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Original Article

Influence of folic acid on suppressive efficacy of methotrexate against mitogen-activated human peripheral-blood mononuclear cells

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ABSTRACT

Folic acid is recommended for rheumatoid arthritis patients to decrease the risk of methotrexate adverse effects. However, modification of immunosuppressive efficacy of methotrexate by folic acid is not precisely understood. We examined the influence of folic acid on the suppressive efficacy of methotrexate against mitogen-activated peripheral-blood mononuclear cells (PBMCs). PBMCs obtained from healthy subjects were cultured in the presence of methotrexate and/or folic acid under stimulation by concanavalin A for 96 h, and cell proliferation was estimated by MTT assay. Concentrations of seven inflammatory cytokines secreted into culture medium were measured by bead array procedures followed by flow cytometry. Percentages of $CD4^+$, $CD4^+/CD25^+$, and $CD4^+/CD25^+/FoxP3^+$ cells in lymphocytes were estimated by staining with specific antibodies. Methotrexate at 1-1000 ng/mL suppressed the proliferation of concanavalin A-activated PBMCs in a concentration-dependent manner, while 0.5-50 ng/mL of folic acid did not significantly disturb the methotrexate suppressive efficacy. Addition of folic acid at 24 h after the beginning of PBMC culture also had no significant effect on the methotrexate efficacy. Methotrexate tended to decrease the secretion of certain inflammatory cytokines, though the effects were not significant. Folic acid showed no significant influence on the methotrexate effects. Methotrexate at 100 ng/mL with or without folic acid significantly decreased the percentages of CD+4 cells in lymphocytes ($p < 0.05$), whereas the percentages of $CD4^+/CD25^+$ and $CD4^+/CD25^+/FoxP3^+$ cells were not affected by methotrexate and/or folic acid. These results suggest that the suppressive efficacies of methotrexate on the activated PBMCs are not attenuated in the presence of folic acid at clinically used concentrations.

KEYWORDS: methotrexate, folic acid, peripheral-blood mononuclear cells, inflammatory cytokines, $CD4^+$ cells, regulatory T cells.

INTRODUCTION

The anti-folate methotrexate has been used as an anchor drug for treatment of rheumatoid arthritis (RA) patients. Folic acid acts as a cofactor to various methyltransferases involved in serine, methionine, thymidine and purine biosynthesis [1]. Methotrexate inhibits dihydrofolate reductase, an enzyme that participates in the reduction of folic acid into the active folate form, and then interferes with thymidylate-purine synthesis to attenuate activation of immune cell proliferation [2]. Methotrexate is also reported to increase extracellular adenosine concentration, which results in inhibition of neutrophil and lymphocyte functions [3].

Adverse effects including myelosuppression and gastrointestinal or hepatic disfunction often occur in RA patients under long-term methotrexate therapy.

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Folic acid is prescribed to prevent and/or relieve these undesirable effects of methotrexate [4, 5]. Thus, RA patients under methotrexate therapy are recommended to be treated with 5 mg/week or less doses of folic acid 24-48h after the methotrexate administration [6]. We suggested in our previous report [7] that the therapeutic efficacies and incidence of hepatotoxic side effect of methotrexate was not basically different between RA patients taking less and more than 5 mg/week of folic acid. However, little has been known whether folic acid at doses of 5 mg/week may influence the immunosuppressive potencies of methotrexate.

Then, we investigated, in the present study, the influence of folic acid at clinically achievable concentrations on methotrexate efficacies to suppress proliferation of T cell mitogen-activated PBMCs of healthy subjects.

MATERIALS AND METHODS

Reagents

RPMI1640 and fetal bovine serum were purchased from Gibco BRL (Grand Island, NY, USA). Concanavalin A was purchased from Seikagaku Kogyo Co., Tokyo, Japan. Methotrexate and folic acid were purchased from Fujifilm Wako Pure Chemical Co. (Osaka, Japan), and were dissolved in 0.025 mol/L NaOH. The working concentrations were prepared after dilution with 0.025 mol/L NaOH. Tetrazolium salt of 3-(4,5-dimethylthiazol-2-yl)-2,5¬diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich Japan Co. (Meguro, Japan). BD Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokine Kit, Human Foxp3 Stain Kit Alexa Fluor, Human CD4 PerCP, Human CD25 PE, and Human CD8 PerCP were obtained from BD Biosciences (San Jose, CA, USA). All other reagents were of the best available grade.

Subjects

The present study was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). The study was approved by the Ethical Committee of Tokyo University of Pharmacy and Life Sciences and written informed consent was obtained from all healthy subjects included in the study. The study included 12 healthy subjects (4 males and 8 females with a mean age of 22.4 years). These subjects had neither a history of immunological disorders nor a history of taking immunosuppressive drugs.

Isolation of PBMCs and evaluation of anti-proliferative effects of methotrexate

Twenty milliliters of venous blood was taken from healthy subjects (see the above section) between 9:00-10:30 in the morning and heparinized. The heparinized blood was loaded onto 3 mL of Ficoll-Hypaque (Nakarai Co., Japan), and centrifuged at 1,300 × g for 20 min. PBMCs were separated and suspended with RPMI 1640 medium containing 10% fetal bovine serum, 100,000 IU/L penicillin and 100 mg/L streptomycin to a final density of 1.075×10^6 cells/mL as we described previously [8, 9]. Cell proliferation was determined by MTT assay procedures [10, 11]. In brief, 186 μL of the cell suspension was placed in each well of a 96-well non-treated microplate. Meanwhile, 186 μL of medium was added to the blank wells. Then, 10 μL of concanavalin A solution was added to each well to give a final mitogen concentration of 5.0 μg/mL. Subsequently, 2 μL of methotrexate solution and/or 2 μL of folic acid solution were added. Four or 6 μL of 0.025mol/L NaOH was added to the corresponding control wells. The plate was mixed on a microshaker for 10 seconds and then incubated for 96 h in an atmosphere of 5% CO_2 at 37 ^oC in a humidified chamber. After the incubation of the culture, 10 μl of 5 mg/ml MTT solution dissolved in saline was added to each well, and then the cultures were re-incubated under 5% CO_2 at 37 °C for 4-5 h [10, 11]. The plates were centrifuged at 375 g for 5 min to precipitate the cells and formazan crystals produced by the proliferated cells. Aliquots of the supernatant were removed from each well, and dimethylsulfoxide was added followed by shaking of the plate on a microshaker for 10 min to dissolve the formazan crystals. Cell growth was determined by measuring the optical density at 550 nm absorbance. Dose-response curves were plotted, and the agent concentration giving 50% inhibition of cell proliferation (IC_{50}) was calculated.

