

RESEARCH ARTICLE

## Immunomodulatory effects of the pentapeptide YGSRS on human peripheral-blood mononuclear cells

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### Abstract

**Context:** The pentapeptide YGSRS is originated from coffee bean, while its pharmacological features have little been examined.

**Objectives:** We investigated the effects of YGSRS on proliferation, cytokine production and CD4+ CD25+ Foxp3+ regulatory T (Treg) cell frequency of human peripheral blood mononuclear cells (PBMCs) activated by T-cell mitogen.

**Materials and methods:** The effects of YGSRS on T-cell mitogen-activated PBMCs were assessed by WST assay procedures. Concentrations of Th1/Th2/Th17 cytokines in the PBMCs culture medium were analyzed with beads-array procedures followed by analysis with flow cytometry. The CD4+ CD25+ Foxp3+ Treg cells in mitogen-activated PBMCs were stained with fluorescence-labeled specific antibodies followed by flow cytometry.

**Results:** YGSRS at 1–10 000 ng/ml (1.56–15 600 nM) has a tendency to promote the mitogen-activated proliferation of PBMCs, but the effects were not statistically significant. YGSRS affect the production of tumor necrosis factor (TNF)  $\alpha$ , interleukin (IL)-4, IL-6 and IL-10 from the activated PBMCs, and statistically significant increase in the concentrations of IL-6 and IL-10 in the medium were observed at 1–1000 ng/ml (1.56–1560 nM) ( $p < 0.05$ ). YGSRS has a tendency to decrease the frequency of Treg cells in the activated PBMCs, but the difference was not statistically significant.

**Discussion and conclusions:** The data suggest that the pentapeptide YGSRS affects the production of several types of cytokines from activated human peripheral T cells, which may modulate Th2 type immunity.

### Keywords

CD4+ CD25+ Foxp3+ regulatory T cells, cytokines, peripheral lymphocytes, T-cell mitogen, YGSRS

### History

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### Introduction

Immunomodulatory and anti-inflammatory activities of several types of naturally occurring or synthetic oligopeptides and peptides have been reported<sup>1–4</sup>. We have also found that certain types of peptide antibiotics suppress proliferation and several cytokine productions in mitogen-activated human peripheral blood mononuclear cells (PBMCs)<sup>5,6</sup>. A well-known immunosuppressive drug cyclosporine widely used for treatment of transplant recipients and patients with autoimmune disorders has also a cyclic peptide structure<sup>7</sup>. Gramicidin D-containing linear pentadecapeptides was reported to have immunosuppressive efficacy, which may be useful as maintenance immunosuppressive therapy for organ transplantations<sup>8</sup>.

The pentapeptide YGSRS was isolated from coffee bean and its chemical structure had been identified, whereas its biological and pharmacological activities have little been investigated so far. The observations concerning immunomodulatory activities of several types of peptides, as described earlier, remind us to examine immunomodulating efficacies of YGSRS using PBMCs. Increasing evidences support that regulatory T (Treg) cells contribute to the suppression of cellular immunity<sup>9</sup>, and thereby the activation and/or increased number of Treg cells may result in therapeutic efficacy against several immunological disorders with progressive autoimmune pathophysiology<sup>10,11</sup>. Thus, Treg cell frequency in patients with autoimmune diseases appears to be critical for the clinical outcome and the efficacy of immunosuppressive therapy in these patients.

The present study was thus conducted to examine the immunomodulatory efficacy of the pentapeptide YGSRS on proliferation, cytokine production and CD4+ CD25+ Foxp3+ Treg cell frequency in human PBMCs activated by T-cell mitogen. Our data suggest that the pentapeptide YGSRS affects the production of several types of cytokines from

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T-cell mitogen-activated human peripheral lymphocytes, which may modulate Th2 type immunity.

