Effect of prednisolone and saireito co-administration on T-cells of rheumatoid arthritis patients

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Abstract

Objective

Rheumatoid arthritis (RA) is an autoimmune disease. Methotrexate (MTX) and prednisolone (PSL) are used in combination for severe RA therapy. However, it can increase the risk of osteoporosis and osteonecrosis. Saireito (114) can be used to reduce PSL dose owing to its immunosuppressive effects. However, the effect of combination therapy of PSL+114 on the immune system of RA patients remains unknown. This study compared the effect of PSL alone and PSL+114 on peripheral blood mononuclear cell (PBMC) proliferation, T-cell subsets, and cytokine production in adult RA patients receiving MTX monotherapy.

Methods

We isolated PBMCs from 14 consenting RA patients, and cultured them with PSL (0.0001–1.0 μM) in combination with or without 114 (300 μg/mL) for 96 h in the presence of concanavalin A. We measured the proliferation rates of PBMC, proportions of $CD4^+$, $CD8^+$, and $CD4^+CD25^+Foxp3^+$T-cells (induced T-regulatory cells), and concentrations of interferon-γ, interleukin (IL)-6, IL-10, IL-17A, and tumour necrosis factor in the culture supernatant.

Results

Compared to the blank, the PBMC proliferation rate significantly decreased at a reduced PSL concentration after 114 administration. The 50% inhibition concentration was 0.43 μM PSL for the PSL-only group as compared to 0.29 μM PSL for the PSL+114 co-administration group. The PSL+114 co-administration group had a significantly higher concentration of IL-6 compared to the PSL-only group.

Conclusion

The use of 114 in combination with low-concentration PSL intensified its immunosuppressive effect. However, the concentration of IL-6 was elevated in the co-administration group, suggesting exacerbation of RA activity.

Key words

rheumatoid arthritis, cytokines, saireito, prednisolone

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Introduction

Rheumatoid arthritis (RA) is an autoimmune form of multiple arthritis characterised by progressive joint destruction and peripheral joint deformity (1). Disease-modifying anti-rheumatic drugs (DMARDs), the main form of treatment for RA, improve symptoms by alleviating synovitis and systemic inflammation. Methotrexate (MTX) is the first-line DMARD (2). If the response to MTX monotherapy is insufficient, a combination of MTX and conventional synthetic DMARDs or glucocorticoids (GCs) is recommended. The drugs most widely used in combination with MTX in clinical practice are GCs, particularly prednisolone (PSL); however, its chronic use may lead to osteoporosis and osteonecrosis (3).

Kampo medication, a traditional Japanese herbal medication, has few side effects, and over 80% of Japanese doctors use it in clinical practice (4). In Japanese healthcare, Kampo medication is often taken in combination with allopathic drugs, and in RA therapy, it is used as complementary medicine for alleviating arthritis, thus reducing the dose of GCs and alleviating RA-associated symptoms (5). A combination of saireito (114), a Kampo medication, with GC has been reported to exert a steroid-like action and an immunosuppressive effect in RA (6).

The proportions of Th1, Th17, and $CD8^+$T-cells in peripheral blood mononuclear cells (PBMCs) have been found to be associated with RA activity, with increased proportions of cells associated with high disease activity (7). Regulatory T-cells (Tregs) play important roles in autoimmune diseases and immune tolerance, and the number of Tregs expressing CD25 and FoxP3 (known as induced Tregs or iTregs) as a proportion of $CD4^+$T-cells is markedly reduced in RA, suggesting that the suppression of Treg function may exacerbate RA symptoms (8). Increasing the number of Tregs is one of the goals of RA treatment. T-cells produce cytokines, among which interleukin (IL)-6 and tumour necrosis factor alpha (TNF-α) are key modulators of disease activity (9). Therefore, T-cells and cytokines are strongly associated with RA and can be used as assessment indices for evaluating response to the therapeutic drugs and for monitoring patients.

In healthy volunteers, PSL+114 treatment of PBMCs increased the 50% inhibition concentration (IC_{50}) of PSL and IL-6, which could interfere with RA treatment and worsen the patients' condition (10). However, to date, no study has addressed the effect of PSL+114 co-administration on cytokine levels as well as its immunosuppressive effects in RA patients. Thus, further research is warranted to elucidate its effect.

