KW***. Tokyo Medical and Dental University (TMDU) receives unrestricted research grants for Departments of Pharmacovigilance with which TMDU pays salary for ***HY***. ***KA*** has received research support from Chugai Pharmaceutical Co., Ltd., Mitsubishi-Tanabe Pharma Co. and AstellasPharma Inc and has received consulting fees or honoraria from Chugai Pharmaceutical Co., Ltd., Mitsubishi-Tanabe Pharma Co., AstellasPharma Inc., Abbvie Pharamaceutical Research & Development., Eisai Co Ltd., Pfizer Inc., Bristol-Myers Squibb. ***YT*** has received consulting fees, speaking fees, and/or honoraria from Mitsubishi-Tanabe Pharma Co., Chugai Pharmaceutical Co., Ltd., Eisai Co., Ltd., Takeda Pharmaceutical Co. Ltd., Astellas Pharma Inc., and Abbott Japan Co., Ltd., Janssen Pharma K.K., Santen Pharma Co., Ltd., Pfizer Japan Inc., Daiichi-Sankyo Co., Ltd., GlaxoSmithKline K.K. Astra-Zaneca., Otsuka Pharma Co., Ltd., Actelion Pharma Japan Ltd., Eli Lilly Japan K.K., Nippon Kayaku Co., Ltd., UCB Japan Co., Ltd., Quintiles Transnational Japan Co. Ltd., Ono Pharma Co., Ltd., and Novartis Pharma K.K and has received research grant support from Mitsubishi-Tanabe Pharma Co., MSD K.K., Astellas Pharma Inc., Chugai Pharmaceutical Co., Abbott Japan Co., Ltd., and Eisai Co., Ltd., Bristol-Myers Squibb., and Janssen Pharmaceutical K.K. ***TS*** has received honoraria from Takeda Pharmaceutical Co. Ltd., Mitsubishi-Tanabe Pharma Co., Chugai Pharmaceutical Co., Ltd., Santen Pharmaceutical Co., Ltd., Astellas Pharma Inc., Bristol-Myers Squibb and Abbvie Japan Co., Ltd. and has received research grant support from Takeda Pharmaceutical Co., Ltd. ***TF*** has received Grant/Research support from Abbott Japan Co., Ltd., Eisai Co., Ltd., Takeda Pharmaceutical Co., Ltd., Mitsubishi-Tanabe Pharma Co., Chugai Pharmaceutical Co., Ltd., Pfizer Japan Inc., AstellasPharma Inc., Bristol-Myers Squibb. ***NM*** has received research grants from Abbott Japan Co., Ltd., Astellas Pharma Inc., Bristol-Myers Squibb, Chugai Pharmaceutical Co., Ltd., Dainihon-Sumitomo Pharma Co. Ltd., Daiichi-Sankyo Co. Ltd., Eisai Co. Ltd., Mitsubishi-Tanabe Pharma Co., Novartis Pharma K.K., Ltd., Takeda Pharmaceutical Co., Ltd., Teijin Pharma Ltd and received Consulting fees or honoraria from Abbott Japan Co., Ltd., Bristol Myers Squibb, Janssen Pharmaceutical KK, and Otsuka Pharmaceutical Co. Ltd. ***MH*** has received research grants from Abbott Japan Co., Ltd., AstellasPharma Inc., Bristol-Myers Squibb., Chugai Pharmaceutical Co., Ltd., Eisai Co., Ltd., Mitsubishi-Tanabe Pharma Co., Pfizer Japan Inc., Sanofi-Aventis KK., Santen Pharmaceutical Co., Ltd., and Takeda Pharmaceutical Co., Ltd. Tokyo Medical and Dental University (TMDU) receives unrestricted research grants for Departments of Pharmacovigilance with which TMDU pays salary for ***MH***.

Appendix

The investigators of the REAL study group and their affiliates who contributed to data collection were Michi Tanaka (Tokyo Medical and Dental University); Shintaro Hirata, Kazuyoshi Saito (University of Occupational and Environmental Health); Taichi Hayashi (University of Tsukuba); Yoshiaki Ishigatsubo (Yokohama City University); Tatsuya Atsumi (Hokkaido University); Shoko

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RESEARCH ARTICLE Open Access

Activation of fibroblast-like synoviocytes derived from rheumatoid arthritis via lysophosphatidic acid–lysophosphatidic acid receptor 1 cascade

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Abstract

Introduction: Lysophosphatidic acid (LPA) is a bioactive lipid that binds to G protein–coupled receptors (LPA_{1-6}). Recently, we reported that abrogation of LPA receptor 1 (LPA_1) ameliorated murine collagen-induced arthritis, probably via inhibition of inflammatory cell migration, Th17 differentiation and osteoclastogenesis. In this study, we examined the importance of the LPA–LPA_1 axis in cell proliferation, cytokine/chemokine production and lymphocyte transmigration in fibroblast-like synoviocytes (FLSs) obtained from the synovial tissues of rheumatoid arthritis (RA) patients.

