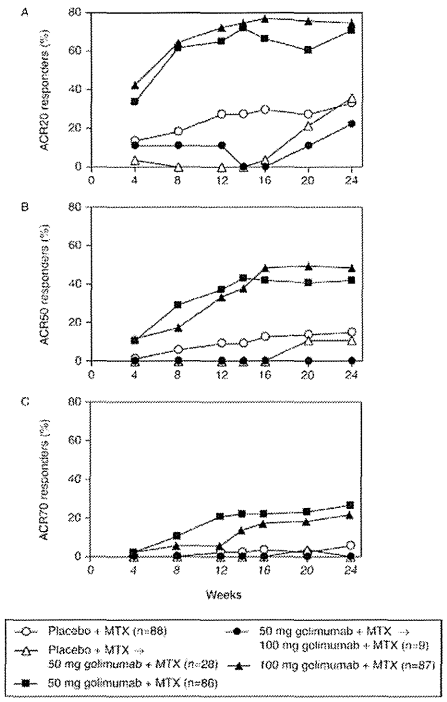

Figure 2 (A) American College of Rheumatology 20% (ACR20), (B) 50% (ACR50) and (C) 70% (ACR70) improvement from baseline through week 24. Note that patients who met the early escape criteria at week 16 and crossed over to golimumab 50 mg or dose escalated from golimumab 50 mg to 100 mg are shown with an open triangle and closed circle, respectively. For the 28 patients in the placebo + MTX group and the nine patients in the golimumab 50 mg + MTX group who met the early escape criteria, week 20 and 24 data were imputed using last observation carried forward methodology, as were other missing data. As such, 88 patients in the placebo + MTX group and 86 patients in the golimumab 50 mg + MTX group were included in these data displays. MTX, methotrexate.

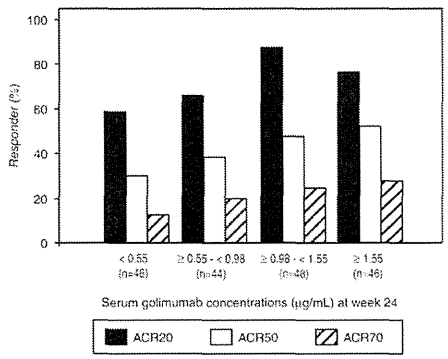

Figure 3 Proportions of patients achieving at least 20%, 50% and 70% improvement in the American College of Rheumatology (ACR20, ACR50, ACR70) response criteria by serum golimumab concentration quartiles (µg/ml) at week 24. The results are from a post hoc analysis of ACR responders in the combined Group 2 (golimumab 50 mg + MTX) and Group 3 (golimumab 100 mg + MTX). MTX, methotrexate.

Radiographic progression

The primary readers exhibited good agreement with regard to vdH-S scores, with ICCs of 0.98 for baseline scores, 0.98 for week 24 scores and 0.80 for the change from baseline to week 24 in vdH-S scores.

Significantly less radiographic progression from baseline to week 24 was observed in patients who received golimumab + MTX (median changes in total vdH-S score of 0.00 (p=0.0009) for combined Groups 2 and 3, 0.00 (p=0.0203) for Group 2 and 0.00 (p=0.0006) for Group 3) versus placebo + MTX (median change 0.25). Treatment group differences in the total vdH-S score were largely attributable to significantly less change in the erosion score with golimumab + MTX therapy. As shown in the cumulative probability plot shown in figure 1 in the online supplement, changes in vdH-S scores were smaller and thus inhibition of radiographic progression was greater in patients treated with golimumab + MTX (Group 2 and Group 3) than in those given placebo + MTX (Group 1).

Significantly greater proportions of patients in combined Groups 2 and 3 (64.7%, p=0.0217) and Group 3 (70.1%, p=0.0066) did not have an increase in the total vdH-S score (ie, change from baseline to week 24 <0) compared with Group 1. The proportions of patients with a change in the total vdH-S score from baseline to week 24 greater than the SDC (3.23) were also significantly lower in combined Groups 2 and 3 (11.0%, p=0.0216) and Group 3 (5.7%, p=0.0023) compared with Group 1 (table 2).

Golimumab pharmacokinetics and antibodies to golimumab

Median serum golimumab concentrations were approximately dose proportional and appeared to have reached steady state by week 14. Median serum golimumab concentrations at weeks 12 and 16 were 0.72 and 0.73 µg/ml, respectively, for Group 2 and 1.28 and 1.16 µg/ml, respectively, for Group 3. These steady state concentrations were maintained at week 24. In Group 2, serum golimumab concentrations in patients who met the EE criteria were approximately 45–82% of those in Group 2 patients who did not meet the EE criteria (data not shown).

In an analysis of week 24 ACR response by week 24 golimumab concentration quartiles, the lowest response rates occurred in patients with serum golimumab concentrations <0.55 µg/ml, followed by concentrations ≥0.55–<0.98 µg/ml (figure 3). No patient developed antibodies to golimumab.

Adverse events

AEs reported at week 16 (fixed treatment regimen study period) and week 24 are summarised in table 3. By week 16, 72.7% (64/88), 75.6% (65/86) and 78.2% (68/87) of patients in Groups 1, 2 and 3, respectively, had AEs. Infections were the most common AEs in Group 1 (35/88, 39.8%), Group 2 (33/86, 38.4%) and Group 3 (29/87, 33.3%) through week 16 and were also the most common AEs at week 24 (table 3).

Serious AEs were relatively uncommon through week 16, occurring in one patient (1.1%) in Group 1 (intervertebral disc protrusion), one patient (1.2%) in Group 2 (ileus) and two patients (2.3%)

Table 3 Summary of safety through weeks 16 and 24 in all randomised patients who received at least one injection of study agent

	Group 1: Placebo+MTX	Group 2: Golimumab 50 mg+MTX		Group 3: Golimumab 100 mg+MTX	Combined Groups 2 and 3	
Week 16						
Number of patients	88	86		87	173	
Patients with AEs	64 (72.7%)	65 (75.6%)		68 (78.2%)	133 (76.9%)	
Patients with SAEs	1 (1.1%)	1 (1.2%)		2 (2.3%)	3 (1.7%)	
Patients with AEs causing study agent d/c	1 (1.1%)	3 (3.5%)		6 (6.9%)	9 (5.2%)	
Patients with infections	35 (39.8%)	33 (38.4%)		29 (33.3%)	62 (35.8%)	
Patients with serious infections	0 (0.0%)	0 (0.0%)		1 (1.1%)	1 (0.6%)	
Patients with injection site reactions*	6 (6.8%)	7 (8.1%)		9 (10.3%)	16 (9.2%)	
Patients with:						
Neoplasia	0 (0.0%)	0 (0.0%)		0 (0.0%)	0 (0.0%)	
Malignancy	0 (0.0%)	0 (0.0%)		0 (0.0%)	0 (0.0%)	
Week 24						
	Group 1: Placebo+MTX	Group 2: Golimumab 50 mg+MTX		Group 3:	Combined	All
	With or without EE Placebo+MTX	With EE Placebo+MTX→ Golimumab 50 mg+MTX	With or without EE Golimumab 50 mg+MTX50 mg→100 mg+MTX	Golimumab 100 mg+MTX	Groups 2 and 3	Golimumab +MTX
Number of patients	88	28	86	87	173	201
Patients with AEs	67 (76.1%)	14 (50.0%)	70 (81.4%)	72 (82.8%)	142 (82.1%)	156 (77.6%)
Patients with SAEs	1 (1.1%)	0 (0.0%)	2 (2.3%)	3 (3.4%)	5 (2.9%)	5 (2.5%)
Patients with AEs leading to d/c of study agent	1 (1.1%)	0 (0.0%)	4 (4.7%)	7 (8.0%)	11 (6.4%)	11 (5.5%)
Patients with infections	39 (44.3%)	4 (14.3%)	36 (41.9%)	34 (39.1%)	70 (40.5%)	74 (36.8%)
Patients with serious infections	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (1.1%)	1 (0.6%)	1 (0.5%)
Patients with injection site reactions*	7 (8.0%)	3 (10.7%)	8 (9.3%)	10 (11.5%)	18 (10.4%)	21 (10.4%)
Patients with:						
Neoplasia	0 (0.0%)	0 (0.0%)	2 (2.3%)†	0 (0.0%)	2 (1.2%)	2 (1.0%)
Malignancy	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)

Data shown are number (%) of patients.
*Injection site reactions were defined as any adverse reaction at a subcutaneous study agent injection site. In the placebo column the reactions are to a placebo injection, in all other columns the reactions are to a golimumab injection.
†The neoplasias included were a non-serious benign breast neoplasm and a serious bone neoplasm determined by histopathological examination to be 'borderline' malignant.
AE, adverse event; d/c, discontinuation; EE, early escape; MTX, methotrexate; SAE, serious adverse event.

in Group 3 (herpes zoster/tendon rupture and aortic dissection). Two additional patients had serious AEs between weeks 16–24, including bone neoplasm (thoracic vertebra tumour (haemangioendothelioma) with 'borderline' or low malignancy potential) in Group 2 and humeral fracture/cruciate ligament injury in Group 3, yielding a total of five (2.5%) patients treated with golimumab + MTX with serious AEs through week 24. No deaths or malignancies were reported.

In addition, by week 16, one (1.1%), three (3.5%) and six (6.9%) patients in Groups 1, 2 and 3, respectively, discontinued the study agent because of an AE. By week 24, 11 (5.5%) of the 201 patients treated with golimumab + MTX had discontinued golimumab due to AEs; these included infection (n=2), skin disorders (n=2), liver function abnormality (n=2), injury (n=2), bone neoplasm (n=1), aortic dissection (n=1), gastrointestinal disorder (n=1) and elevated blood pressure (n=1 in combination with skin disorder).

As noted, infection was the most common system organ class of AEs, occurring in 35 (39.8%), 33 (38.4%) and 29 (33.3%) patients in Groups 1, 2 and 3, respectively, up to week 16. By week 24, 74 (36.8%) patients treated with golimumab + MTX had an infection, most commonly rhinopharyngitis (19.4%, 39/201), gastroenteritis (3.5%, 7/201) and pharyngitis (3.0%, 6/201). No patient developed tuberculosis.

Injection site reactions were reported in six (6.8%), seven (8.1%) and nine (10.3%) patients in Groups 1, 2 and 3, respectively, up to week 16. By week 24, 10.4% (21/201) of all patients treated with golimumab + MTX had an injection site reaction. Erythema at the injection site was the most common of these AEs. All injection site reactions were considered mild and none required cessation of the study agent. No cases of anaphylactic reaction or serum sickness-like reactions were observed.