We conducted this study to investigate the immunosuppressive effect of PSL+114 combination therapy on T-cell subsets. We administered PSL only or PSL+114 to PMBCs obtained from the blood of RA patients and compared their effects on inhibition of PBMC proliferation, proportions of $CD4^+$ and $CD8^+$T-cells, and iTregs in PBMCs, as well as the changes in the concentrations of Th1, Th2, and Th17 cytokines produced by PBMCs.

Materials and methods

Subjects

Fourteen RA patients who attended the Department of Rheumatology of Tokyo Medical University Hospital between May and November 2021 were included. The number of patients was set using Power and Sample Size Calculation, and the PBMC proliferation rate of the blank, PSL single agent group and PSL+114 combination group obtained in the previous study (10), the ratio of $CD4^+$ and $CD8^+$T-cells in PBMC, based on the Th1, Th2, and Th17 cytokine concentrations, the number of samples in each experiment was set to 5 or more with a significance level of 5% and a detection power of 80%. All patients met the American Rheumatism Association (now the American College of Rheumatology) revised classification criteria (11), and were all adults whose disease was well controlled with MTX monotherapy. For RA patients who had a history of receiving PSL, the shortest period after PSL discontinuation was 6 months. Therefore, PSL was no longer present in the blood of these patients and it was considered that a sufficient

washout period had been provided. We evaluated their 28-joint Disease Activity Score based on C-reactive protein (DAS28-CRP) and erythrocyte sedimentation rate (DAS28-ESR) and blood test result during outpatient assessment. Informed consent for this study was obtained in compliance with the ethical standards of the World Medical Association (The Declaration of Helsinki), and the study was approved by the Tokyo University of Pharmacy and Life Sciences Institutional Review Board for Research Use of Human Tissue (approval no. 18-07). It was also approved by the Medical Ethics Committee of Tokyo Medical University (approval no. SH4124).

Table I. Baseline characteristics of the enrolled rheumatoid arthritis (RA) patients.

	RA patient (n=14)
Age, years, median (IQR)	69.5 (66.5-73.8)
Sex, male/female	7/7
Disease duration, years, median (IQR)	8.0 (2.3-11.8)
MTX dosage, mg/week, median (IQR)	8.0 (6.0-8.0)
WBC, /μl, median (IQR)	6450 (4000-7975)
RBC, $\times 10^4$/μl, median (IQR)	430 (352-463)
Hb, g/dL, median (IQR)	13.6 (11.3-14.2)
Plt, $\times 10^4$/μl, median (IQR)	25.4 (17.5-27.7)
ESR, mm/h, median (IQR)*	18.0 (2-41)
CRP, mg/dL, median (IQR)	0.09 (0.02-0.24)
T-P, g/dL, median (IQR)	7.0 (6.3-7.1)
ALB, g/dL, median (IQR)	4.0 (3.7-4.3)
AST, IU/L median, (IQR)	21.0 (12.0-24.5)
ALT, IU/L, median (IQR)	18.0 (9.0-21.8)
LDH, U/L, median (IQR)	195 (139-214)
BUN, mg/dL, median (IQR)	15.5 (10.3-17.0)
CRE, mg/dL, median (IQR)	0.75 (0.53-0.85)
DAS28-CRP, median (IQR)	1.5 (1.1-1.7)
DAS28-ESR, median (IQR)*	2.7 (0.5-2.7)

Blood was collected from RA patients who were outpatients at the Department of Internal Medicine for Rheumatoid Arthritis at Tokyo Medical University Hospital, and laboratory data were obtained (median (IQR)). *ESR and DAS28-ESR were obtained from only 9 patients, so n=9.
RA: rheumatoid arthritis; MTX: methotrexate; WBC: white blood cell; RBC: red blood cell; Hb: haemoglobin; Plt: platelet; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; T-P: total protein; ALB: albumin; AST: aspartate aminotransferase; ALT: aspartate aminotransferase; LDH: lactate dehydrogenase; BUN: blood urea nitrogen; CRE: creatinine; DAS28: 28-joint Disease Activity Score; GC: glucocorticoid; IQR: interquartile range.