Methods: FLSs were prepared from synovial tissues of RA patients. Expression of LPA_{1-6} was examined by quantitative real-time RT-PCR. Cell surface LPA_1 expression was analyzed by flow cytometry. Cell proliferation was analyzed using a cell-counting kit. Production of interleukin 6 (IL-6), vascular endothelial growth factor (VEGF), chemokine (C-C motif) ligand 2 (CCL2), metalloproteinase 3 (MMP-3) and chemokine (C-X-C motif) ligand 12 (CXCL12) was measured by enzyme-linked immunosorbent assay. Pseudoemperipolesis was evaluated using a coculture of RA FLSs and T or B cells. Cell motility was examined by scrape motility assay. Expression of adhesion molecules was determined by flow cytometry.

Results: The expression of LPA_1 mRNA and cell surface LPA_1 was higher in RA FLSs than in FLSs from osteoarthritis tissue. Stimulation with LPA enhanced the proliferation of RA FLSs and the production of IL-6, VEGF, CCL2 and MMP-3 by FLSs, which were suppressed by an LPA_1 inhibitor (LA-01). Ki16425, another LPA_1 antagonist, also suppressed IL-6 production by LPA-stimulated RA FLSs. However, the production of CXCL12 was not altered by stimulation with LPA. LPA induced the pseudoemperipolesis of T and B cells cocultured with RA FLSs, which was suppressed by LPA_1 inhibition. In addition, LPA enhanced the migration of RA FLSs and expression of vascular cell adhesion molecule and intercellular adhesion molecule on RA FLSs, which were also inhibited by an LPA_1 antagonist.

Conclusions: Collectively, these results indicate that LPA–LPA_1 signaling contributes to the activation of RA FLSs.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial hyperplasia with proliferation of fibroblast-like synoviocytes (FLSs), angiogenesis, infiltration of inflammatory cells such as lymphocytes and macrophages, and bone destruction of multiple joints [1]. FLSs are especially responsible for inflammation through cytokine and chemokine production and are also key cells of the invasive synovium, suggesting that they play a major role in the initiation and perpetuation of the destruction of inflamed joints [2].

Lysophosphatidic acid (LPA) is a bioactive lipid that binds to its specific cell surface G protein–coupled receptors (LPA_{1-6}). LPA is generated via the hydrolysis of lysophosphatidylcholine by a secretory protein, autotaxin (ATX), which exhibits lysophospholipase D activity [3]. ATX was shown to be highly expressed in tumor cells, including neuroblastoma, breast cancer and renal cell carcinoma [4-6]. Moreover, LPA was reported to induce

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the production of interleukin 8 (IL-8) and vascular endothelial growth factor (VEGF) by cancer cells, angiogenesis and cancer growth [7-11].

It has previously been shown that expression of ATX by FLSs in the RA synovium and concentration of ATX in the RA synovial fluid are increased [12]. In addition, LPA_{1-3} mRNA has been reported to be expressed in RA FLSs, and incubation with LPA induced cell motility and cytokine expression by the FLSs, indicating that LPA may contribute to the pathogenesis of RA by stimulation of FLSs [13,14]. We recently demonstrated that treatment with an LPA receptor 1 (LPA_1) antagonist, LA-01, ameliorated murine collagen-induced arthritis, probably via inhibition of inflammatory cell migration, Th17 differentiation and osteoclastogenesis [15].

In this study, we extensively analyzed the stimulatory effects of LPA for RA FLSs, as well as the effects of an LPA_1 antagonist, LA-01, against this stimulation.

Methods

Specimens

Synovial tissues were obtained from RA patients (n = 10) who fulfilled American College of Rheumatology criteria [16] and from patients with osteoarthritis (OA) (n = 5). RA patients were a median (range) of 67 years old (45 to 80), and had a disease duration of 14 years (2 to 30) and C-reactive protein level of 0.68 mg/dl (0.0 to 2.85). Seven patients (70%) were positive for rheumatoid factor, and seven (70%) were positive for anticitrullinated protein antibodies. All patients provided informed consent. The experimental protocol was approved by the ethics committee of the Tokyo Medical and Dental University.

Fibroblast-like synoviocytes

Synovial tissues from RA patients were minced and incubated with 0.5 mg/ml collagenase (Sigma-Aldrich, St Louis, MO, USA) for 1 hour at 37°C, then passed through a metal screen to obtain single-cell suspensions. Harvested cells were plated in cell culture plates and incubated with Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich). Adherent cells were maintained in the medium as FLSs and were used after five passages in the following experiments [17].