DISCUSSION

This study evaluated the efficacy of golimumab 50 mg and 100 mg administered subcutaneously every 4 weeks in combination with MTX (6–8 mg/week) versus MTX (6–8 mg/week) monotherapy in Japanese patients with active RA despite MTX therapy. A significantly higher proportion of patients randomised to golimumab 50 mg or 100 mg + MTX (combined Groups 2 and 3) achieved an ACR20 response at week 14 than those receiving MTX monotherapy (73.4% versus 27.3%; p<0.0001). Significantly higher ACR20 response rates were also observed for the individual golimumab dose groups. While the primary endpoint at week 14 did not coincide with trough golimumab concentrations, ACR20 response rates at the time of trough concentrations (week 16) were comparable to those observed at week 14 (ie, 71.7% and 29.5%, respectively, in combined Groups 2 and 3 and Group 1, respectively; data not shown).

These primary endpoint results were consistent with the results of the GO-FORWARD study, a large phase 3 multicentre trial of golimumab encompassing a similar design (primary endpoint at week 14 and treatment change due to EE from week 16 onwards) and a comparable population of patients with RA (approximately 15% of whom were Asian; data on file, Centocor Research & Development) with an inadequate response to MTX.[5] Consistency between our findings and those of the GO-FORWARD study was also observed for improvements in HAQ-DI at week 24.[5]

Significantly less radiographic progression was observed at week 24 with golimumab + MTX than with placebo + MTX, and findings of a post hoc ANOVA analysis of vdH-S scores based on the van der Waerden normal scores were consistent (data not shown). In the GO-FORWARD study, however, minimal radiographic progression was observed in all treatment groups during the same time period, yielding no significant differences between golimumab + MTX and placebo + MTX.[5 16] Minimal radiographic progression was probably related to minimal baseline active inflammation (median CRP 0.8–1.0 mg/dl).[5 16] In a separate study of golimumab, MTX-naïve patients with RA had higher baseline CRP levels (median 1.3–1.4 mg/dl), greater radiographic progression than in the GO-FORWARD study despite less baseline radiographic damage and significantly less radiographic progression at week 28 with golimumab + MTX versus placebo + MTX.[5 16] Thus, CRP is likely to be a more important predictor of radiographic progression than the baseline radiographic score since radiographic progression is less likely if there is no active inflammation, regardless of the amount of baseline radiographic damage.[16] The CRP concentration has also been shown to predict ACR20 response.[17] In this context, the participants in the current study had an intermediate amount of active inflammation at baseline (median CRP 0.9–1.3 mg/dl) and also demonstrated significantly less radiographic progression at week 24 with golimumab + MTX compared with placebo + MTX. In evaluating the radiographic data, it is important to note that the statistically significant differences between the groups are driven by a subset of patients who progress more rapidly than the overall population, and it is in those patients that the treatment effect becomes clinically relevant.

Of note, the MTX dose used in this trial, while consistent with that approved in Japan at the time the trial was planned, was suboptimal (6–8 mg/week) in the context of customary doses elsewhere[18] and as used in the GO-FORWARD study (15–25 mg/week).[16] Evaluation of the efficacy and safety of MTX doses >8 mg/week in Japanese patients with RA has yielded a favourable benefit/risk profile[19] and approved dosing is now extended to up to 16 mg/week. It would therefore be prudent to reassess the responses to golimumab as approved MTX doses in Japan are harmonised with those approved in North America and Europe for RA. These suboptimal MTX doses may explain the higher ACR20 response rates observed in the current golimumab trial (~70%) compared with previously conducted trials of golimumab in RA (~60%) in which more robust ongoing MTX treatment regimens (10–15 mg/week) could have resulted in less room for improvement from baseline.[4 5] It is noteworthy that, when assessing response according to the more stringent ACR50 and ACR70 response criteria, the background MTX dose does not appear to affect the clinical response.[4 5] Similar reasoning may be applied to explain the highly significant difference in radiographic progression observed between placebo + MTX and golimumab + MTX despite only an intermediary level of baseline inflammation compared with previously conducted trials of golimumab.[4 5 16] Finally, more patients met the EE criteria in the golimumab 50 mg + MTX group (Group 2) than in the golimumab 100 mg + MTX group (Group 3), indicating the potential for a dose response.

In interpreting the efficacy findings of this study, it is important to bear in mind that patients could enter this study based on measures of disease activity generally considered to be subjective in nature (ie, tender and swollen joint counts and morning stiffness) or reported from each trial site (ESR) without confirmation by centrally determined parameters such as CRP or erosions. This could have resulted in study enrolment of patients with relatively inactive disease.

Golimumab was generally well tolerated with no unexpected safety issues observed in Japanese patients with RA. By week 24, approximately 10% of all patients treated with golimumab + MTX had an injection site reaction. A variety of dermatological adverse effects, including injection site reactions and dermatitis, have been reported for TNF antagonists such as adalimumab, etanercept and infliximab,[20] as well as for anakinra, a recombinant human form of interleukin-1 receptor antagonist.[21] These dermatological complications typically are well-tolerated, respond to antihistamines and do not necessitate treatment discontinuation.

The incidences of serious AEs, serious infections and malignancies during the fixed treatment regimen period were low and similar with placebo + MTX (1.1%, 0.0% and 0.0%, respectively) and combined golimumab + MTX (1.7%, 0.6% and 0.0%, respectively). These findings indicate a safety profile similar to placebo + MTX (2.3%, 0.8% and 0.0%, respectively) and golimumab + MTX (7.3%, 3.9% and 1.1%, respectively) at week 16 in the GO-FORWARD study.[5] However, these safety findings must be interpreted with caution given the relatively small number of patients evaluated, the lack of power to detect treatment group differences in individual safety events and the relatively short follow-up period. No patients died and no cases of tuberculosis were documented during the 24-week study period.

Taken together, the efficacy and safety findings presented here indicate that golimumab 50 mg + MTX and golimumab 100 mg + MTX were at least as safe and effective in these Japanese patients with active RA despite MTX therapy as they were observed to be when administered to patients with RA who also had an inadequate response to MTX in the GO-FORWARD study.[5]

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ORIGINAL ARTICLE

Efficacy of weekly mizoribine pulse therapy in refractory lupus nephritis

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Abstract
Objective We investigated the efficacy of a high-dose intermittent dosing treatment method (weekly mizoribine pulse therapy) conceived in the hope of achieving better efficacy by increasing the peak blood levels of mizoribine in patients with refractory lupus nephritis.
Methods Seventeen patients with lupus nephritis who had been resistant to corticosteroid and immunosuppressant therapy received weekly mizoribine pulse therapy. Mizoribine (350 mg) was administered three times at 12 h intervals over 2 consecutive days (700 mg for day 1 and 350 mg for day 2), followed by a washout period from day 3 to day 7.
Results This therapeutic strategy enabled the peak blood levels of mizoribine to be increased to more than 3 μg/mL in most of the patients. Although SLEDAI, anti-ds-DNA antibody titer, CH-50, and serum albumin level did not significantly improve, urinary protein levels decreased, and it was possible to taper the dose of concomitant steroids. Using our definition of clinical response, 10 of the 17 patients were responders and 4 of them were nonresponders. The average peak serum mizoribine concentration of the responders was as high as 3.5 μg/mL. Elevation of serum liver enzymes was seen in 1 patient, and hyperuricemia occurred in 4 cases, but none of these adverse events were serious.
Conclusion Intermittent administration of mizoribine can increase blood levels and may be effective for refractory lupus nephritis.

Keywords Lupus nephritis · Mizoribine

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Introduction

Despite recent improvements in therapeutic outcomes for lupus nephritis, a form of nephropathy associated with systemic lupus erythematosus (SLE), the prognosis remains unsatisfactory. Lupus nephritis is primarily treated with corticosteroids, but treatment with corticosteroids alone is often insufficient [1]. In some cases, corticosteroids cannot be used at sufficient doses because of adverse reactions (such as diabetes mellitus, osteoporosis, and avascular necrosis of the femoral head). Therefore, a variety of immunosuppressants are used [2–4], but there have been problems with adverse reactions such as suppression of gonadal function, hemorrhagic cystitis, and renal impairment.

Mizoribine is an immunosuppressant [5] that inhibits inosine monophosphate dehydrogenase (IMPDH) in the same manner as mycophenolate mofetil (MMF) [6, 7]. In Japan, mizoribine has been approved for renal transplant rejection reactions and lupus nephritis, rheumatoid arthritis and nephrotic syndrome. However, in the clinical setting, mizoribine is often ineffective at the approved dose rate (50 mg three times a day) for lupus nephritis and rheumatoid arthritis.

Recently, mizoribine doses have been increased in the hope of achieving higher efficacy, and doses of 6 mg/kg or more per day have sometimes been used in transplant patients [8]. It is therefore possible that dosage and administration have not been adequately studied in the area of collagen vascular diseases.

Here, we investigated the efficacy of a high-dose intermittent dosing treatment method (weekly mizoribine pulse therapy) conceived in the hope of achieving better efficacy by increasing the peak blood levels (C_{max}) of mizoribine in patients with refractory lupus nephritis, with reference to the use of methotrexate (MTX) in the treatment of rheumatoid arthritis.

Materials and methods

Patients

Seventeen patients with refractory lupus nephritis who met the revised classification criteria for SLE (1997) during their whole disease courses were enrolled in this study. All subjects had been resistant to conventional treatment with corticosteroids with or without immunosuppressants at the Department of Rheumatology and Clinical Immunology, Saitama Medical Center, Saitama Medical University, over a 7-year period from May 2003 to April 2009. Patients had been treated for at least 6 consecutive weeks with prednisolone (PSL) ≥30 mg/day with little or no response, or needed to reduce the dose of PSL because of adverse reactions or complications. The following patients were excluded: patients with a creatinine clearance ≤40 mL/min or serum creatinine ≥3.0 mg/dL; patients who were nursing, pregnant, or trying to become pregnant; patients with serious complications such as bone marrow suppression, infections, or peptic ulcers; and patients with hypersensitivity to mizoribine. This study was initiated with the approval of the Ethics Board of Saitama Medical University (approval no. 117/117-II). Written informed consent was obtained in all patients.

Weekly mizoribine pulse therapy

Seven tablets (350 mg) of mizoribine were administered three times at 12 h intervals over 2 consecutive days (700 mg for day 1 and 350 mg for day 2), followed by a washout period from day 3 to day 7. The weekly dose was same as the approved dose (1,050 mg/week).