## Methods

### Materials

RPMI 1640 medium and fetal calf serum were purchased from Gibco Co., Grand Island, NY. Concanavalin A was obtained from Seikagaku Kogyo Co., Tokyo, Japan. YGSRS used in this study is a synthetic linear pentapeptide synthesized at Peptide Institute Inc. (Osaka, Japan) with purity of 99.4%, which was kindly provided by Dr. Henry Aoki, President at YGSRS Co., Acton, MA. The peptide was dissolved in distilled water at a concentration of 0.5 mg/ml as a stock solution. The stock solution was diluted to working concentrations by RPMI 1640 medium before use. Cyclosporine A was provided from Novartis Pharma Co., Tokyo, Japan. The cell proliferation WST-8 assay kits were obtained from Promega Co. (Madison, WI). BD™ cytometric bead array human Th1/Th2/Th17 cytokine kits, Human Foxp3 Stain Kit PE, Human CD4 FITC, Human CD25 allophycocyanin (APC), fluorescein isothiocyanate (FITC) Mouse IgG1 κ Isotype control, APC Mouse IgG1 κ Isotype control and phycoerythrin (PE) Mouse IgG1 κ Isotype control were obtained from BD Biosciences, San Jose, CA. MEMα medium was from Lonza Japan Co. (Tokyo, Japan). Prednisolone, Cell Lysis solution and trypan blue were purchased from Sigma Chemical Co. (St. Louis, MO). Sterilized plastic plates for cell cultures were obtained from Iwaki Co. (Tokyo, Japan). All the 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 (Number: 10-01) and written informed consent was obtained from all the healthy volunteers included in the study. The study includes totally eight healthy subjects (four male and four female; mean age of 30.8 years). These subjects had neither a history of immunological disorders nor a history of taking immunosuppressive drugs including glucocorticoids.

### Isolation of PBMCs and evaluation of anti-proliferative effects *in vitro*

Twenty milliliters of venous blood were taken from healthy subjects between 9:30 and 11:00 in the morning and heparinized. This 20 ml sample size was the smallest possible to examine the effects of the peptide YGSRS, cyclosporine and prednisolone. The heparinized blood was loaded on 3 ml of Ficoll-Hypaque (Nakarai Co., Japan), centrifuged at  $1300 \times g$  for 20 min and PBMCs were separated as described previously<sup>12-14</sup>. For evaluation of the effects of the peptide on PBMCs, the cells were washed and suspended in RPMI 1640 medium containing 10% fetal bovine serum, 100 000 IU/l penicillin and 100 mg/l streptomycin to a final density of  $1 \times 10^6$  cells/ml. About 196 μl of this cell suspension were loaded into wells of a 96-well plate. Concanavalin A, as a T-cell mitogen, was added to each well to a final

concentration of 5.0 μg/ml. Subsequently, 4 μl of an ethanol solution containing the peptide YGSRS were added to give final concentrations of 1–10 000 ng/ml (1.56–15 600 nM). Four microliters of ethanol were added to the control wells. The plate was incubated for 96 h in 5% CO<sub>2</sub>/air at 37° C. After the culture, 20 μl of Cell Counting Kit-8 reagent solution was added to each well, and the plate was incubated for another 2 h. PBMC proliferation was determined by measuring the optical density at 450 nm absorbance (ref. 650 nm).

### MCF-7 cell culture

MCF-7 cells were maintained in MEMα medium containing 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. These cells at densities of  $1-5 \times 10^5$  cells/ml suspended with each medium were incubated in 5% CO<sub>2</sub>/air at 37°C for 3–4 days in a humidified chamber. The cell number of the cell suspension was adjusted to  $1-5 \times 10^5$  living cells/ml, by counting living cells with a trypan-blue dye exclusion test<sup>15</sup>. Subsequently, 98 μl of this cell suspension were placed in each well of a 96-well flat-bottomed plate, and 2 μl of the solution containing YGSRS were added to yield final peptide concentrations of 1–10 000 ng/ml (1.56–15 600 nM), respectively. The cell suspensions were mixed, incubated for 72 h and then the proliferated cells were analyzed with WST assay using Cell Counting Kit-8 as described earlier.