RT-PCR

Total RNA was prepared from the FLSs of RA tissue (n = 10) and OA synovial tissue (n = 5), and first-strand cDNA was synthesized. Quantitative real-time RT-PCR was performed as described previously [18]. cDNA was amplified with primers for LPA_1 (sense, 5′-ACC CAA TAC TCG GAG ACT GAC TGT-3′; antisense, 5′-CGT CAG GCT GGT GTC AAT GA-3′), LPA_2 (sense, 5′-TCA TCA TGG GCC AGT GCT ACT-3′; antisense, 5′-GTG GGA GCT GAG CTC TTT GC-3′), LPA_3 (sense, 5′-CTT GAC TGC TTC CCT CAC CAA-3′; antisense, 5′-CGC ATC CTC ATG ATT GAC ATG-3′), LPA_4 (sense, 5′-TCC TCA GTG GCG GTA TTT CAG-3; antisense, 5′-AAG CAG GTG GTG GTT GCA TT-3′), LPA_5 (sense, 5′-GGT GGT GAG CGT GTA CAT GTG T-3′; antisense, 5′-AGT GGT GCA GTG CGT AG TAG GA-3′), LPA_6 (sense, 5′-AGA ACC AAA AGA AAT GCA AAG ATT G-3′; antisense, 5′-ACG GCG GGT GCA CTT C-3′) and 18S rRNA (sense, 5′-AAC CAG ACA AAT CGC TCC AC-3′; antisense, 5′-ACT CAA CAC GGG AAA CCT CA-3′). 18S rRNA was used as an internal control to standardize the amount of sample mRNA, and the relative expression of real-time PCR products was determined.

Cell surface expression of lysophosphatidic acid receptor 1 on fibroblast-like synoviocytes

FLSs were stained with anti-LPA_1 monoclonal antibody (mAb) (1G6; LSBio, Seattle, WA, USA) as a first antibody, and phycoerythrin-conjugated anti-mouse immunoglobulin G (IgG) antibody (BioLegend, San Diego, CA, USA) as a second antibody. Mouse IgG2b (BioLegend) was used as an isotype control. Cells were then analyzed by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA).

Proliferation assay

FLSs were plated at a density of 2×10^3 cells/well in 96-well flat-bottom plates. Cells were incubated with a selective LPA_1 antagonist (LA-01 (0, 1 or 10 nM); provided by Ono Pharmaceutical, Osaka, Japan) [15,19] for 30 minutes and then stimulated with LPA (Cayman Chemical, Ann Arbor, MI, USA) (0, 1 or 10 μM) in FCS-free DMEM at 37°C for 72 hours. The proliferation of FLSs was measured by using a cell-counting kit with WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt; Dojindo, Kumamoto, Japan) according to the manufacturer's protocol. LPA_1, LPA_2 and LPA_3 share 50% to 57% amino acid identity in humans and comprise the endothelial cell differentiation gene (Edg) family of LPA receptors [20]. The half-maximal inhibitory concentration (IC50) of LA-01 was 0.086, 2.8 and 0.90 μmol/L for LPA_1, LPA_2 and LPA_3, respectively, which was determined by LPA_1-, LPA_2- or LPA_3-transfected CHO cells [15,19]. LPA_{4-6} receptors have been classified into the non-Edg family of LPA receptors and are structurally distant from the Edg family of LPA receptors [20]. The IC_{50} of LA-01 for LPA_{4-6} was not determined. Incubation with LA-01 did not affect viability of the FLSs (data not shown).