Culture medium and reagent preparation

Culture medium was prepared by adding fetal bovine serum (FBS; Gibco, MA, USA) inactivated at 56ºC for 30 min to RPMI 1640 culture medium (Gibco) at a concentration of 10% (v/v) and further by adding 100,000 IU/L penicillin and 100 mg/L streptomycin (Gibco). Concanavalin A (ConA; Wako Pure Chemical Corporation, Kyoto, Japan) was dissolved in RPMI 1640 at a concentration of 1 mg/mL. PSL powder (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan; molecular weight: 365.85) was dissolved in dimethyl sulfoxide (DMSO; FUJIFILM Wako Pure Chemical Corp) at a final concentration of 10 μM, and was serially diluted in RPMI 1640 to obtain final concentrations (after addition to the cell culture system) of 0.0001, 0.001, 0.01, 0.1, 0.25, 0.5, and 1 μM.

Tsumura saireito (TJ114; Tsumura & Co, Tokyo, Japan) and Kracie saireito (KR114; Kracie Pharmaceutical Ltd, Tokyo, Japan) were used in the study. After weighing, the granules were dissolved in RPMI 1640 to obtain a concentration of 10 mg/mL; thereafter, the solution was centrifuged at 3500 rpm and 23ºC for 10 min. Following centrifugation, the supernatant was sterilized via filtration through a syringe fitted with a 0.22-μm membrane filter (AS ONE Corp, Osaka, Japan). After sediment removal, the solution was used as the test Kampo medication.

PBMC isolation

We gently aliquoted 20 mL peripheral venous blood, collected in heparin tubes, from of the 14 patients into lymphocyte separator liquid (Nacalai Tesque, Kyoto, Japan) and centrifuged it at 2,300 rpm for 20 min at 23°C. The PBMC layer was separated into a centrifuge tube using a Pasteur pipette and 10 mL RPMI 1640 medium was added to cleanse the PBMCs. Thereafter, the cells were centrifuged at 1,500 rpm for 10 min at 23°C. The supernatant was removed, and the process was repeated once more. Finally, the PBMCs were suspended in 5 mL RPMI 1640 medium. Cell number was determined using a haemocytometer and a microscope. The cell suspension was diluted with RPMI 1640 medium to reach a concentration of 1×10^6 cells/mL. Cells were plated onto a 96-well plate, followed by addition of 10 μL ConA as the T-cell mitogen (final concentration: 5 μg/mL). Finally, either 20 μL PSL (final concentrations: 0.0001, 0.001, 0.01, 0.1, 0.5, and 1 μM) or 20 μL 114 (final concentration: 300 μg/mL) were added. The concentration of 114 was set according to the clinical concentration in human blood at a steady-state (300 μg/mL) (12).

As a blank for the 114-monotherapy group, we added 170 μL PBMC suspension, 10 μL ConA solution, and 20 μL culture medium. As a blank for the PSL monotherapy and PSL+114 groups, we added 170 μL PBMC suspended solution, 10 μL ConA solution, and 20 μL dimethyl sulfoxide (final concentration: 0.4%). We then cultured the solutions at 37°C for 96 h in the presence of 5% CO_2. Kampo medicine is thought to have weaker actions than allopathic medicine; therefore, we determined the incubation period of the solution to determine the culture duration without affecting the survival of the PBMCs.

PBMC proliferation assessment

Next, 10 μL of Cell Counting Kit-8 Solution (Dojindo Laboratories, Tokyo, Japan) per well was added to cultured PBMCs from 12 RA patients, and the wells were then cultured under 5% CO_2 at 37ºC for 3 h. Absorbance was then measured at 450 nm with a spectral scanning multimode reader. The PBMC proliferation rate at each drug concentration was calculated by dividing the absorbance (nm) of ConA-stimulated PBMCs cultured in the presence of PSL with or without 114 by the absorbance

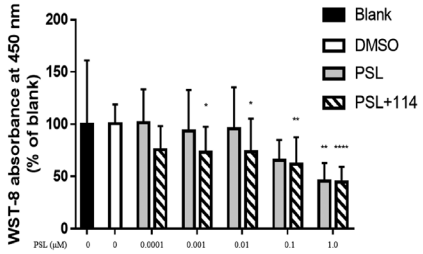


Fig. 1. Inhibition of PBMC proliferation with PSL and 114 treatment. Proliferation of PBMCs stimulated by ConA after addition of PSL and 114. Each treatment group was compared to its respective blank using Dunnett's multiple comparison test (*p<0.05, **p<0.01, ****p<0.0001). The PSL monotherapy and PSL+114 combination therapy groups were compared using the Tukey-Kramer multiple comparison test (mean±SD, n=12). ConA: concanavalin A; PBMCs: peripheral blood mononuclear cells; DMSO: dimethyl sulfoxide; PSL: prednisolone; 114: saireito.