Cytokine analysis

PBMCs were incubated for 96 h in the presence of concanavalin A and methotrexate and/or folic acid as described above, and then the culture supernatants

were stored at -80 °C until measurement of cytokine concentrations. The concentrations of interferon-γ, tumor necrosis factor-α, IL-2, -4, -6, -10 and -17A in the culture supernatant were measured with beads-array procedures using the Human Th1/Th2/Th17 Cytokine Kit, followed by flow cytometry [10], according to the instructions of BD Biosciences (San Jose, CA, USA).

Analysis of $CD4^+$ cells, $CD4^+CD25^+$ cells, and $CD4^+CD25^+Foxp3^+$ cells

The analysis was carried out according to the manufacturer's instructions (BD Biosciences, USA). In brief, PBMCs treated with methotrexate and/or folic acid in the presence of concanavalin A, as described above, were washed twice with phosphate-buffered saline containing 1% fetal bovine serum (PBS) and re-suspended with PBS at a cell density of 1×10^6 cells/mL. For evaluation of $CD4^+$ cells and $CD4^+CD25^+Foxp3^+$ cells, 10 μL of PerCP-CyTM5.5(FITC) Mouse Anti-Human CD4 and 10 μL of BD Pharmingen™ APC Mouse Anti-Human CD25 (BD Biosciences, USA) were added to 1 mL of this cell suspension. Ten microliters of PerCP Mouse IgG1 κ Isotype control and 10 μL of PE Mouse IgG1 κ Isotype control (BD Biosciences, USA) were added to the control cells. These cell suspensions were incubated for 20 min in the dark. After the incubation, the cells were washed with PBS and resuspended with 1 mL of PBS. Then, 1 mL of ten-fold diluted Human Foxp3 Buffer A (BD Biosciences, USA) with distilled water was added to the cell suspension, and incubated for 10 min in the dark. The cells were washed with PBS, re-suspended with 0.5 mL of Human Foxp3 Buffer B (BD Biosciences, USA) diluted with the 10-fold diluted Human Foxp3 Buffer A, and incubated for 30 min in the dark. After the incubation, 1 mL of PBS was added and centrifuged for 5 min. The supernatant was removed and the cells were washed with phosphate-buffered saline. Then, 10 μL of Human BD Pharmingen™ ALexa FLuor® 488 Mouse anti-Human FoxP3 or 10 μL of PE Mouse IgG1 κ Isotype control were added to the control wells. Subsequently, 400 μL of staining buffer was added to the cell suspension, and then analyzed with flow cytometry.

The data were analyzed with a FACSCalibur ™II (BD Biosciences, USA), using CellQuest software (BD, USA) [12]. $CD4^+$ T cells in the lymphocyte fraction were gated, and the percentages of $CD4^+$ $CD25^+$ cells and $CD4^+CD25^+Foxp3^+$ cells (regulatory T cells) in the $CD4^+$ cell fraction were calculated.

Statistics

Differences in the percentages of PBMCs proliferated, amounts of cytokines produced in the culture supernatant, and percentages of $CD4^+$ cells, $CD4^+CD25^+$ cells and $CD4^+CD25^+Foxp3^+$ cells after cell culture in the presence of serial concentrations of methotrexate and/or folic acid were analyzed with Bonnferroni test or Dunnett's multiple comparison test. The differences between the values of any two groups were analyzed with Wilcoxon signed-ranked test. These analyses were performed with GraphPad PRISM 4.0 (GraphPad Software Inc., San Diego, CA, USA). In each case, data were expressed as the mean ± standard deviation (SD), and two-sided P values <0.05 were considered to be significant.

RESULTS

Effect of folic acid on suppressive efficacy of methotrexate against mitogen-activated proliferation of PBMCs

Methotrexate dose-dependently suppressed concanavalin A-activated proliferation of PBMCs with statistically significant decrease at 100 and 1000 ng/mL of the drug ($p < 0.05$) (Fig. 1). Concomitant addition of 0.5, 5, or 50 ng/mL folic acid with methotrexate from the beginning of culture did not change the suppressive efficacies of methotrexate (Fig. 1). Similarly, delayed addition of 0.5, 5, or 50 ng/mL folic acid with methotrexate at 24 h after the culture also did not affect the suppressive efficacies of methotrexate (Fig. 2). The IC_{50} values of methotrexate against the concanavalin A-activated proliferation of PBMCs in the absence or presence of folic acid are listed in Table 1. Folic acid added from the beginning or 24 h after the culture at any concentrations did not significantly change the IC_{50} values of methotrexate.

Influence of folic acid on the effects of methotrexate to modify inflammatory cytokine secretion from mitogen-activated PBMCs

Concentrations of seven inflammatory cytokines in culture medium of concanavalin A-activated