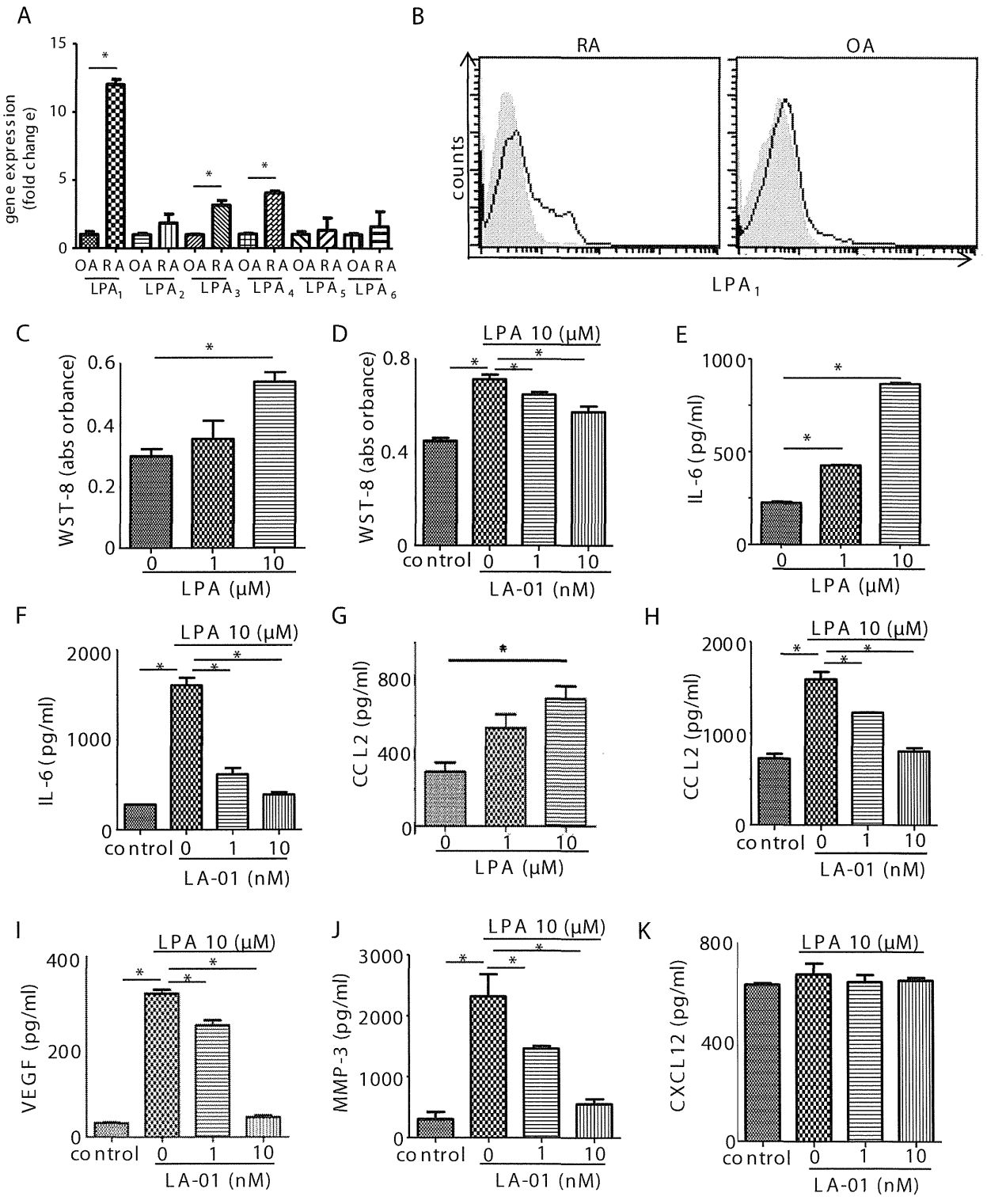

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Figure 1 Expression of lysophosphatidic acid receptors and the effect of lysophosphatidic acid receptor 1 on proliferation and production of inflammatory mediators in rheumatoid arthritis fibroblast-like synoviocytes. The expression levels of lysophosphatidic acid receptor 1 through 6 (LPA_{1-6}) mRNA in fibroblast-like synoviocytes (FLSs) derived from the rheumatoid arthritis (RA) synovium (n = 10) were compared to those in FLSs from osteoarthritis (OA) synovium (n = 5) by real-time RT-PCR **(A)**. Data were derived from samples from multiple individuals. Data are presented as the mean ± SEM. *$P < 0.05$ for RA vs OA. Cell surface expression of LPA_1 on RA (n = 5) and OA (n = 3) FLSs was analyzed by flow cytometry **(B)**. Filled histogram (gray): isotype control; open histogram (black line): LPA_1. Representative histograms are shown. RA FLSs were cultured with lysophosphatidic acid (LPA) for 72 hours **(C)**. FLSs were preincubated with an LPA1 inhibitor, LA-01, for 30 minutes,then stimulated with 10 μM LPA for 72 hours **(D)**. Control: no stimulation with LPA. Cell proliferation was measured by using a cell counting kit **(C)** and **(D)**. RA FLSs were cultured with LPA for 24 hours. Concentrations of interleukin 6 (IL-6) and chemokine (C-C motif) ligand 2 (CCL2) in the culture supernatant were measured by enzyme-linked immunosorbent assay (ELISA) **(E)** and **(G)**. FLSs were preincubated with LA-01 for 30 minutes, then stimulated with 10 μM LPA for 24 hours. Concentrations of IL-6, CCL2, vascular endothelial growth factor (VEGF), matrixmetalloproteinase (MMP-3) and CXCL12 in the culture supernatant were measured by ELISA **(F)**, and (H) through **(K)**. Control: no stimulation with LPA. Data are presented as the means (±SEM) of one of three independent experiments analyzed in triplicate. *$P < 0.05$ vs control or LA-01 0 nM **(C)** through **(K)**.