Clinical assessment was done at 24 months, and the results were compared to the baseline data based on the SLE disease activity index (SLEDAI [9]), serum biochemical analysis, urinalysis, and PSL dose. Missing clinical data were substituted using the last observation carried forward (LOCF) method and employed for statistical analysis (paired t test).

Patients who fulfilled at least one of the following two criteria were considered to be responders: (1) SLEDAI score decreased without increasing the corticosteroid dose; (2) the daily dose of corticosteroid decreased without any elevation in the SLEDAI score. Similarly, nonresponders were defined as patients whose SLEDAI scores and corticosteroid doses were unchanged from the baseline, patients whose SLEDAI scores increased without tapering the dose of corticosteroid, and patients who received an increased corticosteroid dose.

The types of adverse reactions and the actions taken were documented.

To measure the mizoribine blood levels, blood samples were taken 5 times during the treatment course in 10 patients; 2 h after the first dose, immediately before the second dose, and 2 h after the second dose on day 1, and immediately before the third dose and 2 h after the third dose on day 2. Serum samples separated from blood were measured by HPLC at Asahi Kasei Pharma [10].

Results

Demographic features of the 17 patients were as follows: male:female ratio was 4:13; mean age was 33.3 ± 12.2 years; mean duration of illness was 8.2 ± 6.6 years. Renal biopsy revealed type II in 2 cases, type II/III in 1 case, type III/IV in 1 case, type IV in 10 cases, and type V in 2 cases. One case was not tested. Previously used immunosuppressants were cyclophosphamide in 5 cases, cyclosporine in 6 cases, tacrolimus in 5 cases, and azathioprine in 5 cases. At the start of treatment with mizoribine, all of the subjects had been using PSL with a mean dose of 27 ± 17 mg/day (Table 1).

Blood level profiles are shown for the 10 cases in which blood levels were measured over time during the weekly mizoribine pulse therapy (Fig. 1). Mean mizoribine concentrations were 2.4 μg/mL at 2 h after the first dose, 3.3 μg/mL at 2 h after the second dose, and 3.0 μg/mL at 2 h after the third dose.

Changes in clinical parameters at baseline and after 3, 12, and 24 months in subjects undergoing mizoribine pulse therapy are shown in Table 2. Missing clinical data were substituted using the LOCF method and used for statistical analysis (paired t test). The mean SLEDAI score was significantly decreased at 12 months but not at 24 months. Although anti-ds-DNA antibody titer and CH50 did not improve, both urinary protein and the dose of PSL decreased significantly at 24 months. These results suggest

Table 1 Patient demographics

Case	Sex	Age (years)	Duration of illness (years)	Renal biopsy (WHO)	Prior treatment	Mizoribine start date	PSL dose (mg/day)	Concomitant immunosuppressant	Mizoribine treatment time (months)	Mizoribine pulse therapy stopped/continued (reason)
1	F	38	24	V	AZ, POCY	2003.5.12	30	–	80	Continued
2	F	45	4	II/III	–	2005.12.13	30	2009.7.4 ~ CsA	48	Continued
3	F	20	3	IV	CsA, FK-506, AZ, IVCY	2006.10.24	30	–	4	Stopped (liver disorder)
4	F	38	3	III/IV	–	2006.12.11	30	–	36	Continued
5	F	42	8	IV	IVCY, POCY + FK-506	2007.1.15	10	–	22	Continued
6	F	42	8	Not tested	CsA, FK-506	2007.2.9	20	–	4	Stopped (no response)
7	M	25	4	IV	CsA, AZ	2007.5.23	60	–	32	Continued
8	M	29	9	IV	mPSL pulse	2007.6.4	5	2008.5.1 ~ FK-506	30	Continued
9	F	22	5	II	CsA	2007.7.7	10	–	28	Continued
10	F	40	10	IV	CsA, FK-506	2007.2.9	10	–	10	Stopped (no response)
11	F	25	6	V	FK, CsA	2008.7.25	10	2009.5.2 ~ FK-506	16	Stopped (no response)
12	F	56	2	IV	PSL	2008.4.2	40	FK-506	1	Stopped (death by SAH)
13	M	56	11	IV	AZ, POCY	2008.6.30	17.5	–	18	Continued
14	M	21	10	IV	MMF, IVCY, AZ, mPSL pulse	2008.7.4	30	–	9	Stopped (no response)
15	F	23	9	II	AZ, FK-506	2008.9.11	30	2009.9.15 ~ FK-506	15	Continued
16	F	18	1	IV	–	2008.9.29	60	2009.10.7 ~ FK-506	18	Stopped (no response)
17	F	27	24	IV	AZ, FK-506, PE	2009.3.26	50	–	9	Continued

Patient demographics are shown for patients undergoing weekly mizoribine pulse therapy

mPSL methyl prednisolone, *PSL* prednisolone, *CY* cyclophosphamide, *AZ* azathioprine, *CsA* cyclosporine A, *FK-506* tacrolimus, *PE* plasma exchange

the efficacy of concomitant mizoribine pulse therapy. Serum albumin levels increased after 24 months of treatment, but this was not a statistically significant increase.

According to the criteria mentioned above, there were 10 responders and 4 nonresponders (Table 3). Three patients (case nos. 2, 7, and 10) whose SLEDAI scores elevated after successfully reducing the corticosteroid dose could not be classified as either responders or nonresponders. The average peak mizoribine level of the 7 responders was as high as 3.54 μg/mL. One of the 17 subjects undergoing mizoribine pulse therapy experienced liver disorder, resulting in the discontinuation of therapy. Hyperuricemia occurred in four cases. Concomitant treatment with allopurinol was necessary in two of these cases, and this resulted in improvement. Serum creatinine (Cr) levels did not increase in all patients.

Discussion

Mizoribine, a competitive inhibitor of IMPDH, inhibits de novo purine synthesis and reduces the pools of guanine nucleotides, leading to the inhibition of lymphocyte proliferation, like MMF [5]. Mizoribine has been approved for lupus nephritis at a dose of 50 mg three times a day, and enjoys broad clinical use. However, it has been noted clinically that its efficacy is inadequate.

In a placebo-controlled trial on mizoribine in lupus nephritis [11], 6 of the 23 subjects (26.1 %) were rated as "improved or better," although not significantly so, in comparison with the placebo group. Laboratory test results also showed a slight, but not significant, improvement in urinary protein. The incidence of adverse reactions was about the same as for the placebo group. Kuroda et al. [12]

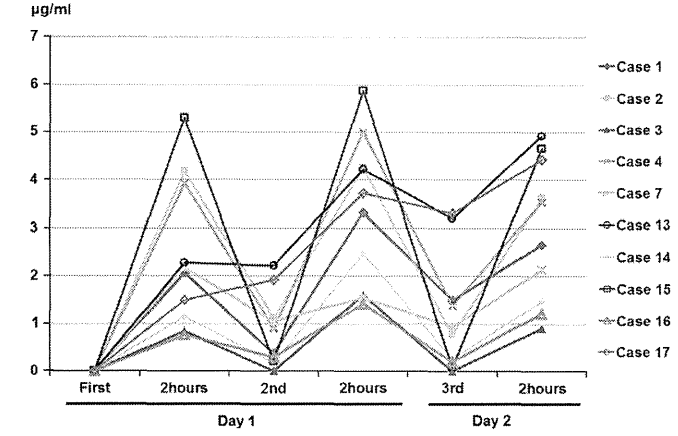

Fig. 1 Blood mizoribine levels in 10 patients Seven tablets (350 mg) of mizoribine were administered three times at 12 h intervals over two consecutive days (700 mg for day 1 and 350 mg for day 2), followed by a washout period from day 3 to day 7. To measure mizoribine blood levels, samples were taken 5 times during the treatment course in 10 patients: 2 h after the first dose, immediately before the second dose and 2 h after the second dose on day 1, and immediately before the third dose and 2 h after the third dose on day 2

Table 2 Clinical assessment of weekly mizoribine pulse therapy

	Baseline (n = 17)	3 months	12 months	24 months
SLEDAI	13 ± 6	10 ± 7	10 ± 7*	10 ± 6
Serum Alb (g/dL)	3.1 ± 0.8	3.4 ± 0.8	3.4 ± 0.9	3.5 ± 0.9
Serum Cr (mg/dL)	1.0 ± 0.5	0.9 ± 0.5	1.2 ± 0.7	1.2 ± 0.7
CH50 (U/mL)	35.4 ± 13.5	37.4 ± 17.4	33.9 ± 18.4	39.7 ± 18.3
Anti-ds-DNA antibody titer (IU/mL)	99 ± 170	89 ± 221	23 ± 27	22 ± 25
Urinary protein (mg/dL)	194 ± 208	157 ± 157	118 ± 108	114 ± 106*
PSL dose (mg/day)	27 ± 17	16 ± 9**	18 ± 12	15 ± 12*

Mean ± SD

CH50 (U/mL), normal = 30–45 U/mL; anti-ds-DNA antibody titer (IU/mL), normal = 0–20 IU/mL

SLEDAI SLE disease activity index

* $p < 0.05$, baseline vs. 24 months; ** $p < 0.01$, baseline vs. 3 months

then studied the association between blood levels and efficacy in patients with lupus nephritis, and showed that the effective range in normal usage is ≥0.66 μg/mL. We therefore suggested that a dose sufficient to enhance efficacy was not established during the development of mizoribine, and it therefore appears that the dose should have been planned accounting for blood levels.