### Cytokine analysis

Peripheral blood mononuclear cells were incubated for 96 h in the presence of concanavalin A and the peptide YGSRS as described earlier, and the culture supernatant was stored at –80°C until measurement of cytokine concentrations. The concentrations of interferon-γ (IFN-γ), tumor necrosis factor (TNF) α, interleukin (IL)-2, IL-4, IL-6, IL-10 and IL-17 in the supernatant of culture medium were measured with bead-array procedures using Human Th1/Th2/Th17 Cytokine Kit, followed by flow cytometry<sup>16</sup>, according to the instructions of BD Biosciences (San Jose, CA).

### CD4+ CD25+ Foxp3+ Treg cell analysis

The analysis was carried out according to the manufacturer's instructions (BD Biosciences), which has been reported in our previous study<sup>17</sup>. In brief, PBMCs treated with the peptide YGSRS in the presence of concanavalin A were washed twice with phosphate-buffered saline (PBS) and re-suspended with PBS at a cell density of  $1 \times 10^6$  cells/ml. To 1 ml of this cell suspension, 20 μl of Human CD4 FITC and 20 μl of Human CD25 APC (BD Biosciences) were added. Twenty microliters of FITC Mouse IgG1 κ Isotype control and 20 μl of APC Mouse IgG1 κ Isotype control (BD Biosciences) were added to the control cells. These cell suspensions were incubated for 20 min in the dark. After incubation, the cells were washed with PBS and resuspended with 1 ml of PBS. Then, 1 ml of 10-fold diluted Human Foxp3 Buffer A (BD Biosciences) 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) diluted with the 10-fold diluted Human Foxp3 Buffer A, and incubated for 30 min in the dark.

After incubation, 1 ml of PBS was added and centrifuged for 5 min. The supernatant was removed and the cells were washed with PBS. Then, 20  $\mu$ l of Human Fc $\gamma$ 3 PE or 20  $\mu$ l of PE Mouse IgG1  $\kappa$  Isotype control for the control wells were added, and the cell suspension was incubated for 30 min in the dark. After washing the cells with PBS, the cells were re-suspended with 0.6 ml of PBS, and then analyzed with flow cytometry.

The data were analyzed with a FACSCan™II (BD Biosciences), using CellQuest software (Becton Dickinson, Franklin Lakes, NJ)<sup>18</sup>. CD4+ cells in the lymphocyte fraction were gated, and the percentages of CD4+ CD25+ Foxp3+ cells, as Treg cells, in the CD4+ cell fraction were calculated.

### Statistics

Differences in the percentages of PBMC-proliferation, amounts of cytokines produced in the culture medium, and percentages of Treg cells in the CD4+ cell fraction after cell culture in the presence of serial concentrations of the peptide YGSRS were analyzed with Bonferroni tests. The correlation coefficients between the concentrations of any two kinds of cytokines in PBMC culture medium were analyzed with the Spearman test. These analyses were performed with GraphPad PRISM 4.0 (GraphPad Software Inc., San Diego, CA). In each case, two-sided *p* values <0.05 were considered to be significant.

## Results

### Effects of the pentapeptide YGSRS on proliferation of mitogen-activated PBMCs

We first examined the effects of the pentapeptide YGSRS on cell proliferation of human PBMCs activated with a T-cell mitogen, concanavalin A, *in vitro*. The PBMCs were cultured in the presence of concanavalin A and serial concentrations of YGSRS for 96 h, and the proliferative response of PBMCs was estimated by WST assay procedures (Figure 1). YGSRS appeared to modulate the mitogen-activated proliferation of PBMCs, but the effects showed wide individual differences between subjects, and no statistically significant change was observed by 1–10 000 ng/ml (1.56–15 600 nM) YGSRS (Figure 1A). Immunosuppressive drugs cyclosporine and prednisolone, used as positive controls, dose-dependently inhibited the concanavalin A-stimulated proliferation of PBMCs (Figure 1B).