Enzyme-linked immunosorbent assay

RA FLSs were cultured overnight in 96-well plates (2×10^4 cells/well), then incubated with LA-01 (0, 1 or 10 nM) or Ki16425 (2 nM) (Cayman Chemical) 30 minutes before stimulation with LPA (10 μM) in FCS-free DMEM at 37°C for 24 hours. Protein levels of IL-6, chemokine (C-C motif) ligand 2 (CCL2), VEGF, matrix metalloproteinase 3 (MMP-3) and chemokine (C-X-C motif) ligand 12 (CXCL12) in the culture supernatant were assessed by using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the instructions supplied by the manufacturer.

Pseudoemperipolesis

FLSs were seeded onto 96-well plates (2×10^4 cells/well) and cultured for 48 hours. CD4- and CD8-positive ($CD4^+$ and $CD8^+$, respectively) T cells and $CD19^+$ B cells were purified from human peripheral blood of healthy volunteers by using MACS microbeads (>95% purity; Miltenyi Biotec, Auburn, CA, USA) and added to the FLS-cultured wells (1×10^5 cells/well). The cells were treated with LA-01 (0, 1 or 10 nM) for 30 minutes, followed by stimulation with LPA (10 μM) in FCS-free DMEM. After 12 hours, the wells were washed three times with medium. Pseudoemperipolesis was assessed by counting the number of cells beneath FLSs in three independent fields under a microscope.

Scrape motility assay

RA FLSs were plated at a density of 1×10^5 cells/ml in 12-well plates in DMEM with 10% FCS. After overnight incubation, FLSs was washed twice with FCS-free medium. The tip of a plastic pipette was drawn across the center of the well to produce a scraped area. Culture wells were washed twice with PBS, and free cells were removed. After pretreatment with LA-01 (0, 1 or 10 nM) for 30 minutes, cells were incubated with LPA (10 μM) in FCS-free DMEM. A cell-free area was measured by using ImageJ software (National Institutes of Health, Bethesda, MD, USA) at 0 and 48 hours, and the ratio was then calculated (cell-free area at 48 hours per cell-free area at 0 hours).

Expression of vascular cell adhesion molecule and intercellular adhesion molecule on RA fibroblast-like synoviocytes

FLSs were stimulated with LPA (10 μM) 30 minutes after adding LA-01 (0, 1 or 10 nM) in FCS-free DMEM at 37°C for 12 hours. Cells were stained with allophycocyanin-conjugated mAb against vascular cell adhesion molecule (anti-VCAM, clone STA; BioLegend) or phycoerythrin-conjugated mAb against intracellular adhesion molecule (anti-ICAM, clone HA58; eBioscience, San Diego, CA, USA). Allophycocyanin- or phycoerythrin-conjugated mouse IgG1 (BioLegend) was used as an isotype control. Cells were then analyzed by flow cytometry (Accuri C6 Flow Cytometer; BD Biosciences).

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). The comparison of the data from the two groups was conducted by using Student's *t*-test. *P*-values less than 0.05 were considered significant.

Results

Expression of lysophosphatidic acid receptors in RA fibroblast-like synoviocytes

The expression of LPA_{1-6} mRNA in FLSs from RA and OA patients was analyzed by quantitative real-time RT-PCR. The expression of LPA_1 mRNA in RA FLSs was significantly higher than that in OA FLSs (Figure 1A). The expression of LPA_3 and LPA_4 was also significantly higher in RA FLSs than that in OA FLSs, although the ratios of LPA_3 and LPA_4 expression in RA FLSs to OA FLSs were smaller than those of LPA_1 expression. Cell surface LPA_1 expression was analyzed by flow cytometry. RA FLSs were expressed LPA_1 on the cell surface, and