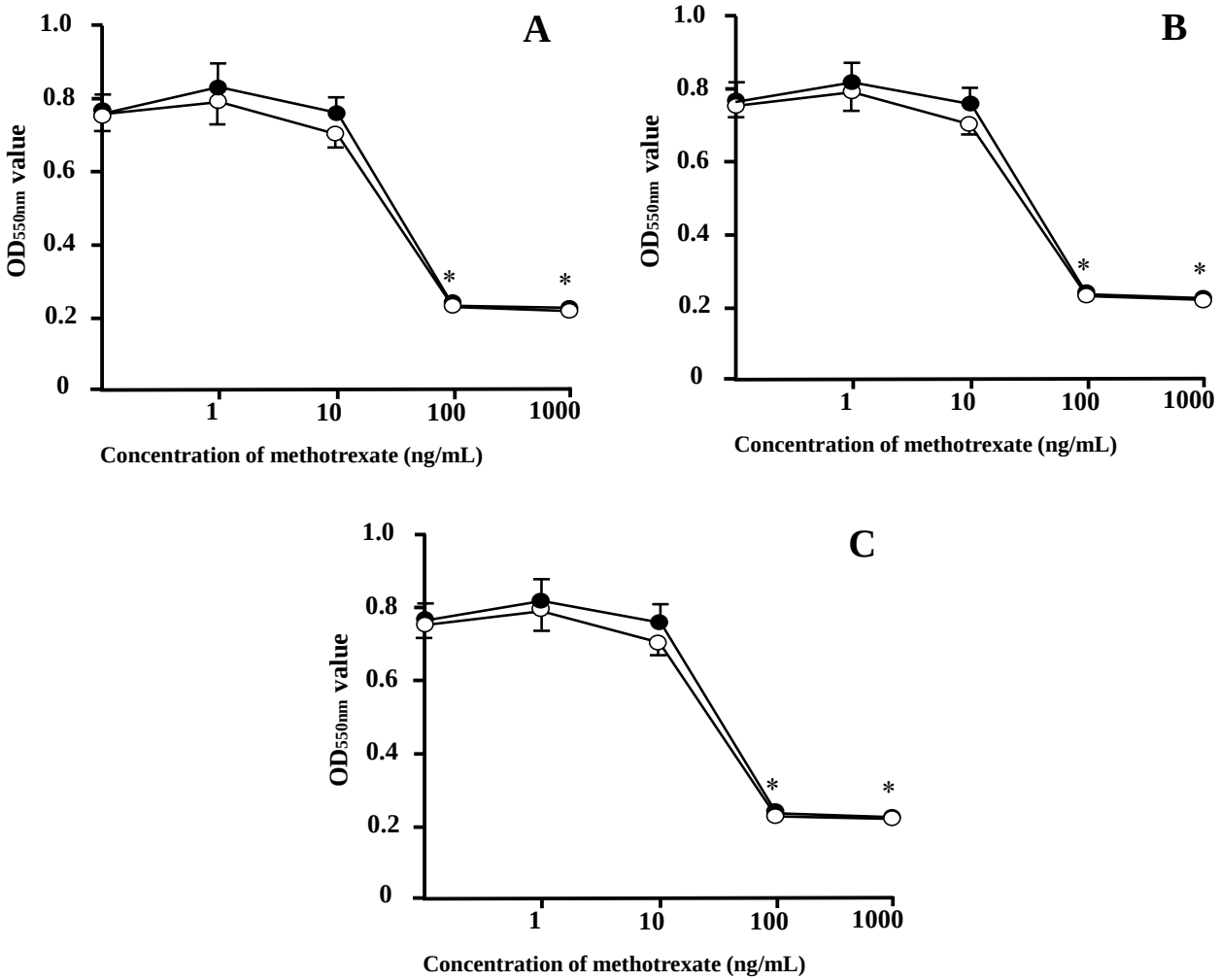

Fig. 1. Effects of methotrexate in the presence or absence of folic acid on concanavalin A-activated proliferation of PBMCs. PBMCs obtained from healthy volunteers were cultured for 96 h under serial concentrations of methotrexate with (solid lines) or without (dashed lines) folic acid in the presence of concanavalin A. After culturing, the proliferated cells were evaluated with MTT assay procedures as described in Materials and Methods. The means (SDs) of the optical density (OD) values read at 550 nm after MTT assay were plotted. *$p < 0.05$ as compared to the control without methotrexate treatment, evaluated by Bonferroni test ($n = 12$). There was no significant difference in the mean OD value between culture wells treated with methotrexate in the absence of folic acid and those treated with methotrexate in the presence of folic acid. Folic acid was added to give final concentrations of 0.5 (A), 5(B), and 50 (C) ng/mL.

PBMCs treated with methotrexate in the absence or presence of folic acid are shown in Figs. 3 and 4. Folic acid added to the culture at the same time with methotrexate (Fig. 3) or 24 h after the addition of methotrexate (Fig. 4) showed no significant influence on the methotrexate effects against the secretion of the inflammatory cytokines.

Methotrexate at concentrations less than 10 ng/mL had no effect on the IL-2 secretion, whereas the drug at 100 ng/mL extensively increased the IL-2 concentration in the medium ($p < 0.05$). Methotrexate dose-dependently increased IL-4 concentration in medium, but these drug effects disappeared in the presence of folic acid despite the time of addition. Methotrexate in the presence or absence of folic acid did not influence on the concentrations of IL-6, IL-10, and interferon γ in the PBMC culture medium. TNF-α concentrations tended to increase