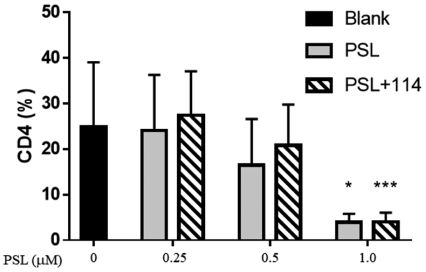
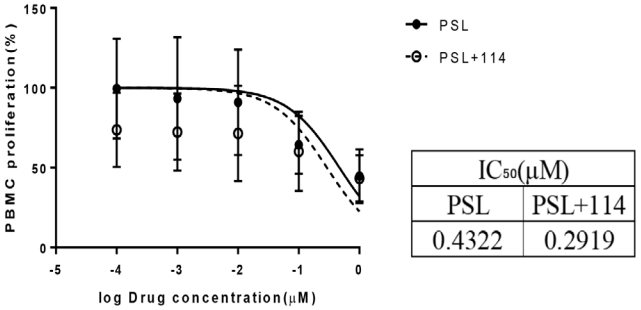


Fig. 3. Cell positive rate of CD4⁺T-cells in PBMCs after PSL and 114 treatments. Ratio of CD4⁺T-cells in PBMCs after PSL and 114 treatments. Each treatment was compared to its respective blank using Dunnett's multiple comparisons test (*p<0.05, ***p<0.001). The PSL monotherapy and PSL+114 combination therapy groups were compared using Tukey-Kramer multiple comparison test (mean±SD, n=5). ConA: concanavalin A; PBMCs: peripheral blood mononuclear cells; PSL: prednisolone; 114: saireito.

of ConA-stimulated PBMCs cultured without drugs and multiplying by 100.

Flow cytometry analysis of CD4⁺ T-cells, CD8⁺ T-cells, and Tregs

Using PBMCs from five RA patients cultured as described in Section 2.3, 10 μL of PerCP-Cy5.5-labelled anti-human CD4 monoclonal antibody solution (BD Pharmingen, San Diego, CA, USA), 5 μL of APC-Cy7-labeled anti-human CD8 monoclonal antibody solution (BD Pharmingen), 10 μL of PE-labeled anti-human CD25 monoclonal antibody solution (BD Pharmingen), and 5 μL of Alexa Fluor 488-labelled anti-human Foxp3 monoclonal antibody solution (BD Pharmingen) were added to each well, and the plate



IC50(μM)	
PSL	PSL+114
0.4322	0.2919

Fig. 2. Typical dose-curves of PSL and PSL+114 for proliferation of ConA-activated PBMCs from RA patients (n=12).
ConA: concanavalin A; PBMCs: peripheral blood mononuclear cells; PSL: prednisolone; 114: saireito.

was incubated under 5% CO_2 at 37ºC for 20 min. After incubation, 1 mL of wash buffer comprising 1% FBS in phosphate-buffered saline (PBS; FUJIFILM Wako Pure Chemical Corp) was added to suspend the cells, and the mixture was centrifuged at 1300 rpm and 23ºC for 5 min and washed. The sediment obtained was resuspended in 400 μL of staining buffer to fix cells. The cell suspension was filtered through a 37-μm nylon mesh (AS ONE Corp, Osaka, Japan) and transferred to polystyrene tubes (FALCON Corp, Aichi, Japan) for flow cytometry analysis. Subsequently, 30,000 stained cells were measured with a FACS Canto™ II (BD Pharmingen). CELLQuest v. 3.1 software (BD Pharmingen) was used for data analysis.

Lymphocyte population gating was conducted with FSC/SSC dot plots, and lymphocyte data were collected. CD4⁺ cell gating was conducted using PerCP-Cy5-5-A as the CD4 detection wavelength, and the resulting cells were categorised as CD4⁺T-cells. CD8⁺ cell gating was conducted using APC-Cy7 as the CD8 detection wavelength, and the resulting cells were categorised as CD8⁺T-cells. After CD4⁺T-cell gating, PE and Alexa Fluor were used as fluorescence wavelengths of CD25 and Foxp3, dot plots displaying CD4⁺T-cells were divided into four, and cells positive for both CD25 and Foxp3 (CD4⁺CD25⁺FoxP3⁺cells) were categorised as iTregs.