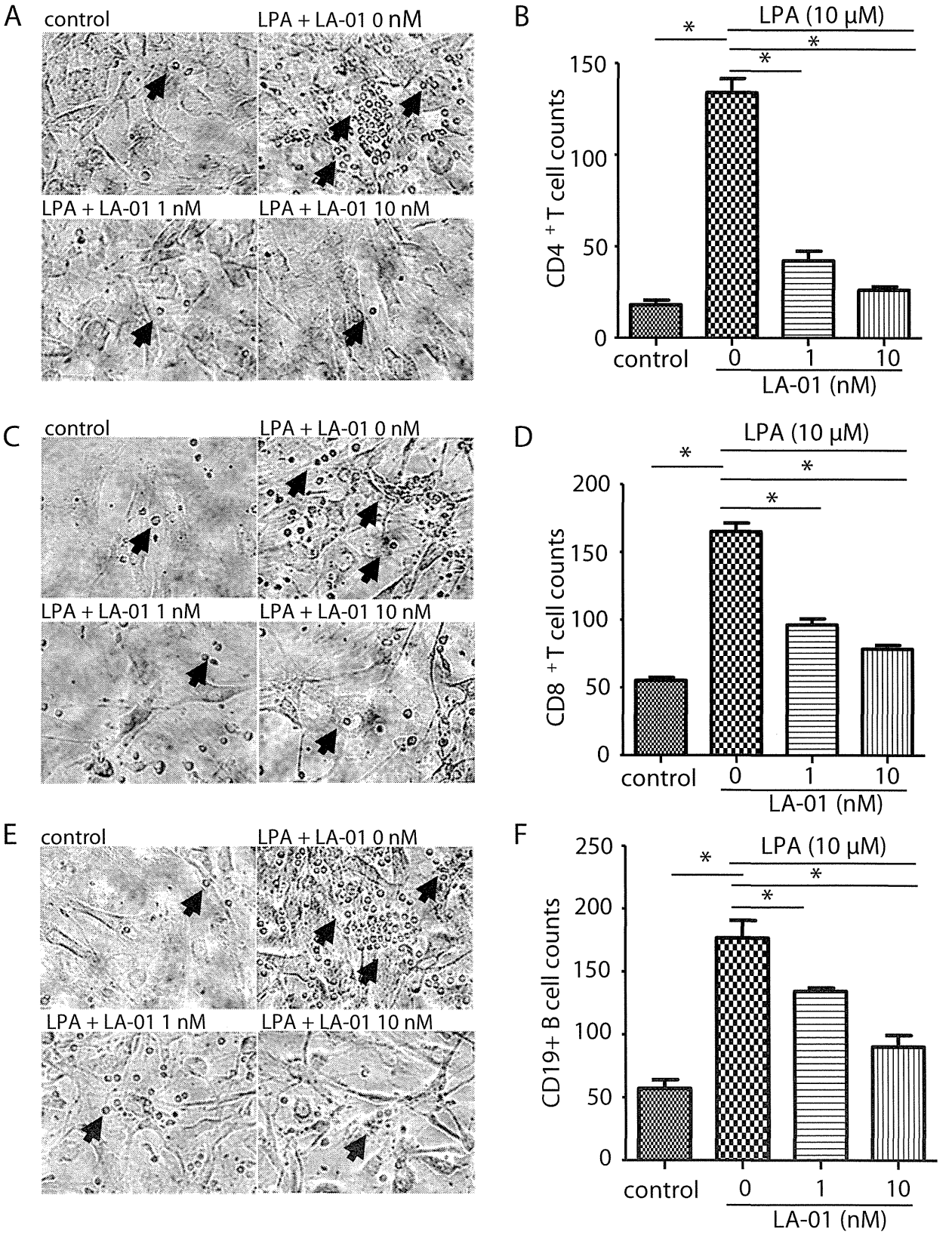

Figure 2 (See legend on next page.)

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Figure 2 Effect of lysophosphatidic acid receptor 1 on pseudoemperipolesis and migration of rheumatoid arthritis fibroblast-like synoviocytes. After preincubation of cocultured rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLSs) and $CD4^+$ T cells **(A)** and **(B)** or $CD8^+$ T cells **(C)** and **(D)** or $CD19^+$ B cells **(E)** and **(F)** with a lysophosphatidic acid (LPA) receptor 1 inhibitor (LA-01; 0, 1 or 10 nM) for 30 minutes, the cells were stimulated with 10 μM LPA for 12 hours. Control: no stimulation with LPA. After the cells were washed, the number of lymphocytes beneath FLSs was counted. Representative photomicrographs of three independent experiments are shown **(A, C and E)**. Arrows indicate the lymphocytes beneath FLSs. Original magnification, ×200. Data on the number of lymphocytes beneath FLSs are presented as one of three independent experiments analyzed in triplicate **(B, D, and F)**. Data are presented as the mean ± SEM. *$P < 0.05$ vs control or LA-01 0 nM **(B, D, F)**.

the expression level was substantially higher than that of OA FLSs (Figure 1B).

Lysophosphatidic acid receptor 1 inhibitor suppressed lysophosphatidic acid–induced proliferation and cytokine production in RA fibroblast-like synoviocytes

We analyzed the effects of LPA on the proliferation and production of inflammatory mediators by RA FLSs. Stimulation with LPA dose-dependently induced the proliferation of FLSs (Figure 1C). LPA stimulation also induced the production of IL-6 and CCL2 from FLSs in a dose-dependent manner (Figures 1E and 1G), which supports a previous report that LPA upregulated IL-6 mRNA expression by RA FLSs [18]. Stimulation with LPA also induced the production of VEGF and MMP-3 by RA FLSs *in vitro* (Figures 1I and 1J).