YGSRS also showed no effects on the *in vitro* growth of cells of a human breast cancer cell line MCF-7 at a concentration range of 1–10 000 ng/ml (1.56–15 600 nM) (Figure 1C).

### Effects of YGSRS on cytokine production from mitogen-activated PBMCs

The effects of YGSRS on the production of Th1/Th2/Th17 cytokines from the T-cell mitogen-activated PBMCs were

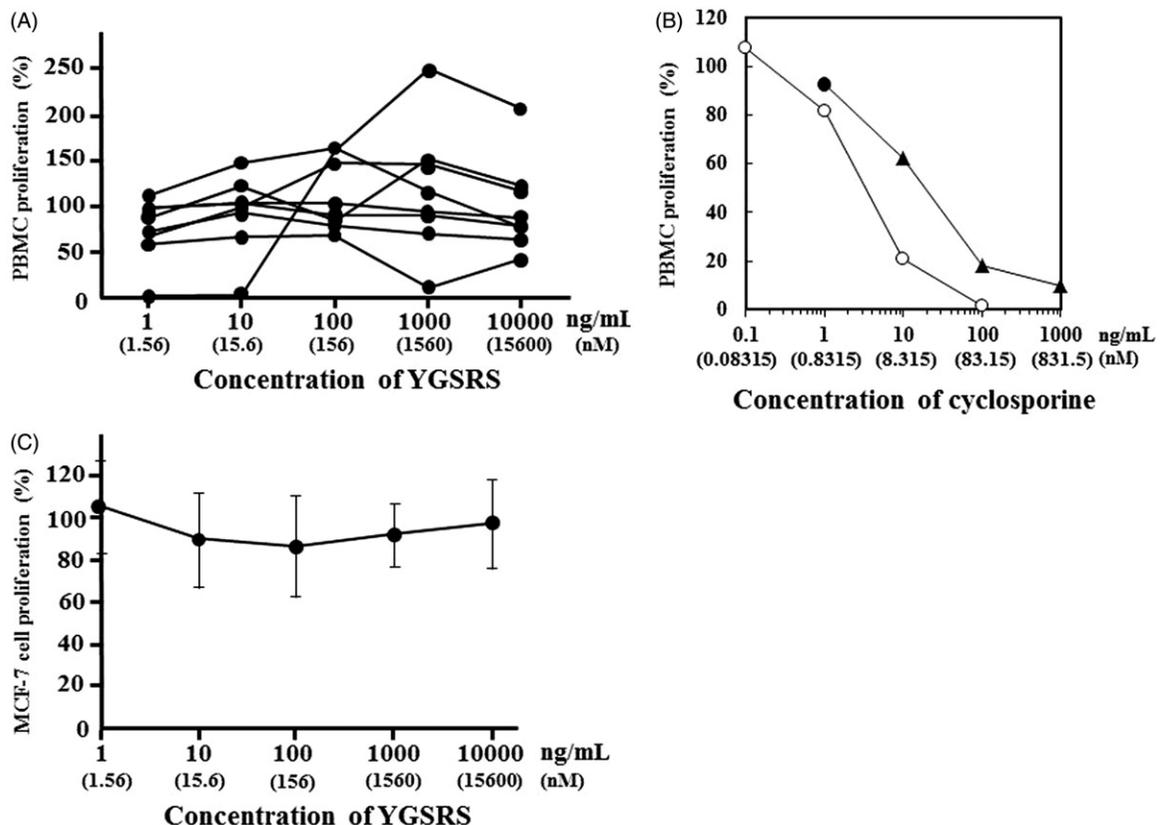


Figure 1. Effects of YGSRS on proliferation of human PBMCs activated with concanavalin A and cells of a breast cancer cell line MCF-7 *in vitro*. (A) PBMCs were cultured with concanavalin A and serial concentrations of YGSRS for 96 h, and the cell proliferation was analyzed as described in “Materials and methods” section. Dose–response curves of the peptide on the proliferation of PBMCs obtained from eight individual healthy subjects were expressed. Statistically significant changes by YGSRS, as compared to control (0 ng/ml), were not observed. (B) Effects of cyclosporine (open circle) and prednisolone (closed circle) on the proliferation of human PBMCs activated with concanavalin A. Typical data of each drug were shown. (C) Dose–response curve of MCF-7 cell proliferation in the presence of YGSRS. The mean (SD) of five independent experiments were shown. Statistically significant changes by YGSRS, as compared to control (0 ng/ml), were not observed.