Each T-cell subset was calculated using the following formulas:

CD4⁺T-cells = the number of CD4⁺ stained cells / the number of lymphocytes × 100;

CD8⁺T-cells = the number of CD8⁺ stained cells / the number of lymphocytes × 100;

iTregs = the number of CD25⁺ and Foxp3⁺ stained cells / the number of CD4⁺ stained cells × 100.

Cytokine concentration assessment

IFN-γ, IL-6, IL-10, IL-17A, and TNF-α concentrations in the supernatant were measured with a Human Flex Set (BD Pharmingen). To each sample tube, we sequentially added 50 μL of capture beads for each cytokine, 50 μL of detection reagent, and 50 μL of culture supernatant or standard solution, and the tubes were then incubated in the dark at 15–25°C for 3 h. The samples were then washed in wash buffer and centrifuged. After centrifugation, the supernatant was discarded, each sample was then resuspended in an additional 300 μL of wash buffer, and the specimens were measured with a FACS Canto™ II. Individual cytokine concentrations were indicated in terms of fluorescence intensity. The standard solution was serially diluted to prepare a calibration curve to determine the concentration of each cytokine in the culture supernatant. FACP Array™ software (BD Pharmingen) was used for data analysis.

Statistical analysis

The means of the results obtained from TJ114 and KR114 were defined as the results for 114. Dunnett's multiple comparisons test was used to test for significant differences between the blank, PSL-only group, and PSL+114 co-administration group, and Tukey's multiple comparisons test was used for comparisons between the PSL-only group and PSL+114 co-administration group. In all cases, p<0.05 was considered significant. GraphPad PRISM ver. 8.0 software (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analysis.

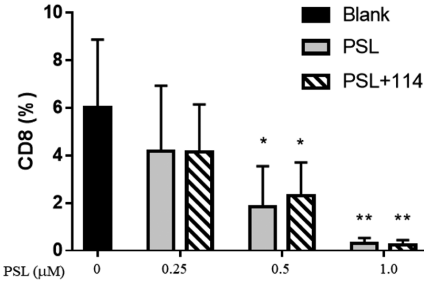


Fig. 4. Cell positive rate of CD8⁺T-cells in PBMCs after PSL and 114 treatment. Ratio of CD8⁺T-cells in PBMCs after PSL and 114 treatment. Each treatment group was compared to its respective blank using Dunnett's multiple comparisons test (*p<0.05, **p<0.01), and the comparison of PSL monotherapy and PSL+114 combination therapy groups was conducted using Tukey-Kramer multiple comparison test (mean±SD, n=5). ConA: concanavalin A; PBMCs: peripheral blood mononuclear cells; PSL: prednisolone; 114: saireito.

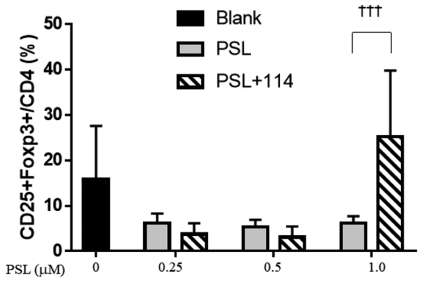


Fig. 5. Cell positive rate of iTregs in PBMCs after PSL and 114 treatments. Ratio of iTreg cells in PBMCs stimulated by ConA after PSL and 114 treatments. Each treatment group was compared to its respective blank using Dunnett's multiple comparisons test. The PSL monotherapy and PSL+114 combination therapy groups were compared using Tukey-Kramer multiple comparison test (†††p<0.001; mean±SD, n=5). ConA: concanavalin A; PBMCs: peripheral blood mononuclear cells; PSL: prednisolone; 114: saireito.

Results

Patient baseline characteristics

The baseline characteristics and clinical laboratory values of 14 RA patients are shown in Table I. Patients comprised 7 men and 7 women with a median age of 69.5 (66.5–73.8) years. The median disease duration of RA was 8.0 years, and the median MTX dose was 8 mg/week. Three of the 14 patients had previously received PSL treatment. The median DAS28-CRP and DAS28-ESR scores were 1.5 (1.1–1.7) and 2.7 (0.5–2.7), respectively.