Next, we analyzed the effect of an LPA_1 inhibitor on LPA stimulation for RA FLSs. Enhanced cell proliferation by 10 μM LPA was significantly suppressed by LA-01, the LPA_1-selective antagonist (Figure 1D). The treatment with LA-01 significantly reduced the production of IL-6, CCL2, VEGF and MMP-3 by LPA-stimulated RA FLSs (Figures 1F and 1H through 1J). In contrast, the production of CXCL12 by RA FLSs was not altered by stimulation with LPA (Figure 1K). We used Ki16425, another LPA_1 antagonist, to confirm the effects of LPA_1 inhibition on IL-6 production from LPA-stimulated RA FLSs. Incubation with Ki16425 suppressed IL-6 production from LPA-stimulated RA FLSs as well as LA-01 (IL-6 concentrations: vehicle = 299.413 ± 28.084 pg/ml; Ki16425 = 116.785 ± 11.162 pg/ml ($P < 0.05$ vs vehicle); LA-01 = 145.715 ± 15.921 pg/ml ($P < 0.05$ vs vehicle)). These results suggest that LPA–LPA_1 signaling plays important roles in proliferation and cytokine production of RA FLSs *in vitro*.

LPA–LPA_1 signaling promoted pseudoemperipolesis

RA FLSs have been shown to promote the spontaneous migration of leukocytes beneath them, a process termed *pseudoemperipolesis* [21]. We examined the effect of LPA on pseudoemperipolesis. Stimulation with 10 μM LPA significantly increased the number of $CD4^+$ and $CD8^+$ T cells, as well as $CD19^+$ B cells, beneath RA FLSs (Figures 2A to 2F). Moreover, incubation with LA-01 suppressed the LPA-enhanced pseudoemperipolesis of $CD4^+$ and $CD8^+$ T and $CD19^+$ B cells (Figures 2A through 2F), suggesting that interaction of LPA and LPA_1 promotes pseudoemperipolesis of leukocytes.

LPA–LPA_1 signaling promoted cell motility of RA fibroblast-like synoviocytes

We also analyzed the effect of LPA_1 on RA FLS migration by scrape motility assay. Incubation with 10 μM LPA significantly decreased the cell-free area, indicating that LPA induced cell migration *in vitro* (Figures 3A and 3B), as reported previously [22]. In addition, LA-01 significantly increased the cell-free area of RA FLSs (Figures 3A and 3B), suggesting that LPA–LPA_1 signaling also contributes to the promotion of RA FLS motility.

LPA–LPA_1 signaling induced adhesion molecule expression on RA fibroblast-like synoviocytes

It has been reported that signaling from VCAM and ICAM in RA FLSs supports pseudoemperipolesis [21]. Therefore, we next analyzed the expression of VCAM and ICAM on RA FLSs by flow cytometry. We found that stimulation with 10 μM LPA induced the expression of VCAM and ICAM on RA FLSs (Figure 4). Moreover, LA-01 decreased the expression of VCAM and ICAM induced by LPA on RA FLSs (Figure 4). However, the expression of E-selectin on RA FLSs was not altered by LPA simulation (data not shown).

Discussion

In this study, we found that LPA_1 was highly expressed in RA FLSs. LPA stimulated RA FLSs to enhance proliferation, production of inflammatory mediators, pseudoemperipolesis, migration and the expression of adhesion molecules, which are attributable to signaling through LPA_1.

RA FLSs express inflammatory cytokines, chemokines and matrix-degrading enzymes, which contribute to the pathogenesis of RA. LPA has been reported to induce IL-6 mRNA expression on RA FLSs, as well as cell motility [13]. However, the corresponding LPA receptor on RA FLSs has not been identified. We show that LPA augmented IL-6, CCL2, VEGF and MMP-3 production by RA FLSs. Moreover, the LPA-induced production of the inflammatory mediators was inhibited by a LPA_1-selective inhibitor. Therefore, the LPA–LPA_1 cascade plays