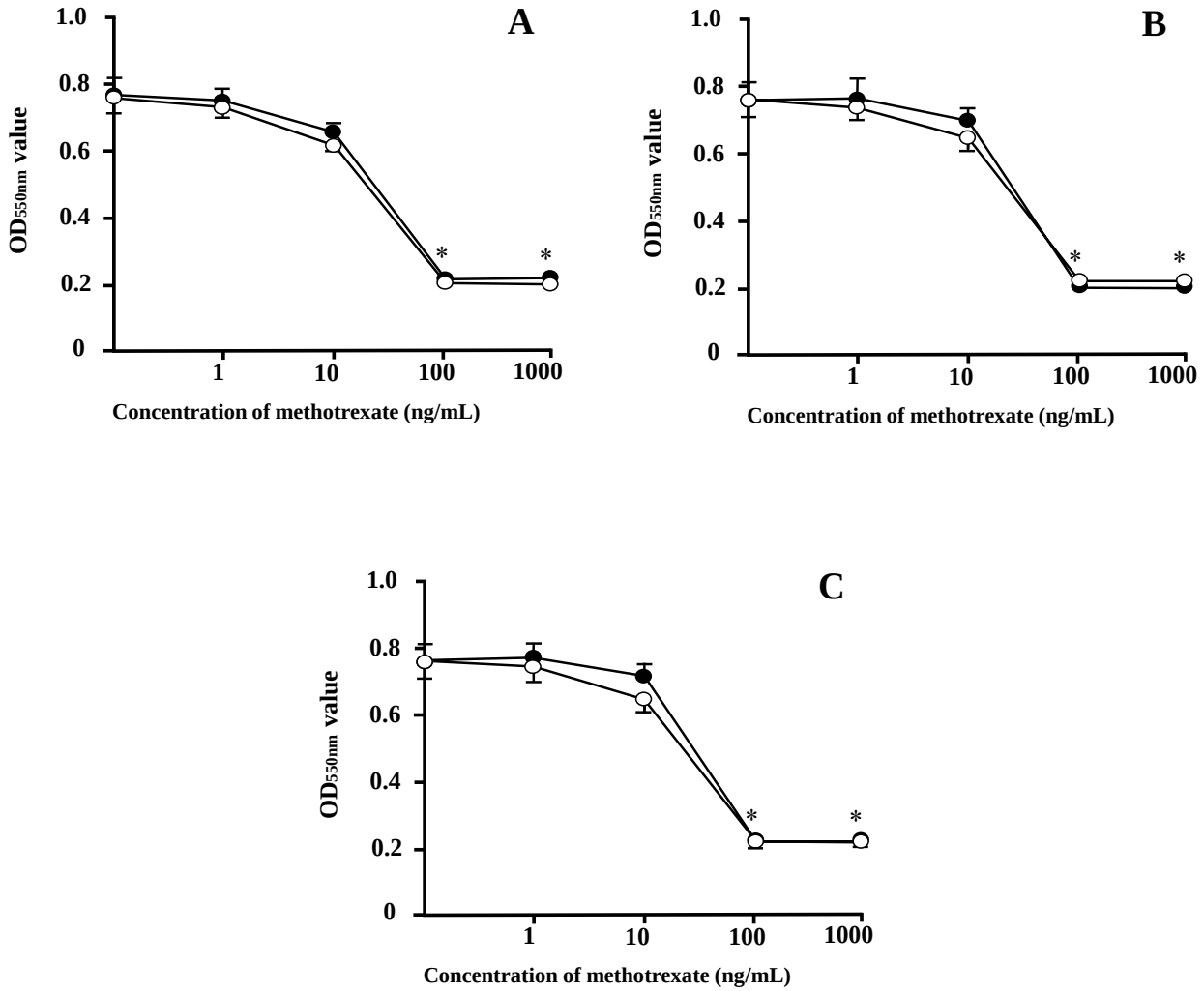

Fig. 2. Effects of folic acid added 24 h after beginning of methotrexate treatment on the methotrexate effects against concanavalin A-activated proliferation of PBMCs. PBMCs obtained from healthy volunteers were cultured for 24 h under serial concentrations of methotrexate in the presence of concanavalin A. Subsequently, folic acid or vehicle was added to the culture to give final concentrations of 0.5 (A), 5(B), and 50 (C) ng/mL. Then, the cells were cultured for an additional 72 h with (solid lines) or without (dashed lines) folic acid. After culturing, the proliferated cells were evaluated with MTT assay procedures as described in Materials and Methods. The means (SDs) of the OD values read at 550 nm after MTT assay were plotted. *$p < 0.05$ as compared to the control without methotrexate treatment, evaluated by Bonferroni test ($n = 12$). There was no significant difference in the mean OD value between culture wells treated with methotrexate in the absence of folic acid and those treated with methotrexate in the presence of folic acid.

after treatment by 100 ng/mL of methotrexate, in the presence or absence of folic acid, while the changes were not statistically significant. IL-17 concentrations in the culture medium tended to decrease after treatment by higher concentrations of methotrexate, in the presence or absence of folic acid, though the changes were not significant.

Influence of folic acid on the effects of methotrexate to modify percentages of $CD4^+$, $CD4^+CD25^+$ and $CD4^+CD25^+FoxP3^+$ cells in mitogen-activated PBMCs

Percentages of $CD4^+$, $CD4^+CD25^+$ and $CD4^+CD25^+FoxP3^+$ cells in the activated lymphocytes treated with methotrexate in the absence or presence of folic acid are shown in Figs. 5 and 6.

Table 1. IC_{50} values of methotrexate against the concanavalin A-activated proliferation of PBMCs in the presence of folic acid.

Addition of folic acid (hours after culturing)	Mean (SD) IC_{50} ng/mL
0h Folic acid concentration (ng/mL)	
0	23.28 (4.55)
0.5	46.47 (67.05)
5	45.54 (73.42)
50	37.77 (45.23)
24h Folic acid concentration (ng/mL)	
0	19.28 (6.29)
0.5	40.14 (58.62)
5	30.09 (28.01)
50	34.19 (60.78)

Methotrexate at concentrations less than 10 ng/mL had no effect on the percentage of $CD4^+$ cells in lymphocytes, whereas the drug at 100 ng/mL significantly decreased the percentage of $CD4^+$ cells ($p < 0.05$). Folic acid added to the culture at the same time with methotrexate (Fig. 5) or 24 h after the addition of methotrexate (Fig. 6) showed no significant influence on the methotrexate effects to decrease the $CD4^+$ cell rates. Methotrexate in the presence or absence of folic acid did not influence the percentages of $CD4^+CD25^+$ and $CD4^+CD25^+$ $FoxP3^+$ cells in the activated lymphocytes.

DISCUSSION

Folic acid is recommended for RA patients under methotrexate therapy to decrease risks of methotrexate adverse effects including myelosuppression and hepatotoxicity. Whereas, immunosuppressive efficacy of methotrexate may also be attenuated by concomitant use of folic acid. Thus, the present study was conducted to examine the influence of folic acid on the immunosuppressive efficacy of methotrexate using an *in vitro* model of mitogen-activated human PBMC cultures.

We used in the present study the human-PBMC culture system stimulated by concanavalin A as a T cell mitogen, since this culture system has been demonstrated to reflect the clinical efficacies of immunosuppressive drugs in patients with several autoimmune diseases and recipients of organ transplantations [13]. Our present data using this cell culture system demonstrated that, although methotrexate concentration-dependently suppressed the concanavalin A activated proliferation of human PBMCs, folic acid at concentrations of 0.5, 5, and 50 ng/mL did not influence the suppressive efficacies of methotrexate. Folic acid is generally administrated 24 h after intake of methotrexate in RA patients to relieve the methotrexate side effects. Then, we also examined in the present study the influence of folic acid added into PBMC culture 24 h after administration of methotrexate. We demonstrated again that the delayed addition of folic acid did not influence the suppressive efficacy of methotrexate against the mitogen-activated proliferation of PBMCs. The dose of folic acid clinically used in RA patients are less than 5 mg/day, which are suggested to give average serum concentrations of folic acid at 6.78 ± 2.88ng/mL [14]. Thus, the concentration range of the folic acid we used in the present study appears to be appropriate to simulate the blood concentration of folic acid in RA patients.