Inhibition of PBMC proliferation with PSL and 114

In the PSL-only group, PBMC proliferation was inhibited in a concentration-dependent manner, with proliferation significantly inhibited as compared to the blank at ≥1.0 μM (Fig. 1). In PSL+114, concentration-dependent inhibition of PBMC proliferation was evident, but proliferation was significantly inhibited at ≥0.001 μM PSL (Fig. 1). No significant difference was observed between the PSL-only and PSL+114 groups. The IC_{50} of PSL-only group was 0.43 μM, but the IC_{50} of PSL+114 was 0.7 of this value at 0.29 μM (Fig. 2).

Changes in the proportion of CD4⁺T-cells in PBMC following addition of PSL and 114

In the PSL-only group, CD4⁺T-cell proportion tended to decrease in a concentration-dependent manner compared to the blank, with the proportion of CD4⁺T-cells in PBMCs significantly decreasing from 24.8% to 4.3% at 1.0 μM, the highest concentration used in this study (Fig. 3).

In PSL+114, CD4⁺T-cell proportion decreased in a concentration-dependent manner compared to the blank, with the proportion of CD4⁺T-cells decreasing to 3.97% in PSL 1.0 μM+114 (Fig. 3). No significant difference was observed in CD4⁺T-cell proportion between PSL-only and PSL+114 groups at any concentration (Fig. 3). Further, no significant difference was observed in CD4⁺T-cell proportion between PSL+TJ114 and PSL+KR114 at any concentration (Supplementary Fig. S1).

Changes in CD8⁺T-cell proportion in PBMCs following addition of PSL with or without 114

In the PSL-only group, CD8⁺T-cell proportion decreased in a concentration-dependent manner, with CD8⁺T-cell proportion in PBMCs decreasing significantly compared to the blank at 0.25-1.0 μM to a minimum of 0.3% (Fig. 4).

In PSL+114, CD8⁺T-cell proportion tended to decrease in a concentration-dependent manner compared to the blank. In PSL 0.5 μM+114 and PSL 1.0 μM+114 groups, CD8⁺T-cell proportion in PBMCs decreased significantly to a minimum of 0.24% compared to the blank (Fig. 4). No significant difference was observed in CD8⁺T-cell proportion between PSL-only and PSL+114 groups at any concentration (Fig. 4). Moreover, no significant difference was observed in CD8⁺T-cell proportion between PSL+TJ114 and PSL+KR114 at any concentration (Suppl. Fig. S2).

Changes in iTreg proportion in PBMCs following addition of PSL with or without 114

In the PSL-only group, iTreg proportion in CD4⁺T-cells tended to decrease compared to the blank at 0.25 μM, and it significantly decreased to 6.15% at the maximum concentration of iTreg proportion in CD4⁺T-cells (Fig. 5).

In PSL+114, iTreg proportion in CD4⁺T-cells tended to decrease in a concentration-dependent manner compared to the blank at ≤0.5 μM; however, at 1.0 μM, iTreg proportion in CD4⁺T-cells increased to 25.6%, although the difference was not significant. At 1.0 μM, the iTreg proportion in PSL+114 was approximately 4.1 times higher than that in the PSL-only group (Fig. 5). Further, no significant difference was observed in iTreg proportion between PSL+TJ114 and PSL+KR114 at any concentration (Suppl. Fig. S3).

Th1, Th2, and Th17 cytokine production in PBMC culture supernatant after addition of PSL with or without 114

We measured the concentrations of IFN-γ, IL-6, IL-10, IL-17A, and TNF-α in the PBMC culture supernatant of PSL-only and PSL+114 groups (Fig. 6). IFN-γ concentration decreased at 0.25 μM and 0.5 μM PSL, and although it increased following 114 co-administration, the difference was not significant (Fig. 6A). IL-6 concentration was higher in PSL+114 than in the PSL-only group, with the median value significantly increasing from 9.5 pg/mL to 10975.4 pg/mL at 0.25 μM PSL and from 8.9 pg/mL to 8764.6 pg/mL at 0.5 μM PSL (Fig. 6B).