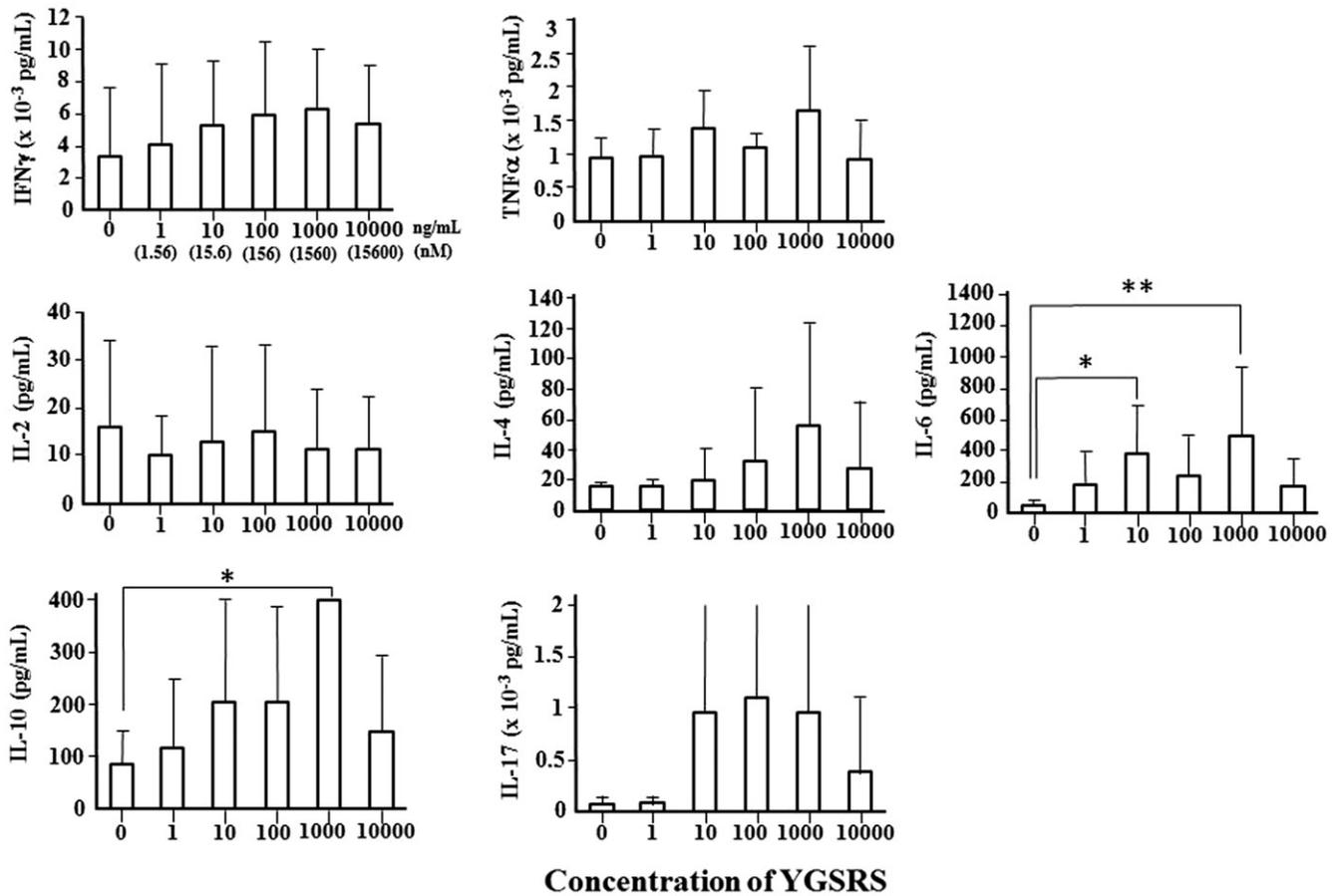


Figure 2. Cytokine concentrations in the supernatant of T-cell mitogen-activated PBMCs treated with YGSRS. PBMCs were stimulated with concanavalin A for 96 h in the absence (0 ng/ml) or presence (1–10 000 ng/ml) (1.56–15 600 nM) of YGSRS. Then, the culture supernatant was analyzed for concentrations of IFN- $\gamma$ , TNF- $\alpha$ , IL-2, IL-4, IL-6, IL-10 and IL-17 ( $n=4$  for each) using beads-array procedures followed by flow cytometry. \* $p < 0.05$  and \*\* $p < 0.01$ , as compared to each control.

examined (Figure 2). YGSRS significantly increased the concentration of IL-6 and IL-10 in the medium of the mitogen-activated PBMC culture at 10–1000 ng/ml (15.6–1560 nM) ( $p < 0.05$  or  $p < 0.01$ ). YGSRS also appeared to affect the production of IFN- $\gamma$ , TNF- $\alpha$ , IL-4 and IL-17A from the activated PBMCs, but the changes were not statistically significant.

The concentrations of IL-6 and IL-10 in the PBMC culture medium stimulated with concanavalin A in the presence of several concentrations of YGSRS were significantly correlated each other, as can be seen in Figure 3. Whereas, no significant correlation was observed between concentrations of any other two types of cytokines produced in the presence of YGSRS ( $r = 0.6167$ ,  $p = 0.0004$ ).