Whittle and Hughes suggested that folate supplementation at usual doses do not disturb the therapeutic efficacy of methotrexate in RA patients under methotrexate treatment [15]. Dhir *et al*. reported that, even with the high doses of methotrexate used in current practice, there was no additional benefit or harm of a higher dose of folic acid over a usual dose [6]. We also suggested in our previous study that high-dose folic acid did not influence the methotrexate efficacies and safety in Japanese RA patients under methotrexate treatment [1]. The *in vitro* data of the present study support these previous findings.

The observations in the effects of methotrexate on the secretion of inflammatory cytokines were somewhat complex. One of the reasons for these observations are suggested to be due to the PBMC incubation period, since the optimal incubation time for the mitogen-activated PMBCs to produce several inflammatory cytokines are not the same. The incubation time of PBMCs in the present study to produce the inflammatory cytokines was

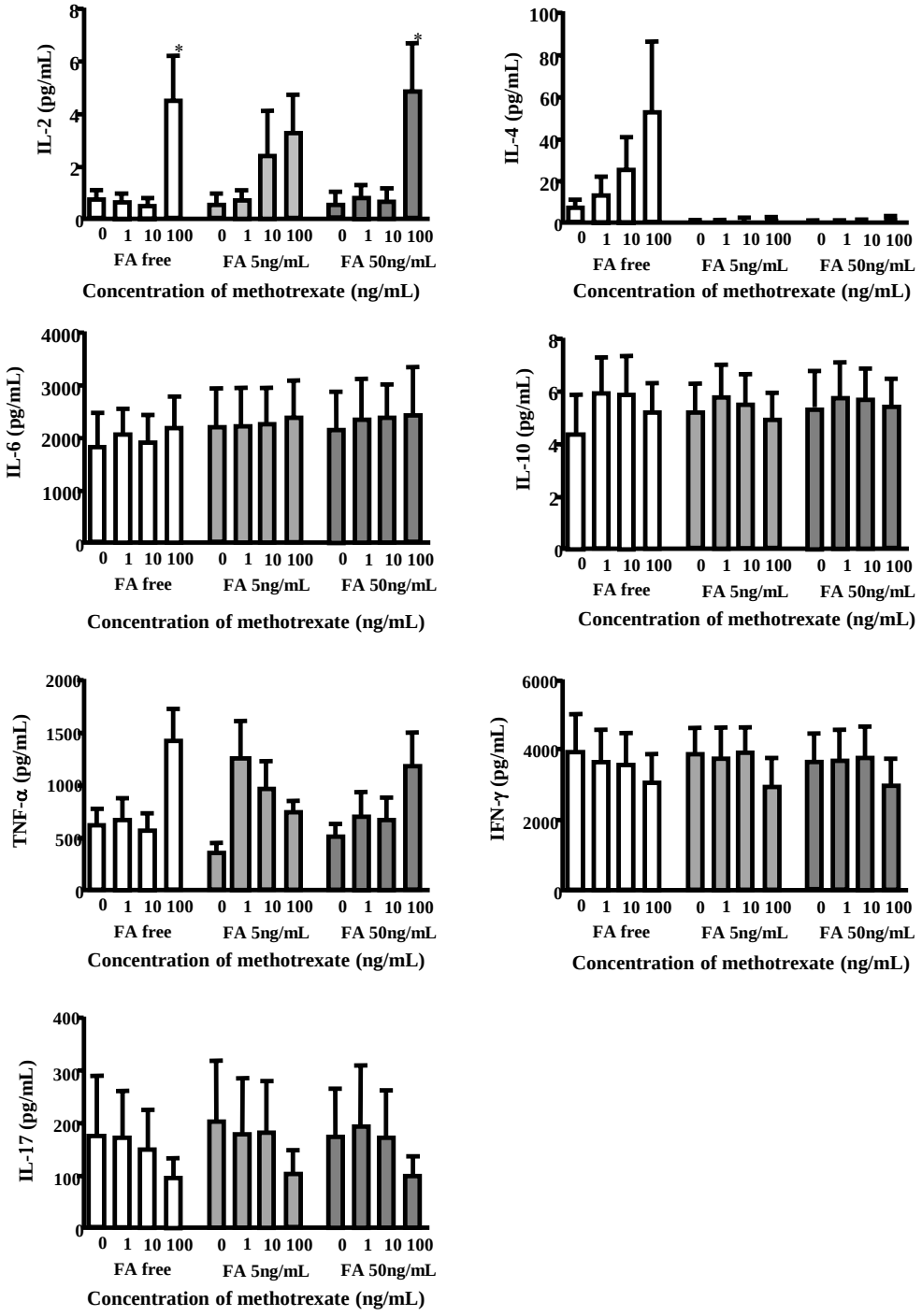

Fig. 3. Effects of methotrexate in the presence of folic acid on secretion of inflammatory cytokines from concanavalin A-activated PBMCs. PBMCs were cultured for 96 h under serial concentrations of methotrexate without (open bars) or with (shaded bars) folic acid in the presence of concanavalin A. After culturing, concentration of Th1/Th2/Th17 cytokines in medium was evaluated with bead array/flow cytometry procedures as described in Materials and Methods. The means (SDs) of cytokine concentrations were plotted. *$p < 0.05$ as compared to the control without methotrexate treatment, evaluated by Bonferroni test ($n = 12$). There was no significant difference in the cytokine concentration between culture wells treated with methotrexate in the absence of folic acid and those treated with methotrexate in the presence of folic acid.