IL-10 concentration decreased in the PSL-only group, but the median significantly increased from 6.7 pg/mL to 43.5 pg/mL at 0.25 μM PSL in PSL+114 compared to that in the PSL-only group (Fig. 6C). IL-17A concentration increased to 0.25 μM in the PSL-only group, but

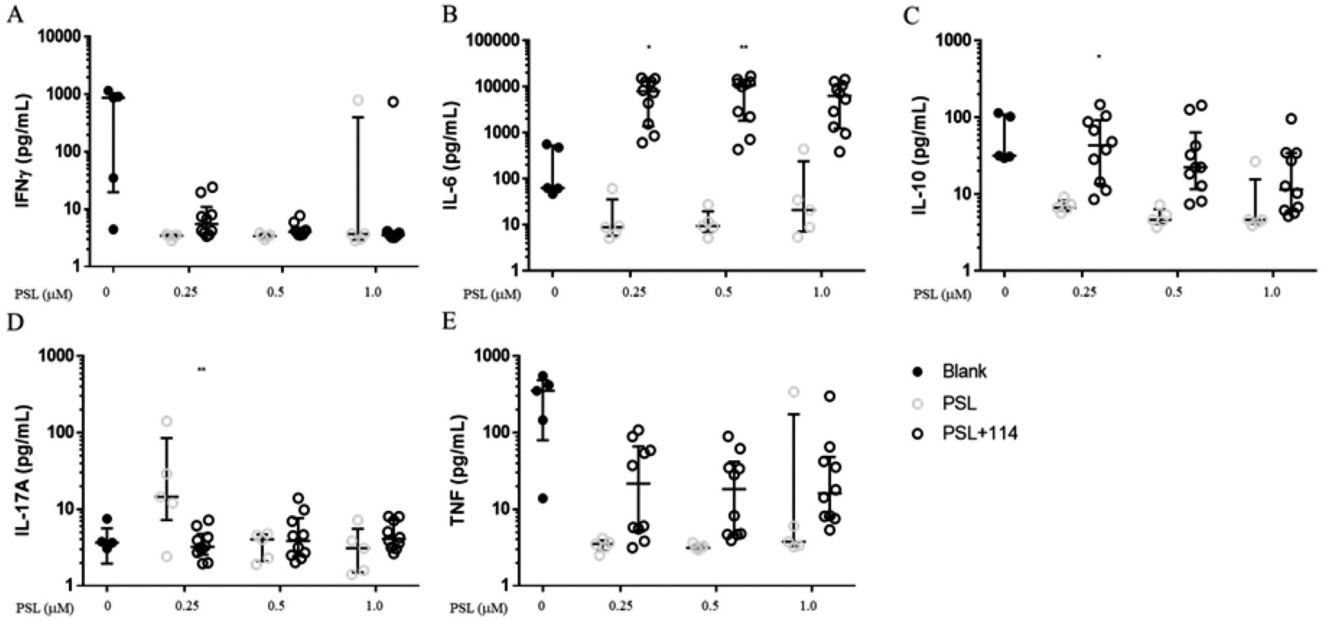


Fig. 6. Concentration of cytokines in PBMCs after PSL and 114 treatments. ConA, PSL, and 114 were added to PBMCs, and the solution was incubated for 96 h. For flow cytometry, beads were used to analyse the concentration of IFN-γ (**A**), IL-6 (**B**), IL-10 (**C**), IL-17A (**D**), and TNF-α (**E**) in the culture supernatant. The PSL monotherapy and PSL+114 combination therapy groups were compared using Tukey-Kramer multiple comparison test (*p<0.05, **p<0.01; median with IQR, n=5). ConA: concanavalin A; PBMCs: peripheral blood mononuclear cells; PSL: prednisolone; 114: saireito.

the median significantly decreased from 16.4 pg/mL to 3.2 pg/mL in PSL+114 (Fig. 6D). Moreover, TNF-α concentration was not significantly different between PSL+114 and the PSL-only group at any concentration. However, at 0.25 μM and 0.5 μM PSL, it tended to increase in PSL+114 compared to the PSL-only group (Fig. 6E).

Discussion

We used PBMCs from RA patients to compare the effects of administration of PSL alone and PSL+114 on immune cells. The parameters investigated comprised the antiproliferative effects of 114 on PBMCs, changes in T-cell subsets, and cytokine concentrations.

PSL suppresses PBMC proliferation in RA patients (13), and similar results were obtained in our study. Additionally, co-administration of 114 and PSL did not attenuate the latter's antiproliferative effect on PBMCs, suggesting that PBMC proliferation was inhibited at a lower PSL concentration as compared to the blank. However, no additive or synergistic antiproliferative effect of 114 was observed at high PSL concentrations. This is because the action of 114 at 300 μg/mL, which assumes a clinical steady-state blood concentration, is mild compared to PSL; therefore, at low concentrations, the antiproliferative effect of 114 in combination therapy is seen. However, the effect of PSL was remarkable at high concentrations and the combined effect of 114 was diminished. Additionally, co-administration of 114 and PSL reduced the latter's IC_{50} by half, as described above. Saikosaponin-a, a major component of 114, induces dose-dependent apoptosis in ConA-activated T-cells (14). In this study, the inhibitory effect of 114 combination on PBMC proliferation may be caused by the apoptotic effects of saikosaponin-a.