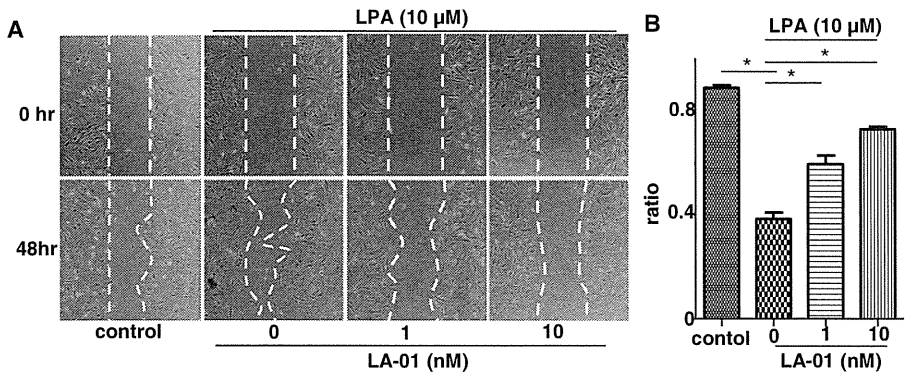

Figure 3 The effect of lysophosphatidic acid receptor 1 on the migration of rheumatoid arthritis fibroblast-like synoviocytes. A scraped cell-free area was created on cultured RA FLSs. After preincubation with a lysophosphatidic acid (LPA) receptor 1 inhibitor (LA-01; 0, 1 or 10 nM) for 30 minutes, cells were stimulated with 10 μM LPA for 48 hours. Control: no stimulation with LPA. **(A)** Representative photomicrographs of three independent experiments are shown. Original magnification, ×40. **(B)** The cell-free area was assessed, and a ratio (cell-free area in 48 hours to cell-free area in 0 hours) was defined. Data are presented as the means (±SEM) of one of three independent experiments analyzed in triplicate. *$P < 0.05$ vs control or LA-01 0 nM. Upper dashed line indicates the cells are stimulated with LPA 10 uM, and lower dashed line indicates LA-01 is added with indicated concentration.

an important role in cytokine, chemokine and matrix-degrading enzyme production by RA FLSs. Although IC_{50} of LA-01 was 86 nM, which was determined by using LPA_1-transfected CHO cells, 10 nM LA-01 significantly inhibited stimulation of LPA in RA FLSs. The IC_{50} may be dependent on cell type or on the expression level of LPA_1.

Pseudoemperipolesis contributes to the chronic inflammation induced by lymphocyte recruitment in the inflamed joints and protects lymphocytes from apoptosis [21,23,24]. We show that LPA enhanced the pseudoemperipolesis of T and B cells, which is also attributable to LPA_1. It has been reported that stimulation with CXCL12 and signaling from VCAM and ICAM in RA

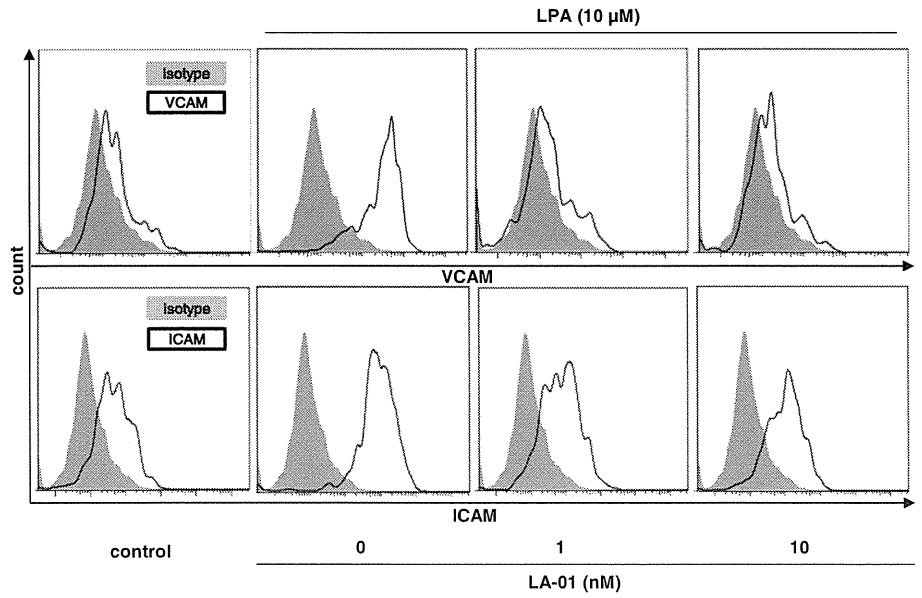

Figure 4 The effect of lysophosphatidic acid receptor 1 on the expression of adhesion molecules on fibroblast-like synoviocytes. Rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLSs) were pretreated with a lysophosphatidic acid (LPA) receptor 1 inhibitor (LA-01; 0, 1 or 10 nM) for 30 minutes, then the cells were stimulated with 10 μM LPA for 12 hours. Cells were stained with allophycocyanin-conjugated monoclonal antibody (mAb) against vascular cell adhesion molecule (anti-VCAM) or phycoerythrin-conjugated mAb against intercellular adhesion molecule (anti-ICAM). Allophycocyanin- or phycoerythrin-conjugated mouse immunoglobulin G1 (IgG1) was used as a control. The expression of VCAM and ICAM on FLSs was analyzed by flow cytometry. Filled histogram (gray): isotype control; open histogram (black line): VCAM or ICAM.