#### Effects of YGSRS on the frequency of CD4+ CD25+ Foxp3+ Treg cells in mitogen-activated PBMCs

The percentages of CD4+ CD25+ Foxp3+ Treg cells in mitogen-activated PBMCs cultured in the presence or absence of YGSRS for 96 h were analyzed with flow cytometry. CD4+ cells in the lymphocyte fraction of the T-cell mitogen-activated PBMCs were gated, and the percentages of CD25+ Foxp3+ cells in the CD4+ cell fraction (Treg cells) were calculated (Figure 4). YGSRS at concentrations >1 ng/ml tended to decrease the percentages of CD4+ CD25+ Foxp3+ Treg cells in CD4+ cells in lymphocytes, but the changes

were not statistically significant (Figure 4). We also examine the relationship between mitogen-stimulated PBMC proliferation and the ratios of CD4+ CD25+ Foxp3+ Treg cells treated by YGSRS, but there was no significant correlation (Figure 5).

#### Discussion

Several types of naturally occurring or synthetic oligopeptides and peptides have been reported to show immunomodulatory and anti-inflammatory activities<sup>1–4</sup>. Clinically used immunosuppressive drug cyclosporine has a cyclic peptide structure<sup>7</sup>. We have also reported that gramicidin D, which contains linear pentadecapeptides, shows immunosuppressive efficacy in rat heterotopic heart transplantation model<sup>8</sup>. The present study was undertaken to examine the immunomodulatory efficacies of a linear pentapeptide YGSRS *in vitro* using PBMCs obtained from healthy subjects. Although precise underlying mechanism remains to be elucidated, our data suggest that YGSRS affects the production of several types of cytokines from activated human peripheral T cells, which may modulate Th2 type immunity.