The doses at which mizoribine is used are too low in comparison to MMF. A clinical study was recently conducted in renal transplant patients that used MMF doses as a reference, and high doses of 6–12 mg/kg were shown to be as effective as MMF [8], showing that the effects of mizoribine are related to blood levels. The dose relationship of mizoribine to immunocompetent cells has been studied using various methods, and it has been shown that concentrations of 1–5 μg/mL result in 50 % inhibition of nucleic acid synthesis in human lymphocytes [13–15]. Mizoribine has also been reported to bind to 14-3-3 proteins, enhancing glucocorticoid receptor transcriptional activity, but this effect was significantly enhanced at mizoribine concentrations of ≥10 μM (2.6 μg/mL) [16]. In light of these findings, mizoribine should be administered at a concentration of more than 3 μg/mL [17]. A simulation study of mizoribine in pediatric patients with renal disease showed that the peak serum concentration of mizoribine administered orally once a day at doses of between 2.5 and 13.5 mg/kg (mean 6.14 mg/kg) was as high as 6 μg/mL [17]. Another pharmacokinetic study was done in male healthy volunteers, where 12 mg/kg/day (6 mg/kg twice daily) of mizoribine was well tolerated and C_{max} reached

Table 3 Peak mizoribine concentrations of nonresponder and responder patients

Case no.	PSL	SLEDAI	Mizoribine peak level (μg/mL)
Nonresponders			
6	Increased	Decreased	nd
11	Increased	Unchanged	nd
12	Unchanged	Unchanged	nd
14	Unchanged	Unchanged	2.48
Responders			
1	Decreased	Decreased	3.32
3	Decreased	Unchanged	1.61
4	Decreased	Decreased	3.93
5	Unchanged	Decreased	nd
8	Unchanged	Decreased	nd
9	Unchanged	Decreased	nd
13	Decreased	Decreased	4.23
15	Decreased	Decreased	5.88
16	Decreased	Decreased	1.42
17	Decreased	Decreased	4.42
			(3.54 ± 1.73)

4.6 ± 1.6 μg/mL [18]. The therapeutic window of mizoribine for suppressing acute-phase rejection following transplantation has been defined in terms of the trough concentration as 0.5–3.0 μg/mL. This was achieved using a dosing regimen of 6 mg/kg twice daily [18]. Yumura et al. reported on the pharmacokinetics of mizoribine in patients with lupus nephritis. Blood kinetics were compared at three mizoribine doses: 150 mg once a day, 100 mg twice a day, and 50 mg three times a day. At a dose of 150 mg, the mean peak blood level was 1.8 μg/mL. In contrast, the peak blood level at three doses of 50 mg was less than 1 μg/mL [19].

Recently, the efficacy of mizoribine pulse therapy (500 mg twice a week) has been reported in a patient with focal segmental glomerulosclerosis [20]. Good results have also been reported with mizoribine pulse therapy in pediatric lupus nephritis [21, 22]. A peak mizoribine blood level of 3 μg/mL was targeted in these reports as well. The outcomes were similar to those reported here, and the results fully support our approach.

In light of the fact that weekly administration of MTX at 12 h intervals was widely reported to be effective for rheumatoid arthritis, and considering the previous case reports on the effectiveness of high-dose mizoribine therapies for lupus nephritis, we developed weekly mizoribine pulse therapy, in which the weekly dose would be given over 2 days in order to get sufficient or higher blood levels of mizoribine. Seventeen patients with active refractory lupus nephritis who had been resistant to corticosteroids and immunosuppressants were enrolled in this study. The high doses of mizoribine administered, 350 mg at a time (maximum daily dose of 700 mg), led to peak blood levels of ≥3 μg/mL. Although clinical markers for lupus activity such as SLEDAI, anti-dsDNA titer, and serum complement level did not change, corticosteroid doses were significantly reduced. Ten of the 17 subjects were considered responders with peak blood levels of mizoribine as high as 3.5 μg/mL, which also supports the idea that peak blood levels of mizoribine are an important factor in the efficacious treatment of lupus nephritis. Although the number of patients analyzed was very small, peak mizoribine concentration may be related to clinical response. Some subjects were still on mizoribine monotherapy for more than 2 years after being weaned from corticosteroids (Fig. 2). No serious adverse reactions were observed in our 17 patients. However, mild liver dysfunction occurred in 1 case, and hyperuricemia occurred in 4 cases.

A number of immunosuppressants and a wide range of treatment options that can be used for lupus nephritis are currently available in Japan. Tacrolimus is used to treat refractory lupus nephritis, but is not always effective [23]. Multi-target therapy has recently been proposed, and the results of combination treatment with MMF and tacrolimus have been published [24]. Based on that research, combination therapy with mizoribine and tacrolimus has been studied in Japan, with good results [25]. Such a combination therapy may be a good treatment option for remission induction therapy and lead to better outcomes for lupus nephritis.

Mizoribine is a IMPDH inhibitor that causes the inhibition of purine synthesis by lymphocytes. As shown by the efficacies of various immunosuppressive therapies, such as cyclophosphamide and MMF, it is necessary to suppress lymphocyte function to control lupus nephritis. In this study, immunological markers such as anti-dsDNA titer and serum complement level did not improve significantly. This may be because they were already suppressed by a previous long-term immunosuppressive treatment, including moderate to high dose steroid treatment. Our weekly mizoribine pulse therapy yielded a C_{max} of mizoribine of 3 μg/mL or more, which the approved dose regimen (50 mg three times a day) cannot reach, leading to the suppression of lymphocyte function and enabling us to reduce the dose of concomitant corticosteroid administered to control lupus activity. In this therapeutic strategy, serum mizoribine levels are almost zero from day 4 to day 7. Given that the clinical efficacy of intermittent mizoribine pulse therapy has been demonstrated in previous reports [20, 21] as well as in our 10 responders, it appears to be important to obtain a sufficiently high peak serum level (more than 3 μg/mL), even only transiently.

Concomitant immunosuppressive agents should be used to reduce the risk of adverse effects of long-term steroid

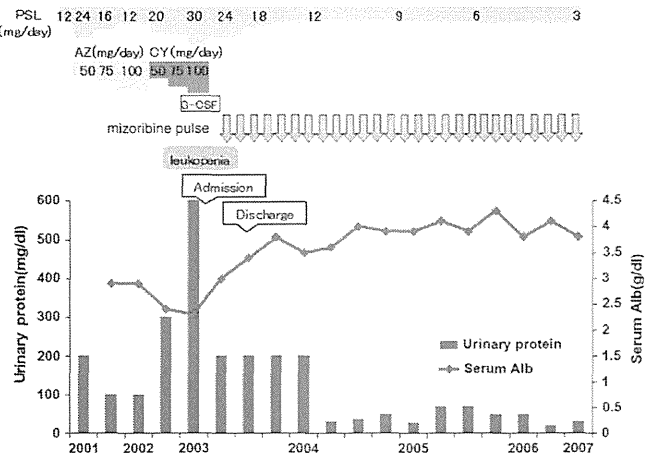

Fig. 2 Clinical course of a representative case (case 1)

treatment in the management of collagen vascular diseases. This mizoribine pulse therapy seems to be effective, and should be attempted instead of the approved mizoribine therapy for refractory lupus nephritis. A prospective comparative study between mizoribine pulse therapy and the standard approved therapy should also be performed to confirm the efficacy of this therapeutic strategy.

Conflict of interest None.

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A merged presentation of clinical and radiographic data using probability plots in a clinical trial, the JESMR study

In terms of the relationship between synovial inflammation and radiographic changes, including both joint damage repair and progression,[1] in rheumatoid arthritis (RA), pre-existing joint damage and persistent synovitis may promote joint destruction, while in the absence of synovitis, damaged joints may heal.[2 3] Although presentation of radiographic results using cumulative probability plots has substantially improved understanding of clinical trial data,[4] the effects of treatments on radiographic progression and improvement (regression) in individual RA patients has not yet been fully explained.

In the JESMR study,[5 6] 151 active RA patients unresponsive to treatment with methotrexate (MTX) were randomised into 1 of 2 treatment groups: etanercept (ETN) 50 mg/week with 6–8 mg/week of MTX (the E+M group), or ETN alone (the E group). Radiographs of the hands and feet before ETN (baseline) and during the first year of treatment were available from 53 (72%) and 68 (88%) patients in the E and E+M groups, respectively. Baseline characteristics of patients were comparable between those with and without available radiographic data in each treatment group (data not shown). However, most patients without data did not complete the study up to Week 52 as per protocol, chiefly due to lack of efficacy in the E group.[6] The mean baseline total Sharp-van der Heijde score (TSS)[7] was 114.5 in the E group and 113.1 in the E+M group (disease duration: 10.0 years and 8.4 years, respectively), and the smallest detectable change (SDC) in TSS over 52 weeks was 1.9.

Cumulative probability plots provided by the American College of Rheumatology (ACR)-N[8] clearly demonstrated a superior response (figure 1A,B) and a significantly greater ACR50 response rate in the E+M group at week 52 (76.5% vs 50.9%, p=0.0041, Fisher's exact test). Merged probability plots of individual radiographic change over 52 weeks (ΔTSS) suggested preferential existence of aggressive radiographic progressors among ACR50 non-responders in the E group. The relationship among treatment, clinical disease activity, and radiographic change was further addressed using time-averaged disease activity score of 28 joints (DAS28) over 52 weeks in place of ACR-N at Week 52 (figure 1C,D). Significant correlation between time-averaged DAS28 and ΔTSS was observed in the E (r^2=0.097, p=0.023) but not the E+M group (r^2=0.019, p=0.26). Aggressive radiographic progression was preferentially observed among patients with moderate or high activity on average in the E group (figure 1C), while in the E+M group, radiographic progression among these patients seemed to be balanced by radiographic regression among those in remission or with low disease activity (figures 1D–F).

The absence of radiographic regressors (>SDC) among clinical responders in the E group (figure 1A,C,E) was surprising, although 18.2% of those patients showed regression within the SDC. This may be partly explained by the limitations of the study due to the small number of patients involved. Another limitation was much lower MTX dose at study enrolment than the current global standard dosage: 7.0±1.4 (the mean±SD) and 7.4±1.1 in the E and E+M groups, respectively.

In summary, we first demonstrated the relationship between individual clinical responses and radiographic changes by merging cumulative probability plots of ACR-N or time-averaged DAS28 and ΔTSS. These presentations clearly show the relationships between two parameters as a whole, facilitating further post hoc analyses of clinical trials. Further, merged presentation of probability plots is useful in comparing a single parameter (eg, health assessment questionnaire-disability index: HAQ-DI) before and after treatments (figure 2). However, merged presentation of probability plots must be followed by statistical analyses after being classified into binary or ternary categories, as we showed here.