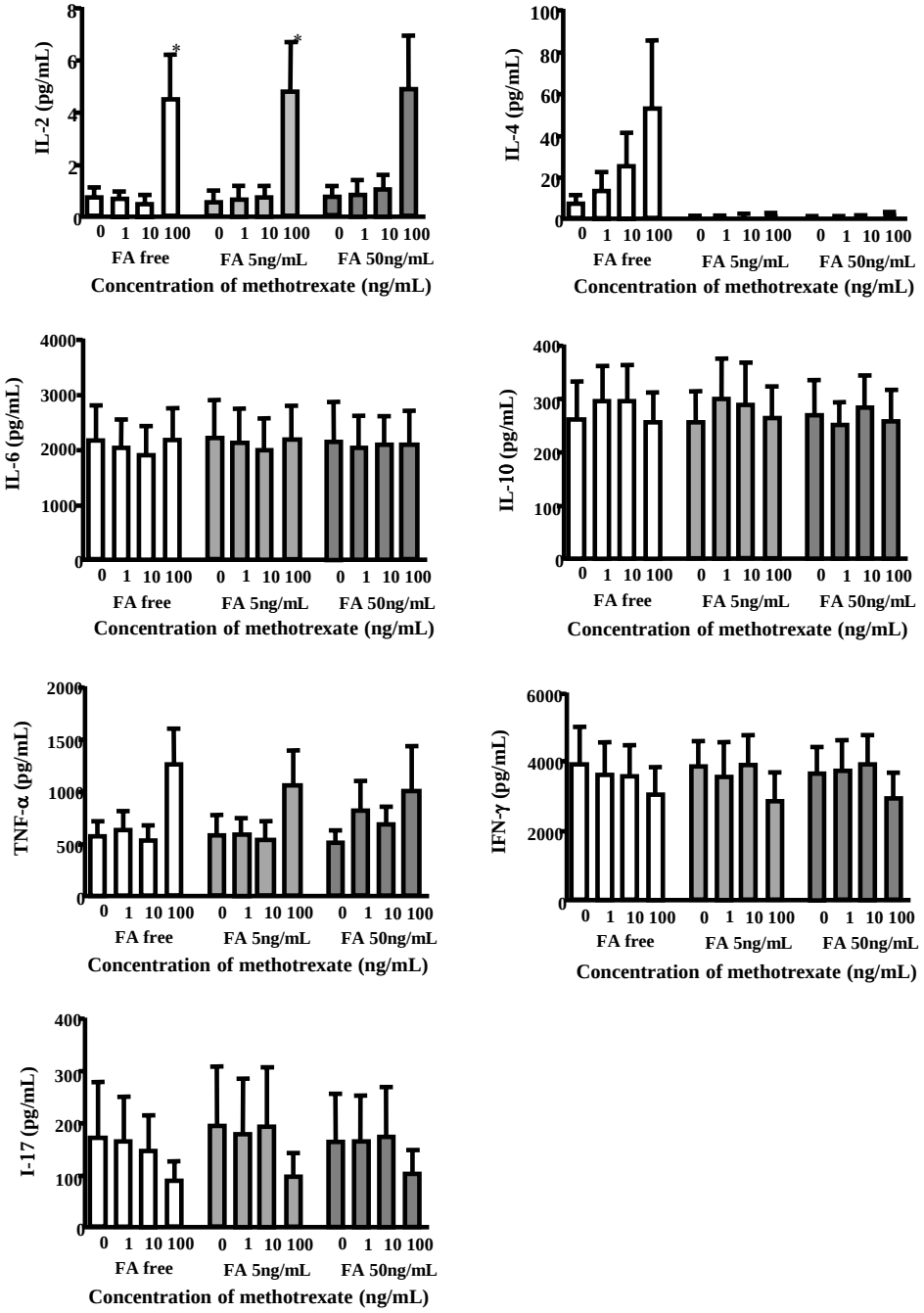

Fig. 4. Effects of folic acid added 24 h after beginning of methotrexate treatment on the effects of methotrexate against secretion of inflammatory cytokines from concanavalin A-activated PBMCs. PBMCs were cultured for 24 h under serial concentrations of methotrexate in the presence of concanavalin A. Subsequently, vehicle (open bars) or folic acid (shaded bars) was added to the culture to give final concentration of 5 or 50 ng/mL. Then, the cells were cultured for more 72 h with or without folic acid. After culturing, concentration of Th1/Th2/Th17 cytokines in medium was evaluated with bead array/flow cytometry procedures as described in Materials and Methods. The means (SDs) of cytokine concentrations were plotted. *$p < 0.05$ as compared to the control without methotrexate treatment, evaluated by Bonferroni test ($n = 12$). There was no significant difference in the cytokine concentration between culture wells treated with methotrexate in the absence of folic acid and those treated with methotrexate in the presence of folic acid.