Helper T (Th) cells play critical roles in orchestrating adaptive immune responses. They exert such functions mainly through secreting cytokines and chemokines that activate and/or recruit target cells. An imbalance between Th1 and Th2 cytokine production has been shown to play a key role in the

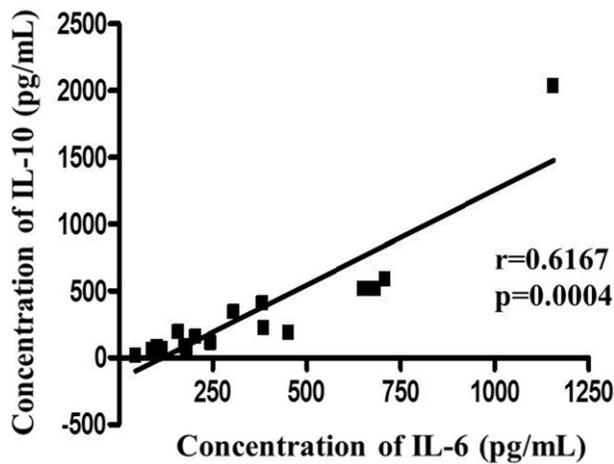


Figure 3. Correlation between concentrations of IL-6 and IL-10 in the PBMC culture medium stimulated with concanavalin A in the presence of several concentrations (1–10 000 ng/ml) (1.56–15 600 nM) of YGSRS. A statistically significant positive correlation was observed between the two indices ( $r = 0.6167$ ,  $p = 0.0004$ ).

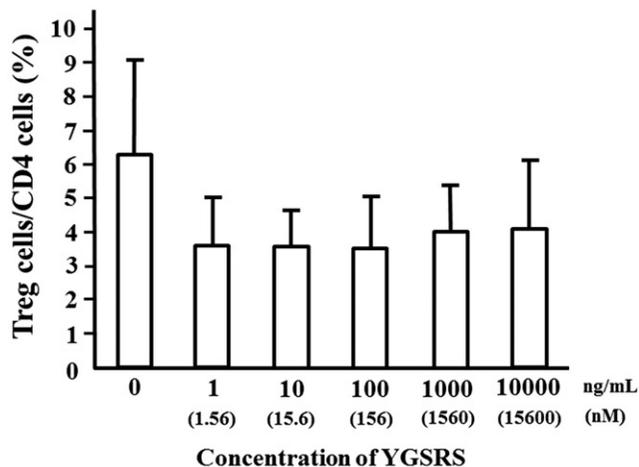


Figure 4. Flow cytometric analysis of CD4+ CD25+ Foxp3+ cells (Treg cells) in concanavalin A-stimulated PBMCs treated with YGSRS. The mean percentages of CD4+ CD25+ Foxp3+ Treg cells in concanavalin A-stimulated PBMCs cultured in the presence or absence of serial concentrations (1–10 000 ng/ml) (1.56–15 600 nM) of YGSRS for 96 h were shown. The data were indicated as the mean (SD) percentages of six independent experiments using PBMCs obtained from six individual healthy subjects. Statistically significant changes by YGSRS, as compared to control (0 ng/ml), were not observed.

induction and development of autoimmune diseases<sup>19–22</sup>. The pentapeptide YGSRS was isolated from coffee bean and its chemical structure had been identified, whereas its biological and pharmacological activities have little been investigated so far. The present data suggest that YGSRS promoted, rather than suppressed, many types of Th1/Th2/Th17 cytokines, especially IL-6 and IL-10. Although individual differences were observed between the PBMC donors in the effects of YGSRS on the proliferation of T-cell mitogen-activated PBMCs, the effects of YGSRS appeared to be also promotive, rather than suppressive, on the PBMC proliferation. These observations suggest that the modulatory effects of YGSRS on cytokine production are related to the effects of the peptide on T-cell proliferation. Thus, the data suggest that YGSRS