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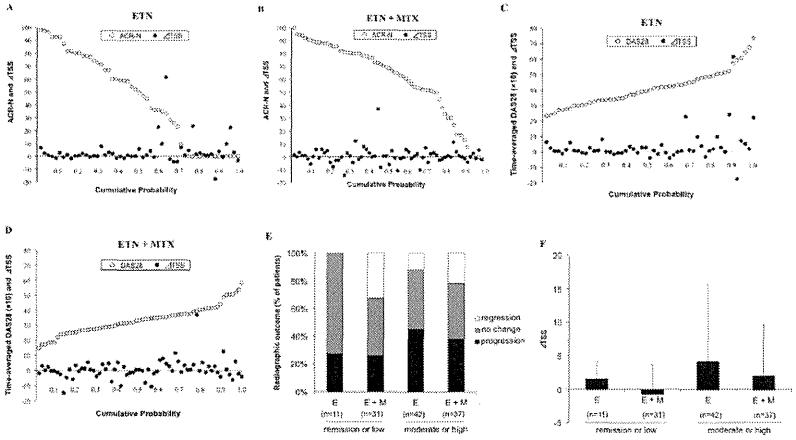

Figure 1 Cumulative probability plot analysis of ACR-N (A,B) or time-averaged DAS28 (C,D) and radiographic changes in the E (A,C) and E+M groups (B,D), merged to keep same patients on the vertical line, followed by the radiographic outcomes (E) and changes (F) stratified by the treatment and time-averaged disease activity state. Time-averaged DAS28 was calculated by the area under the curve of DAS28 at weeks 0, 2, 4, 8, 12, 24 and 52, divided by 52. No significant differences were observed between groups using Pearson's test (E) and Kruskal-Wallis test (F). ACR, American College of Rheumatology; DAS28, disease activity score of 28 joints; ETN, etanercept; MTX, methotrexate; TSS, total Sharp-van der Heijde score.

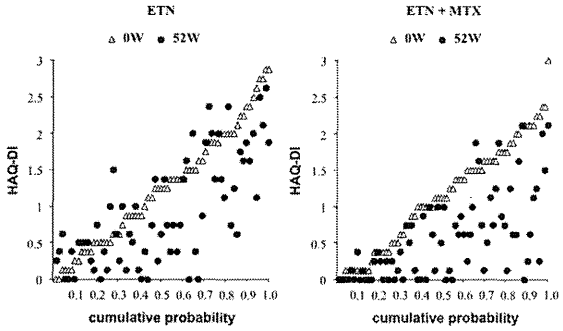

Figure 2 Merged probability plots of individual health assessment questionnaire-disability index (HAQ-DI) scores at baseline (open triangle) and Week 52 (closed circle) in the E (left) and E+M groups (right). Subsequent analyses included comparison of the rate of HAQ-DI≤0.5 at 52 weeks in patients with baseline HAQ-DI>1.5. None of 15 patients (0.0%) in the E group and 6 of 23 patients (26.1%) in the E+M group, respectively; p=0.037 by Fisher's exact test (one-sided). ETN, etanercept; MTX, methotrexate.

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ORIGINAL ARTICLE

Pulmonary infections following immunosuppressive treatments during hospitalization worsen the short-term vital prognosis for patients with connective tissue disease-associated interstitial pneumonia

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Abstract

Objective. Connective tissue disease-associated interstitial pneumonia (CTD-IP) significantly affects the mortality of patients with CTD. The purpose of the present study is to identify causes and risk factors for death during hospitalization for immunosuppressive treatment of CTD-IP.

Methods. A multicenter, retrospective study was conducted that collected data from patients with CTD who had been hospitalized for commencing or intensifying immunosuppressive treatment of CTD-IP using a standardized case report form. Risk factors were identified using the Cox proportional hazard regression model.

Results. A total of 322 CTD-IP patients were enrolled with rheumatoid arthritis ($n = 84$), systemic lupus erythematosus ($n = 13$), polymyositis ($n = 33$), dermatomyositis ($n = 69$), systemic sclerosis ($n = 55$), mixed connective tissue disease ($n = 21$), microscopic polyangiitis ($n = 19$), and overlap syndrome ($n = 28$). Of the 42patients who died during hospitalization, 22 died from CTD-IP, 15 from CTD-IP and pulmonary infection, 2 from pulmonary infection, and 3 from other causes. Age ≥ 65 years and development of pulmonary infections after commencing or intensifying immunosuppressive treatments were identified as risk factors for death during hospitalization after adjusting for covariates.

Conclusion. Careful consideration of the benefit–risk balance of immunosuppressive treatment for CTD-IP is indispensable for improving the short-term vital prognosis of these patients.

Keywords

Connective tissue disease, Interstitial pneumonia, Immunosuppressive treatments, Pulmonary infections, Vital prognosis

History

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Introduction

Among the varieties of lung involvements in patients with connective tissue diseases (CTD), CTD-associated interstitial pneumonia (CTD-IP) is prevalent and has considerable influence on morbidity and mortality [1]. In clinical practice, CTD-IP is frequently observed in patients with rheumatoid arthritis (RA), polymyositis (PM)/dermatomyositis (DM), and systemic sclerosis (SSc). The prevalence of clinically definitive CTD-IP in RA, PM/DM, and SSc has been reported to be 7–14% [2], 5–46% [3], and 40–80% [1]; and 5-year survival rates were 40–90% [2,4–6], 50–87% [7,8], and 80–90% [9,10], respectively. Some studies even show that CTD-IP has a more unfavorable prognosis than idiopathic interstitial pneumonia when adjusted for age and gender [11,12].

Patients with active CTD-IP often receive treatments with corticosteroids with or without other immunosuppressants. The efficacy of immunosuppressive treatments depends on the type of CTD, imaging pattern or pathological classification of CTD-IP, residual pulmonary function, and disease activity of CTD-IP [5,10,12–16]. Patients with CTD-IP sometimes develop life-threatening pulmonary complications, such as severe pulmonary infections [17–20] and mediastinal emphysema [21] during immunosuppressive treatment. To improve long-term survival of patients with CTD-IP, achieving better short-term survival is indispensable after the initial or remission induction treatment of CTD-IP. Few studies have reported short-term survival rates of patients with CTD-IP after commencing or intensifying immunosuppressive treatments [22–24], and little is known about the risk factors associated with death during treatment.

The present study reports the results of a multicenter, retrospective study of patients with CTD-IP who required hospitalization for immunosuppressive treatment. The purpose of this study was to identify causes and risk factors of death during hospitalization of CTD-IP patients with an emphasis on pulmonary infections occurring after commencing or intensifying immunosuppressive treatments.

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Materials and methods

Patients

Ten university hospitals and one national hospital participated in this study. The retrospective cohort of this study consisted of patients with RA, systemic lupus erythematosus (SLE), PM, DM, SSc, mixed connective tissue disease (MCTD), microscopic polyangiitis (MPA), or overlap syndrome who required hospitalization for treatment of CTD-IP between April 2004 and March 2007. All participating hospitals searched their admission logs and enrolled virtually all patients eligible for this study. The diagnoses of CTDs were made by the attending rheumatologists with reference to the classification or diagnostic criteria of these diseases [25–30]. When one patient concurrently had two or more of the above-mentioned CTDs, the patient was classified as having overlap syndrome. The diagnosis of CTD-IP was determined by the attending physicians and investigators in the participating hospitals based on clinical manifestations, images on chest X-ray and thoracic computed tomography (CT), and laboratory tests, and confirmed by M.T. using medical records for each patient.

Collection of clinical data

Clinical data were systematically extracted for each patient using a standardized case report form and included age, gender, disease duration in months for each CTD, clinical characteristics of CTD-IP (i.e., new-onset or recurrent and presence or absence of mediastinal emphysema), details of treatment for CTD-IP after admission [i.e., maximum prednisolone (PSL)-equivalent daily dosage of oral corticosteroid, use of methylprednisolone pulse (mPSL pulse) therapy, and use of immunosuppressants], pulmonary infections after commencing or intensifying immunosuppressive therapy for CTD-IP, and the status of the patient with CTD-IP at discharge by the attending physician's global assessment (improved, unchanged, deteriorated, or death). These data were based on medical records obtained during hospitalization and outpatient visits after discharge. Causes of death were determined by two board-certified rheumatologists (M.T. and M.H.) and a board-certified specialist of infectious diseases (R.K.) based on medical records during hospitalization and the outpatient clinic. The start date of the observation period was the date immunosuppressive treatment for CTD-IP was commenced or intensified after hospitalization. Observation was stopped either on the date of death, loss-to-follow-up, or on March 30, 2007, whichever came first.

Statistical analysis

For group comparisons involving categorical variables, the chi-square or Fisher's exact test was used. Continuous variables were compared using the Mann–Whitney U test. To identify risk factors for death during hospitalization, the multivariate Cox proportional hazards regression model was used with the forced entry procedure. In addition, we used Benjamini and Hochberg (BH) method [31] to correct for multiple comparisons. BH method is one of the approaches to multiple comparison problems by controlling the false discovery rate (FDR). All analyses were performed using SPSS software, version 17.0 (SPSS Japan, Tokyo, Japan).

Ethics

This study was approved by the ethics committees of the Tokyo Medical and Dental University Hospital and other participating hospitals. The guidelines of the Helsinki Declaration and the ethics guidelines for epidemiologic research in Japan were followed. The ethics guideline for epidemiological research in Japan requires notifying eligible patients of the study and allows implementation of that study without obtaining individual written informed consent. This study was publicized by leaflets or posters in outpatient clinics of each participating hospital. Patients were excluded from the study if they expressed unwillingness to participate.

Results

Clinical characteristics of patients with CTD-IP

We enrolled 322 patients who were hospitalized for treatment of CTD-IP between April 2004 and March 2007. The numbers of cases with each CTD were 84 RA (26.1%), 13 SLE (4%), 33 PM (10.2%), 69 DM (21.4%), 55 SSc (17.1%), 21 MCTD (6.5%), 19 MPA (5.9%), and 28 overlap syndrome (8.7%). The median (range) observation and hospitalization periods of the patients were 1.1 (0–3.2) years and 1.8 (0–32.1) months, respectively. Demographic and clinical features of the patients at admission for each CTD-IP are summarized in Table 1. The mean age of patients with MPA was highest and that of patients with SLE lowest. The proportion of female patients with MPA was significantly lower than those for other diseases ($p = 0.001$, chi-square test). Patients with PM, DM, and MPA tended to have shorter disease duration. The rate of newly developed CTD-IP in patients with SLE and DM was significantly higher ($p = 0.002$, chi-square test) and that in RA patients was significantly lower ($p = 0.002$, chi-square test) compared with those with other diseases.

Treatment of CTD-IP

Following admission, immunosuppressive treatments for CTD-IP were commenced or intensified in all patients, using oral corticosteroids, mPSL pulse therapy, intravenous cyclophosphamide therapy (IVCY), and/or other immunosuppressants (Table 1). Patients with RA were more frequently treated with mPSL pulse therapy ($p = 0.001$, chi-square test) and less frequently with IVCY ($p < 0.001$, chi-square test). Patients with SSc were treated less frequently with mPSL pulse therapy ($p < 0.001$, chi-square test) and oral corticosteroids ($p = 0.008$, chi-square test) and more frequently with IVCY ($p < 0.001$, chi-square test). Patients with MCTD were treated less frequently with mPSL pulse therapy ($p < 0.001$, chi-square test). In addition to IVCY, the main immunosuppressants used for CTD-IP were cyclosporine (68/133; 51.1%), tacrolimus (48/133; 36.1%), and azathioprine (10/133; 7.5%).