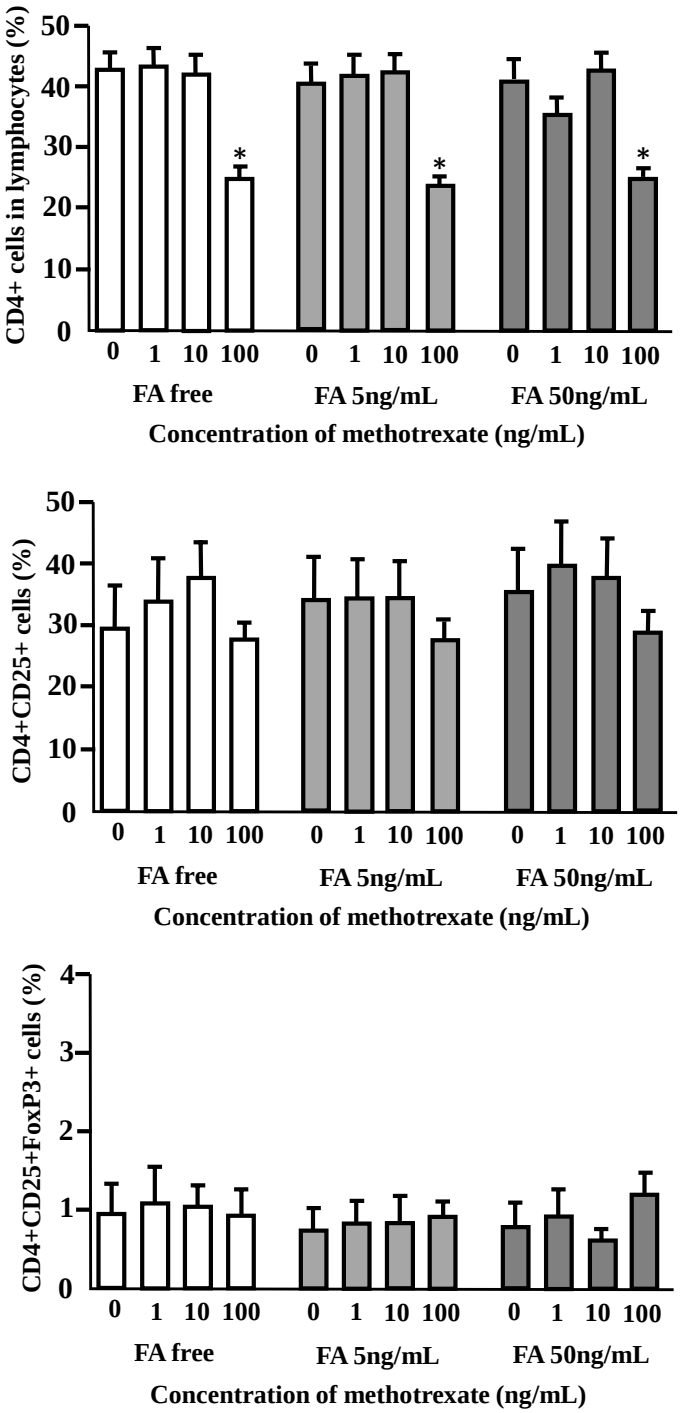

Fig. 5. Effects of methotrexate in the presence of folic acid on percentages of $CD4^+$, $CD4^+CD25^+$ and $CD4^+CD25^+FoxP3^+$ cells in concanavalin A-activated PBMCs. PBMCs were cultured for 96 h under serial concentrations of methotrexate without (open bars) or with (shaded bars) folic acid in the presence of concanavalin A. After culturing, percentages of $CD4^+$, $CD4^+CD25^+$ and $CD4^+CD25^+FoxP3^+$ cells in lymphocyte fraction were evaluated as described in Materials and Methods. The means (SDs) of the percentages of cells in lymphocyte fraction were plotted. *$p < 0.05$ as compared to the control without methotrexate treatment, evaluated by Bonferroni test ($n = 12$). There was no significant difference in the cell percentage between PBMCs treated with methotrexate in the absence of folic acid and those treated with methotrexate in the presence of folic acid.

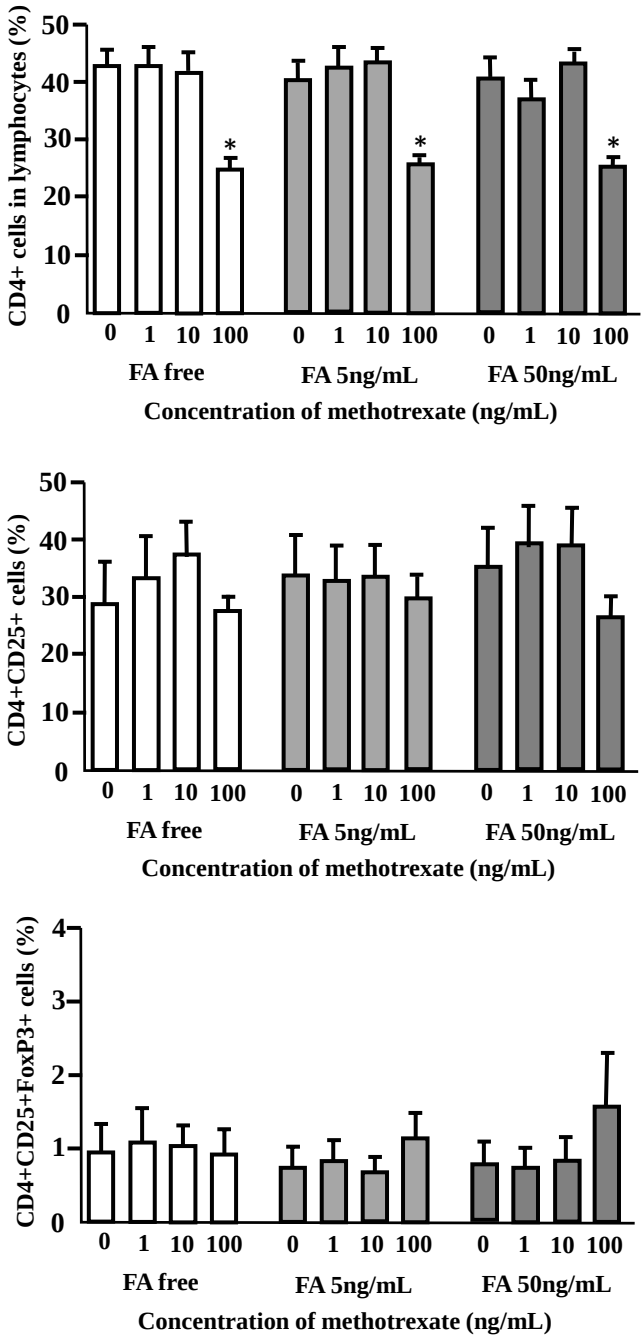

Fig. 6. Effects of folic acid added 24 h after beginning of methotrexate treatment on the influences of methotrexate against percentages of $CD4^+$, $CD4^+CD25^+$ and $CD4^+CD25^+FoxP3^+$ cells in concanavalin A-activated PBMCs. PBMCs were cultured for 24 h under serial concentrations of methotrexate in the presence of concanavalin A. Subsequently, vehicle (shaded bars) or folic acid (open bars) was added to the culture to give final concentration of 5 or 50 ng/mL. Then, the cells were cultured for an additional 72 h with or without folic acid. After culturing, percentages of $CD4^+$, $CD4^+CD25^+$ and $CD4^+CD25^+FoxP3^+$ cells in lymphocyte fraction were evaluated as described in Materials and Methods. The means (SDs) of the percentages of cells in lymphocyte fraction were plotted. *$p < 0.05$ as compared to the control without methotrexate treatment, evaluated by Bonferroni test ($n = 12$). There was no significant difference in the cell percentage between PBMCs treated with methotrexate in the absence of folic acid and those treated with methotrexate in the presence of folic acid.

96 h, which is too long to detect certain kinds of cytokines such as IL-2. The incubation time of 96 h to examine inflammatory cytokines was selected referring the incubation time of PBMCs for the cell proliferation assay. Thus, further studies are required to examine the absolute effect of methotrexate on the secretion of each cytokine from activated PBMCs. Methotrexate at the highest concentration (100 ng/mL) significantly increased IL-2 secretion in the PBMC culture medium from baseline levels, despite the presence of folic acid. Kremer *et al*. reported that significant amounts of IL-2 were released from PBMCs during the treatment by methotrexate *ex vivo* [16], the observation which is consistent with our present data. Though the mechanism of methotrexate to increase the IL-2 release from PBMCs is not clearly understood, they observed an inverse correlation between IL-2 in the sera and patient evaluation of pain [16]. Anyhow, our present data revealed that folic acid at concentrations up to 50 ng/mL did not modify the effects of methotrexate on cytokine secretion from concanavalin A-activated PBMCs.