The $CD4^+$T-cell count decreases after 24 h of PSL administration in RA patients (15). In this study, PSL+114 co-administration increased the proportion of $CD4^+$T-cells as compared to PSL alone at ≤0.5 μM, but at a high PSL concentration of 1.0 μM, the effect of 114 co-administration might be attenuated. Other studies have found that 114 increases the proportion of $CD4^+$T-cells in PBMCs (16), and the increased proportion of $CD4^+$T-cells observed on co-administration of 114 with low-concentration PSL might be due to the action of 114.

The administration of PSL alone decreased $CD8^+$T-cell proportion in a dose-dependent manner. Co-administration of 114 and PSL had no effect on the decrease in $CD8^+$T-cell proportion observed after treatment with PSL alone. Saikosaponin d, the active ingredient of 114, decreases $CD8^+$T-cells (17), and its co-administration with PSL would be expected to exert a synergistic antiproliferative effect on cells; however, our results suggested that the co-administration of 300 μg/mL of 114 with PSL is unlikely to intensify the inhibition of $CD8^+$T-cells.

In the PSL-only group, iTregs were suppressed as compared to the blank. PSL suppresses the production of both Tregs and $CD4^+$T-cells in mice (18), and similar results have been obtained from studies on RA patients. However, co-administration of 114 and PSL (1.0 μM) significantly increased iTreg proportion as compared to the PSL-only group. In mice, 114 induces iTreg production in the spleen (19), and it may also induce iTregs in PMBCs. Thus, the marked reduction in iTregs observed on administration of 1.0 μM PSL alone might have been reduced due to the co-administration of 114, thereby increasing iTreg proportion in $CD4^+$T-cells. The percentage of iTreg in RA patients is negatively correlated with disease activity, ESR, CRP and RF (IgM) (20). Therefore, increasing the percentage of iTreg with 114 combination is considered to lead to the improvement of disease activity.

In this study, no significant difference was observed in TNF-α and IFN-γ concentrations between the PSL-only and PSL+114 co-administration groups at any concentration, suggesting that 114 might have little effect on the action of PSL on Th1-generated cytokine production. However, the production of Th2-generated cytokines IL-6 and IL-10 significantly increased in PSL+114 co-administration group as compared to PSL-only group at 0.25 μM and 0.5 μM. Thus, an increased IL-6 production induced by 114 co-administration might exacerbate RA disease activity. Two herbal medicines, shosaikoto and goreisan, constitute 114. IL-6 induction has been reported for shosaikoto. Shosaikoto induces Th2 differentiation. Generally, IL-6 is produced from Th2 (21).

At 0.25 μM, IL-17A significantly decreased in the PSL+114 co-administration group as compared to that in the PSL-only group. In cells from one patient, there was a large increase in IL-17A concentration in the PSL-only group, indicating the involvement of this mechanism.

One limitation of this study was that it was an *in vitro* study. Drugs undergo the process of drug disposition during absorption, distribution, metabolism, and excretion *in vivo*, and thus, further investigation using *in vivo* models should be conducted. The standard treatment for our patient population was MTX; however, there is scope for investigation of their treatment histories and concomitant use of other drugs, including non-steroidal anti-inflammatory drugs.

Our results showed that PSL+114 co-administration reduced IC_{50} compared to PSL alone, suggesting that the use of 114 for RA might enhance the immunosuppressive action and enable dose reduction of PSL. Additionally, for the first time, we reported that 114 co-administration increases IL-6 concentration in RA patients. The significance of 114 use is to improve patients' symptoms. Thus, when used with PSL, 114 can reduce the side effects of PSL and improve patient's quality of life. Thus, disease activity must be carefully monitored on continuous administration of PSL, and its withdrawal should be considered in case of exacerbation. Our results showed that kampo medications could exacerbate disease activity through mechanisms such as induction of cytokines, thus the patient's condition must be monitored with great care during prolonged administration.

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