Prognosis and causes of death of CTD-IP patients

At discharge, 223 cases (69.3%) showed improvement, 54 cases (16.8%) had no change, 3 cases (0.9%) deteriorated according to the

Table 1. Clinical characteristic of patients with CTD-IP.

	Age (years)	Gender (Female)	Disease duration (months)	Newly developed	Treatments for CTD-IP during hospitalization: mPSL pulse	CS	IS	IVCY
RA ($n = 84$)	65.4 ± 9.1	57.1%	123.7 ± 128.7	42.9%‡	49.3%§	93.3%	37.8%	6.8%¶
SLE ($n = 13$)	43.9 ± 16	92.3%	73.6 ± 128.9	84.6%†	23.1%	100%	25%	23.1%
PM ($n = 33$)	56 ± 10	81.8%	26.7 ± 59.7	69.7%	35.5%	96.8%	51.6%	16.1%
DM ($n = 69$)	54.8 ± 12	65.2%	23.6 ± 42.3	72.1%†	35.9%	98.4%	59.4%	22.2%
SSc ($n = 55$)	56 ± 16	58.2%	71.5 ± 98.6	46.3%	14%¶	80.4%¶	43.1%	56.9%**
MCTD ($n = 21$)	54.8 ± 13.4	61.9%	55.7 ± 74.3	57.1%	10%¶	95%	60%	15%
MPA ($n = 19$)	73.2 ± 8.1	42.1%*	18.9 ± 21.3	52.6%	21%	100%	36.8%	15.8%
Overlap ($n = 28$)	52.6 ± 11.7	92.9%	49.9 ± 76.5	64.3%	23.1%	88.5%	42.3%	42.3%

mPSL pulse methylprednisolone pulse therapy, *CS* corticosteroid, *IS* immunosuppressants other than IVCY, *IVCY* intravenous cyclophosphamide, *RA* rheumatoid arthritis, *SLE* systemic lupus erythematosus, *PM* polymyositis, *DM* dermatomyositis, *SSc* systemic sclerosis, *MCTD* mixed connective tissue disease, *MPA* microscopic polyangiitis, *Overlap* overlap syndrome.
Statistical significance was defined as $p < 0.05$ and adjusted residual as absolute value more than 2.00.
*Significantly lower percentage of female ($p = 0.001$; chi-square test).
†Significantly higher percentage of newly developed CTD-IP ($p = 0.002$; chi-square test).
‡Significantly lower percentage of newly developed CTD-IP ($p = 0.002$; chi-square test).
§Significantly higher percentage of concomitant use ($p = 0.001$; chi-square test).
¶Significantly lower percentage of concomitant use ($p = 0.001$; chi-square test).
‖Significantly lower percentage of concomitant use ($p = 0.008$; chi-square test).
**Significantly higher percentage of concomitant use ($p < 0.001$; chi-square test).

attending physicians' global assessment, and 42 cases (13%) died during hospitalization (Table 2). In-hospital mortality rates were significantly higher for RA (20.2%) and DM (21.7%) and lower for SSc and overlap syndrome, compared with those for other diseases ($p < 0.001$, chi-square test). Of the 42 deaths during hospitalization, the causes of death were CTD-IP for 22 cases, CTD-IP and pulmonary infection for 15, pulmonary infection for 2, CTD-IP and pulmonary hypertension for 1, pulmonary hypertension for 1, and pulmonary hemorrhage for 1. Six patients died after discharge from the hospital and before the end of the observation period. The cause of death was unknown in 5 of these cases and was heart failure in 1 case.

Because 17 deaths during hospitalization were totally or partially attributed to pulmonary infection after immunosuppressive treatment for CTD-IP was initiated, according to the attending physician, we examined the prognosis for the 43 cases that developed pulmonary infections. Of these 43 cases, 17 died before discharge, including 7 with DM; 4 with RA; and 2 each for PM, SSc, and MPA. The mortality rate for each CTD-IP ranged from 40 to 67% (Table 2). The types of the pulmonary infection in these 43 cases were mixed pulmonary infection for 13 cases, bacterial pneumonia for 12 cases, *Pneumocystis jirovecii* pneumonia for 6 cases, bronchitis for 3 cases, *P. jirovecii* pneumonia and *Cytomegalovirus* pneumonia for 2 cases, *Cytomegalovirus* pneumonia for 1 case, fungal pneumonia for 1 case, non-tuberculous mycobacterial infection for 1 case, influenza for 1 case, and unknown for 3 cases. Because we did not collect information about prophylaxis, we were unable to examine its association with development of pulmonary infection.

Risk factors for death during hospitalization

The 42 patients who died during hospitalization accounted for 87.5% of all 48 deaths during the observation period of this study,

Table 2. Status of patients with CTD-IP at discharge.

	Status of CTD-IP patients at discharge: Improved	Unchanged	Deteriorated	Deceased	Development of pulmonary infections‡
RA ($n = 84$)	61	6	0	17*	10 (4)
SLE ($n = 13$)	11	1	0	1	2
PM ($n = 33$)	24	4	0	5	3 (2)
DM ($n = 69$)	49	5	0	15*	15 (7)
SSc ($n = 55$)	27	25*	1	2†	4 (2)
MCTD ($n = 21$)	18	1	2	0	3
MPA ($n = 19$)	18	2	0	2	5 (2)
Overlap ($n = 28$)	18	10	0	0†	1
All	223	54	3	42	43 (17)

The status of CTD-IP patients at discharge is summarized according to the attending physicians' global assessment as improved, unchanged, deteriorated, or deceased.
RA rheumatoid arthritis, *SLE* systemic lupus erythematosus, *PM* polymyositis, *DM* dermatomyositis, *SSc* systemic sclerosis, *MCTD* mixed connective tissue disease, *MPA* microscopic polyangiitis, *Overlap* overlap syndrome.
Statistical significance was defined as $p < 0.05$, and adjusted residual as absolute value more than 2.00.
Numbers in parentheses are numbers of deaths during hospitalization.
*Significantly higher percentage ($p < 0.001$; chi-square test).
†Significantly lower percentage ($p < 0.001$; chi-square test).
‡Development of pulmonary infections after new or additional immunosuppressive treatments for CTD-IP.

Table 3. Univariate analyses for death during hospitalization of patients with connective tissue disease-associated interstitial pneumonia.

	Survived cases ($n = 263$)	Deceased cases ($n = 31$)	p value
Characteristics of the patients			
Age (years)*	57 ± 13.8	66.2 ± 11.9	< 0.001†
Age (= or > 65 y/o)	31.9%	64.5%	< 0.001‡
Gender (female)	65%	67.7%	0.76‡
Disease duration of each CTD (months)*	62.7 ± 96.2	66.5 ± 106.3	< 0.81†
Newly developed CTD-IP	58.4%	40%	0.054‡
Development of mediastinal emphysema during hospitalization	5%	10.1%	0.016‡
Development of pulmonary infections during hospitalization	10%	50.0%	< 0.001 =
New or additional treatments for CTD-IP after admission			
Concomitant use of mPSL pulse therapy	26%	80.6%	< 0.001‡
Concomitant use of CS	94.3%	83.9%	0.029‡
Maximum dosage of CS (mg/day of PSL equivalent)*	38.7 ± 18.2	58 ± 43.4	0.008†
Concomitant use of immunosuppressant other than IVCY	48.3%	35.5%	0.17‡
Concomitant use of IVCY	24.1%	22.6%	0.85‡

CTD connective tissue disease, *CTD-IP* connective tissue disease associated interstitial pneumonia, *CS* corticosteroid, *PSL* prednisolone, *IVCY* intravenous cyclophosphamide.
*Mean ± SD. p values were calculated using the Mann–Whitney test (†) or chi-square test (‡).

indicating that clinical management during hospitalization is important to improve short-term vital prognosis of patients with CTD-IP. We, therefore, examined risk factors for death during hospitalization in 294 patients who had detailed information about immunosuppressive treatment for CTD-IP. We compared surviving and deceased cases using univariate analyses (Table 3) and selected variables for the multivariate Cox regression hazard analysis to evaluate the risk factors for death during hospitalization.

Based on the results of univariate analyses (Table 3), we applied age (≥ 65 years old), development of mediastinal emphysema, development of pulmonary infection after commencing or intensifying immunosuppressive treatments, concomitant use of mPSL pulse therapy, and the maximum daily dosage of oral corticosteroids into multivariate Cox proportional hazards regression models by the forced entry procedure. Age (≥ 65 years old; $p = 0.001$), development of pulmonary infection ($p = 0.004$), and concomitant use of mPSL pulse therapy ($p = 0.032$) were identified as significant risk factors for death during hospitalization (Table 4). After corrections for multiple comparisons using FDR and BH methods [31], age (≥ 65 years old) and development of pulmonary infection remained significant. Because we observed a significant association between use of mPSL pulse therapy and maximum daily dosage of oral corticosteroids, we used "mPSL pulse therapy or maximum daily dosage of oral corticosteroids ≥ 40 mg/day" with the other three factors in Table 3 as independent variables and performed a multivariate Cox proportional hazards regression analysis. This second model also identified age (≥ 65 years old) and development of pulmonary infection as significant risk factors (data not shown).

Discussion

This multicenter, large-scale, retrospective analysis of CTD-IP patients in Japan was implemented to determine the short-term vital prognosis and to identify risk factors for death after commencing or intensifying immunosuppressive treatments for CTD-IP. There are three major findings from our study. First, the overall mortality rate of patients with CTD-IP during hospitalization for immunosuppressive treatment for IP was 13% (42/322). Second, CTD-IP patients with RA and DM had higher in-hospital mortality rates following immunosuppressive treatments. Third, advanced age (≥ 65 years old) and development of pulmonary infection were significant risk factors for death during hospitalization after corrections for multiple comparisons.

In clinical practice, patients with CTD-IP often develop a pulmonary infection and sometimes die from this complication. To the best of our knowledge, this is the first study that demonstrates an association with statistical significance between development of pulmonary infections after commencing or intensifying immunosuppressive treatment and death during hospitalization. Several investigators have reported IP as a risk factor for infection or serious infection in patients with CTD [19,32–35]. These data strongly indicate the importance of prophylaxis, monitoring, and early diagnosis of pulmonary infection during immunosuppressive treatment of CTD-IP.