In the present study, we also examined the effects of methotrexate on the ratio of $CD4^+$, $CD4^+$ $CD25^+$ and $CD4^+CD25^+FoxP3^+$, which reflect helper T cells, activated $CD4^+$ cells, and regulatory T cells in lymphocytes, respectively. Methotrexate at the highest concentration (100 ng/mL) significantly decreased the ratio of $CD4^+$ cells, whereas the drug did not significantly influence the ratio of $CD4^+CD25^+$ cells and regulatory T cells. Folic acid even at the highest concentration (50 ng/mL) did not change the effect of methotrexate to decrease the ratio of helper T cells. Regulatory T cells have been reported to play an important role in controlling immune responses, and the frequency of regulatory T cells is known to decrease in many autoimmune diseases, including RA [17]. Therapeutic drugs which may increase the frequency of regulatory T cells would be of benefit for the treatment of RA patients [17]. Methotrexate is reported to restore regulatory T cell function through demethylation of the FOXP3 upstream enhancer in patients with RA [18]. However, the present data suggest that methotrexate, in spite of the presence or absence of folic acid, does not modify the frequency of regulatory T cells.

CONCLUSIONS

Methotrexate at 1-1000 ng/mL dose dependently suppressed the proliferation of concanavalin A-activated PBMCs, while 0.5-50 ng/mL of folic acid did not significantly disturb the methotrexate suppressive efficacy. Addition of folic acid at 24 h after the beginning of PBMC culture also had no significant effect on the methotrexate efficacy. Folic acid showed no significant influence on the methotrexate effects against the secretion of inflammatory cytokines from the activated PBMCs. Methotrexate at 100 ng/mL with or without folic acid significantly decreased the percentages of CD+4 cells in lymphocytes, whereas the percentages of $CD4^+/CD25^+$ and $CD4^+/CD25^+/FoxP3^+$ cells were not affected by methotrexate and/or folic acid. We concluded that the suppressive efficacies of methotrexate on human PBMCs activated by T cell mitogen are not attenuated by clinically used concentrations of folic acid.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest to disclose.

ABBREVIATIONS

PBMCs	:	Peripheral-blood mononuclear cells
RA	:	Rheumatoid arthritis
MTT	:	3-(4,5-dimethylthiazol-2-yl)-2,5¬diphenyltetrazolium bromide
IL	:	Interleukin

REFERENCES

1. Tchantchou, F. and Shea, T. B. 2008, In: G. Litwack (Ed.), Vitamins and Hormones. Elsevier, 79, 83.
2. Schweitzer, B. I., Dicker, A. P. and Bertino, J. R. 1990, FASEB J., 4, 2441.
3. Shea, B., Swinden, M. V., Tanjong Ghogomu, E., Ortiz, Z., Katchamart, W., Rader, T., Bombardier, C., Wells, G. A., and Tugwell, P. 2013, Cochrane Database of Systematic Reviews, Issue 5, ArtNo.:CD000951
4. Suzuki, Y., Uehara, R., Tajima, C., Noguchi, A., Ide, M., Ichikawa, Y. and Mizushima, Y. 1999, Scand. J. Rheum., 28, 273.
5. Whittle, S. L. and Hughes, R. A. 2004, Rheumatology (Oxford), 43(3), 267.

6. Dhir, V., Sandhu, A., Kaur, J., Pinto, B., Kumar, P., Kaur, P., Gupta, N., Sood, A., Sharma, A. and Sharma, S. 2015, Arthritis Research & Therapy, 17, 156.
7. Kameyama, S., Kase, Y., Kurihara, S., Yoshida, F., Noda, M., Iiduka, T., Horiguchi, M., Sugiyama, K. and Hirano, T. 2017, Drug Res., 67(12), 705. doi: 10.1055/s-0043-117498. Epub 2017 Sep 12
8. Xu, W., Wang, X., Tu, Y., Masaki, H., Tanaka, S., Onda, K., Sugiyama, K., Yamada, H. and Hirano, T. 2019, Phytother. Res., 33(1), 187.
9. Sugiyama, K., Wada, S., Tanaka, S., Urai, K. and Hirano, T. 2019, Curr. Top. Pharmacol., 23, 93.
10. Meng, K., Xu, W., Miura, T., Suzuki, S., Chiyotanda, M., Tanaka, S., Sugiyama, K., Kawashima, H. and Hirano, T. 2018, Exp. Dermatol., 27, 1058.
11. Kusano, J., Tanaka, S., Matsuda, H., Hara, Y., Fujii, Y., Suzuki, S., Sekiyama, M., Ando, E., Sugiyama, K. and Hirano, T. 2018, J. Clin. Pharm. Ther., 43(6), 895.
12. Hatanaka. H., Ishizawa, H., Nakamura, Y., Tadokoro, H., Tanaka, S., Onda, K., Sugiyama, K. and Hirano, T. 2014, Life Sci., 99, 61.
13. Hirano, T. 2007, Int. Immunopharmacol., 7, 3.
14. Hiraga, Y., Yuhki, Y., Itoh, K., Tadano, K., Takahashi,Y. and Mukai, M. 2004, Modern Rheum., 14, 135.
15. Whittle, S. L. and Hughes, R. A. 2004, Rheumatology, 43, 267.
16. Kremer, J. M., Lawrence, D. A., Hamilton, R. and McInnes, I. B. 2016, RMD Open, 2(1), e000287. doi: 10.1136/rmdopen-2016-000287
17. Meyer, A, Wittekind, P. S., Kotschenreuther, K., Schiller, J., von Tresckow, J., Haak, T. H. and Kofler, D. M. 2020, Ann. Rheum. Dis., Published Online First: 21 Jan 2020. doi: 10.1136/annrheumdis-2019-216598
18. Cribbs, A. P., Kennedy, A., Penn, H., Amjadi, P., Green, P., Read, J. E., Brennan, F., Gregory, B. and Williams, R. O. 2015, Arthritis Rheum., 67, 1182. doi:10.1002/art.39031