Our study identified older age (≥ 65 years old) as a significant risk factor for death during hospitalization for immunosuppressive treatment of CTD-IP. Kocheril et al. [12] performed a case–control study of patients with CTD-ILD (interstitial lung disease)

Table 4. Multivariate Cox proportional hazards regression analysis for death during hospitalization of patients with CTD-IP.

Risk factors	Hazard ratio	95% CI	p value
Age (≥ 65 years old)	3.98	1.70–9.32	0.001*
Development of pulmonary infections after new or additional immunosuppressive treatments for CTD-IP	3.40	1.49–7.72	0.004*
Concomitant use of mPSL pulse therapy	2.86	1.09–7.50	0.032
Maximum dosage of CS (mg/day of PSL equivalent)‡	1.01	0.996–1.02	0.16
Development of mediastinal emphysema	1.35	0.45–4.06	0.60

95% CI 95% confidence interval, *CTD-IP* connective tissue disease-associated interstitial pneumonia, *CS* corticosteroid, *PSL* prednisolone.
Significant risk factors for death during hospitalization for immunosuppressive treatment of CTD-IP were identified using Cox proportional hazards regression models.
*These p values were statistically significant after corrections for multiple comparisons using FDR and BH methods [31].

and idiopathic interstitial pneumonia and found that the hazard of death increased by 4% per 1-year increment in age at the diagnosis of CTD-ILD. Other studies, however, have failed to find a significant association between age and prognosis of collagen vascular disease-IP (CVD-IP) in patients with PM/DM [36,37] or SSc [38] following treatment for CVD-IP. The association of age with vital prognosis may be altered by other factors, such as types of CTD and treatment provided.

Several studies have investigated the long-term vital prognosis for patients with CTD-IP. Su et al. [9] estimated the survival of patients with CTD-ILD using the Stanford ILD database and reported that 1-year, 3-year, and 5-year survival rates at the last follow-up from diagnosis of ILD were 88%, 61%, and 53%, respectively. This and other studies showed that the probability of survival of patients with CTD-IP greatly decreased during the first and second years after diagnosis and tended to plateau after that [4,11,12]. A study of patients with acute exacerbation of CTD-IP (6 with RA, 6 with DM, and 3 with SSc) found that the 90-day survival rate after hospital treatment for acute exacerbation of CTD-IP was only 33% [39]. These data indicate that patients with CTD-IP have an unfavorable short-term vital prognosis especially after initiation of therapy for CTD-IP. Altogether, these results are compatible with the results of our study.

A number of studies have found that RA patients with CTD-IP have a poor vital prognosis [1,2,4–6]. Hakala [40] analyzed the clinical course of 49 RA patients admitted to their hospital with interstitial lung fibrosis, and reported a poor prognosis, with a median survival of 3.5 years and a 5-year survival rate of 39%. Rajaskekaran et al. [5] reported a similarly poor prognosis for 18 patients with RA-ILD, with a 5-year survival rate of 44%. Park et al. [4] reported that the survival of RA patients with CVD-IP was lower than that for patients with other CVD-IPs. The high in-hospital mortality rate of RA patients with CTD-IP in our study is in agreement with these previous reports of long-term vital prognosis.

The presence of ILD in patients with PM/DM resulted in increased mortality [7,8,13]. Marie et al. [13] reported that survival of PM/DM patients with ILD (PM/DM-ILD) was 94.4%, 90.4%, and 86.5% at years 1, 3, and 5, respectively. Fujisawa et al. [7] compared the prognosis of ILD between patients with PM and DM. They reported that DM patients with ILD had significantly shorter survival rates than PM patients with ILD (5-year survival, 55.6% vs. 87.1%, respectively), and that most of the deaths in patients with DM-ILD were from respiratory failure due to deterioration of ILD. In our study, 15 of 69 DM patients (21.7%) and 5 of 33 PM patients (15.2%) died during hospitalization. The cause of death in patients with DM was CTD-IP for 8 cases, CTD-IP and pulmonary infection for 6, and pulmonary infection for 1. These results support a shorter vital prognosis for CTD-IP in DM compared with that in PM and other CTDs.

There are certain limitations in our study. First, the patients with CTD-IP enrolled in this study were limited to hospitalized patients, who might have a more severe or treatment-resistant CTD-IP than non-hospitalized patients. Those patients with less severe CTD-IP not requiring immunosuppressive treatments with hospitalization were excluded from our study. Second, the observation period of our study was shorter than those of previous reports. However, the probability of survival after treatment with any immunosuppressants in PM/DM [22,37] or SSc [23,24] patients tended to plateau after two years of follow-up. Therefore, careful clinical management during hospitalization would be important not only for short-term, but also for mid- to long-term vital prognosis of patients with CTD-IP. Third, we could not collect previously reported risk factors for an unfavorable prognosis [5,10,12–16], such as chest X-ray, thoracic CT images, and results of pulmonary function tests. Additional risk factors might have been identified if we had collected and applied these data to this study.

In conclusion, proper management of patients with CTD-IP with careful consideration of benefit–risk balance for immunosuppressive treatments is necessary to improve the short-term prognosis of these patients. Because the development of pulmonary infections after the initiation of immunosupression has a substantial influence on the mortality rate of patients with CTD-IP, physicians should pay special attention to evaluation of the risk for the pulmonary infections and consider initiating preventive measures before starting immunosuppressive treatment for CTD-IP.

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Conflict of interest

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REVIEW ARTICLE

Could retinoids be a potential treatment for rheumatic diseases?

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Abstract Retinoid, a derivative of vitamin A, is a general term used to describe compounds that bind to and activate retinoic acid receptors [RARs (RARα, RARβ, and RARγ)] and/or retinoid X receptors [RXRs (RXRα, RXRβ, and RXRγ)]. They have been shown to surpress the differentiation of Th1/Th17 cells and induce the development of Th1/regulatory T cells. They also affect the proliferation of B cells as both an inducer and suppressor. Furthermore, retinoids may induce the maturation of dendritic cells and production of interleukin-10 from monocytes/macrophages. We recently demonstrated that retinoids suppressed the production of reactive oxygen species, the release of elastase from neutrophils by inhibiting mitogen-activated protein kinase signals, and both the migration speed and chemotaxis directionality of neutrophils. Retinoids, such as all-*trans* retinoic acid and tamibarotene, were previously shown to have positive effects on animal models of several rheumatic diseases, including arthritis, myositis, and vasculitis in vivo. Moreover, retinoids have been used in a pilot study to effectively treat patients with lupus nephritis and systemic sclerosis. We herein reviewed the effects of retinoids on immune cells, animal models of rheumatic diseases, and rheumatic patients.

Keywords Retinoid · T cells · B cells · Rheumatoid arthritis · Rheumatic diseases

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Introduction

Biological drugs, such as anti-tumor necrosis factor (TNF) monoclonal antibodies, were recently shown to markedly improve arthritis and inhibit bone destruction associated with rheumatoid arthritis (RA) [1, 2]. However, some patients do no respond to these treatments, and biological agents have been shown to increase the risk of severe infection [3, 4]. Other rheumatic diseases, such as myositis and vasculitis, are treated with prednisolone (PSL) monotherapy or PSL combined with immunosuppressive therapy, which can also increase the risk of infection. Previous studies reported that biological drugs may be effective for vasculitis and myositis [5, 6]; however, these treatments have not yet been established. Therefore, therapies urgently need to be developed that are more effective, cheaper, and safer than conventional treatments.

RA was previously treated with retinoids, but was unsuccessful because of severe adverse events and low efficacy [7, 8]. Mucida et al. demonstrated that retinoids regulated the differentiation of T helper (Th) cells in 2007 [9], which led to a marked increase in the number of studies examining the immunoregulatory effects of retinoids. We previously reported that the synthetic retinoid, Am80, attenuated arthritis, myositis, and vasculitis in the respective murine models [10–12]. In addition to all-*trans* retinoic acid (ATRA), tamibarotene (Am80) was approved for the treatment of acute promyelocytic leukemia (APL) in Japan in 2005. We herein reviewed the immunological function of retinoids, and their potential as therapeutic agents in the treatment of rheumatic diseases.

Retinoids

Retinoid, a derivative of vitamin A, is a general term used to describe compounds that bind to and activate retinoic acid receptors [RARs (RARα, RARβ, and RARγ)] and/or retinoid X receptors [RXRs (RXRα, RXRβ, and RXRγ)], members of the nuclear receptor superfamily [13]. RARs and RXRs are transcriptional regulators that bind to specific retinoic acid response elements present in the promoters of their target genes. Retinoids are critically involved in embryonic development, organogenesis, tissue homeostasis, cell proliferation, differentiation, and apoptosis [13]. A previous study showed that retinoids also contributed to immune regulation through RARs and RXRs [14], including Th differentiation and B cell activation [15]. Etretinate has been used clinically for the treatment of cutaneous inflammatory disorders such as psoriasis and acne [16, 17]. ATRA, which is a ligand for RARα, β, and γ, and Am80, which is a specific ligand for RARα and β, but not for RARγ [18], are also used to treat APL [19, 20].

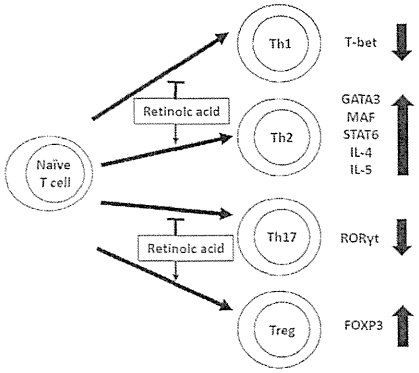


Fig. 1 Regulation of Th differentiation by retinoic acid. Retinoic acid enhances the differentiation of Th2 by inducing the expression of GATA3, MAF, STAT6, IL-4, and IL-5, and also Treg differentiation through the expression of FOXP3. In contrast, retinoic acid suppresses Th1 and Th17 differentiation by downregulating the expression of T-bet and RORγt, respectively

Effects of retinoids on immune cells

T cells

T cells play an important role in the immune system. Signals from dendritic cells (DCs), macrophages, and cytokines induce the differentiation of cells into Th1, Th2, Th17, or regulatory T (Treg) cells. ATRA has been shown to inhibit differentiation into Th1 cells by downregulating T-box expressed in T cells (T-bet) expression and promotes the differentiation of Th2 cells by inducing the expression of GATA-binding protein-3 (GATA3) and MAF as well as activating STAT6 in vitro [21] (Fig. 1). A deficiency in vitamin A was shown to result in an environment that was conducive to the differentiation of naive precursor $CD4^+$ T cells into interferon (IFN) γ–secreting Th1 cells [22]. In addition, ATRA directly induced the differentiation of Th2 cells via RAR [21] and indirectly promoted that of Th2 cells by increasing the production of interleukin (IL)-4 and IL-5 from Th2 cells, which are important cytokines for Th2 differentiation [23]. ATRA can also inhibit differentiation into Th17 cells by downregulating the expression of retinoid-related orphan receptor-gamma t (RORγt) and induce forkhead box P3 (FOXP3)-positive Treg by upregulating the expression of FOXP3 in vitro [24].

All-*trans* retinoic acid strongly enhances the production of IL-2 from T cells, which, in turn, induces the proliferation of T cells [25, 26]. A previous study demonstrated that ATRA regulated the migration of T cells into the gut by inducing the expression of α4β7-integrin and CC chemokine receptor 9 (CCR9) on T cells [27]. These findings indicated that retinoids could regulate Th differentiation as well as the proliferation and migration of T cells.

B cells

The proliferation of B cells is induced by stimulating the B cell receptor (BCR), CD38, CD40, CD19, Toll-like receptor (TLR) 4, and TLR9 [28–30]. ATRA can also regulate B cell proliferation as both an inducer and suppressor. The incubation of B cells with ATRA inhibited their proliferation due to the stimulation of BCR and TLR4 [31, 32]. In contrast, ATRA enhanced the proliferation of memory B cells by stimulating TLR9 [33]. The effects of retinoic acid on B cell proliferation may depend on the B cell subpopulation and co-stimulations.

Activation-induced cytidine deaminase (AID) is expressed in germinal center B cells and leads to the somatic hypermutation and class switch recombination of immunoglobulin genes. The expression of AID in B cells is induced by stimulations with lipopolysaccharide (LPS), IL-4, transforming growth factor-β (TGF-β), IFN-γ, and the CD40 ligand [34]. ATRA also increased the expression of AID in BCR-stimulated B cells, which suggested that it plays a positive role in regulating somatic hypermutation and class switch recombination [31]. A previous study showed that retinoic acid increased TGF-β-promoted

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].

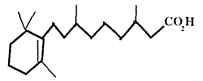
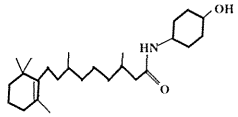
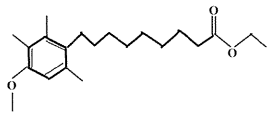
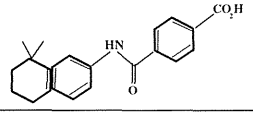
Thirty-one patients with systemic sclerosis (7 were treated with etretinate monotherapy, 5 with etretinate plus immunosuppressive therapies, 13 with immunosuppressive therapy only, and 6 with no treatment) were evaluated using the modified Rodnan total skin thickness score [58]. A significant improvement 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 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.

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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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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 Toyama,Yoshinari Takasaki (JuntendoUniversity); Naoichiro Yukawa, Tsuneyo Mimori (Kyoto University); Yukitaka Ueki (Sasebo Chuo Hospital), Shigeto Tohma (Sagamihara National Hospital), Kazuya Michishita, Kazuhiko Yamamoto (The University of Tokyo), Akira Hashiramoto, Syunichi Shiozawa (Kobe University); Akitomo Okada, Atsushi Kawakami (Nagasaki University); Tetsuji Sawada (Tokyo Medical University Hospital); Kazuhiko Ezawa (Kurashiki Kohsai Hospital); Sae Ochi (Tokyo Metropolitan Bokutoh Hospital); Hideto Kameda, Yuko Kaneko, Tsutomu Takeuchi (Keio University); Kenji Nagasaka (Ome Municipal General Hospital); Yoshinori Nonomura (Tokyo Kyosai Hospital); Kiyoshi Migita (National Hospital Organization Nagasaki Medical Center); Ayako Nakajima, Hisashi Yamanaka (Tokyo Women's Medical University). We appreciate Ms. Marie Yajima (Tokyo Medical and Dental University) for her contributions to data collection and the maintenance of the REAL database. Yokohama City Minato Red Cross Hospital is also a member of the REAL study group, but was not involved in this study. We sincerely thank all the rheumatologists and others caring for RA patients enrolled in the REAL.

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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).

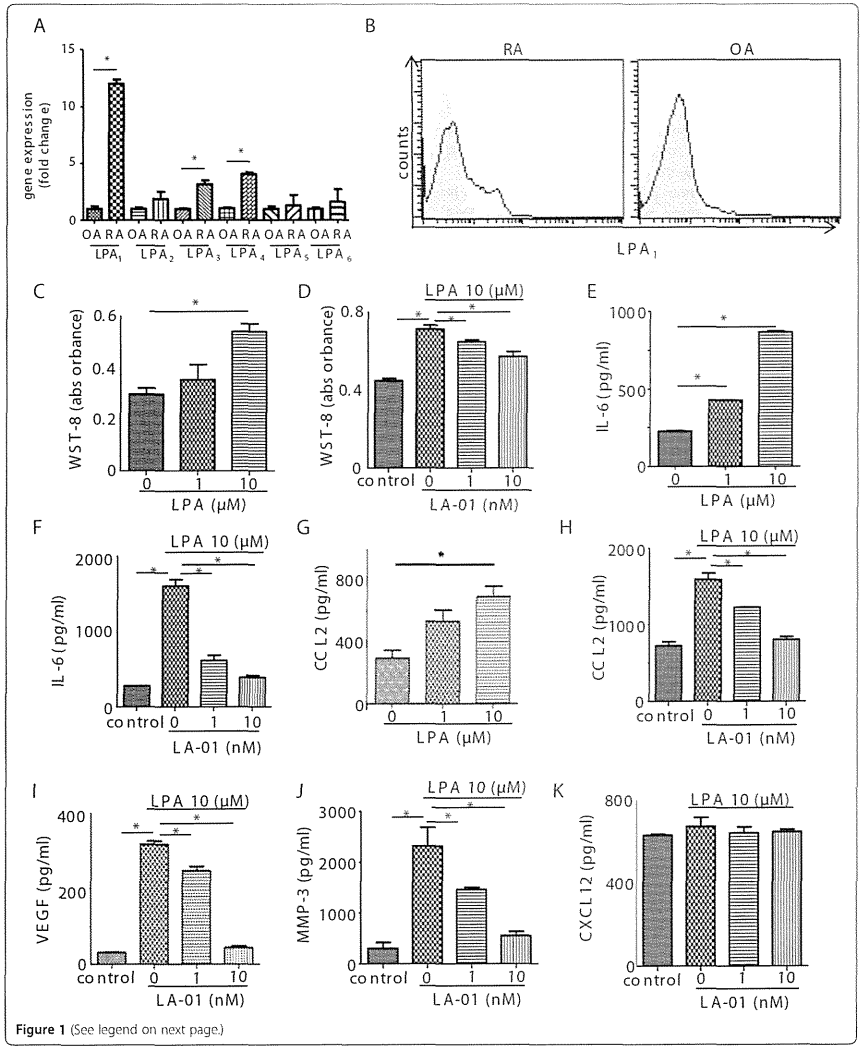

Figure 1 (See legend on next page.)

(See figure on previous page.)
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

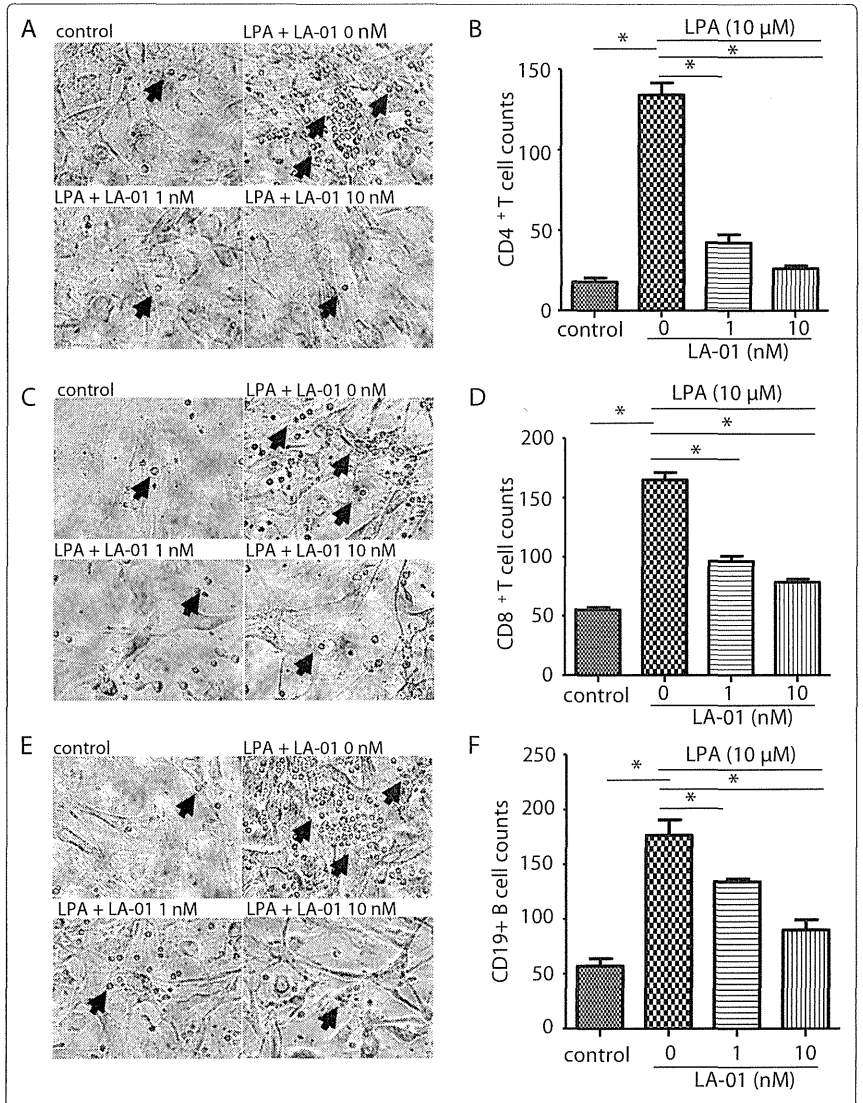

Figure 2 (See legend on next page.)