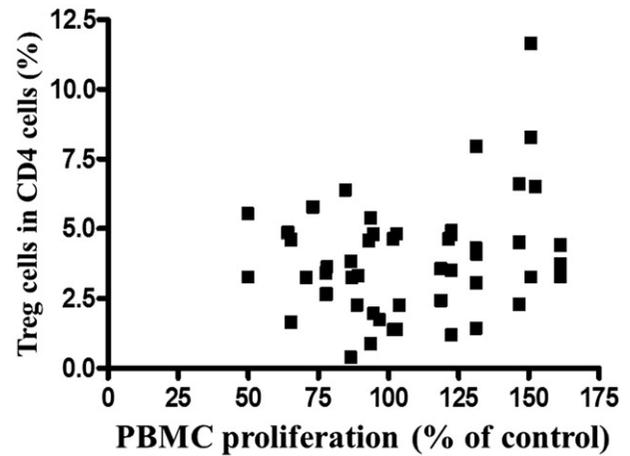


Figure 5. Relationship between the PBMC proliferative indices (% of control: see Figure 1) and CD4+ CD25+ Foxp3+ cell ratios (Figure 4) under stimulation by concanavalin A in the presence of several concentrations (1–10 000 ng/ml) (1.56–15 600 nM) of YGSRS. Statistically significant correlation was not observed.

affected function of helper T cells in PBMCs. Cyclosporine is known to inhibit production of several cytokines including IL-2 from human PBMCs activated by T-cell mitogen<sup>8</sup>, and this agent suppressed the concanavalin A-induced PBMC proliferation at concentrations <10 ng/ml dose-dependently in the present assay system (Figure 1B). As described earlier, we observed a promotive effect of YGSRS on activated PBMC proliferation, as well as an enhancement of secretion of IFN- $\gamma$ , TNF- $\alpha$ , IL-4 and IL-17A, up to 1000 ng/ml (1560 nM). Although these effects of the peptide were not statistically significant, the data suggest immune enhancing efficacies of YGSRS. These effects are possibly implicated in anticancer effects of this peptide by modulating tumor immunity, and are thus worth pursuing of this peptide in future studies.

CD4+ Th cells can be induced to differentiate toward Treg and Th17 phenotype according to the local cytokine milieu<sup>23</sup>. Treg provide a substantial component of the autoimmune counterbalance, and the thymus plays a role in Treg development and induction of tolerance<sup>24</sup>. Treg cells are reported to have a suppressive role on cellular immunity<sup>9</sup>, and therefore, Treg cell frequency in patients with autoimmune or malignant diseases appears to be critical for the clinical outcome of these patients. However, YGSRS did not significantly change the relative percentages of Treg cells in CD4+ cells in peripheral lymphocytes (Figure 4). Moreover, the relative percentages of Treg cells in CD4+ cells did not correlate with the proliferation indices of mitogen-stimulated PBMCs in the presence of serial concentrations of YGSRS (Figure 5). We detected Treg cells in the peripheral-blood samples of healthy subjects as CD4+ CD25+ Foxp3+ lymphocytes in the present study, since Foxp3 has been reported to define identity and function of Treg in these lymphocytes as described earlier<sup>25</sup>. Thus, we thought that the CD4+ CD25+ Foxp3+ lymphocytes we detected in the present study have typical Treg features and functions. The increase in the prototypical Treg cytokine IL-10 by treatment with YGSRS may relate to increased frequency of Treg, but we did not observe such correlation in the present study as

described earlier. Thus, we suggest that the immunomodulation by YGSRS is independent of Treg frequency in PBMCs.

Our present study, in summary, raises the possibility that the pentapeptide YGSRS affects the production of several types of cytokines from T-cell mitogen-activated human peripheral lymphocytes, which may modulate Th2 type immunity.

## Conclusions

This study was conducted to examine the effects of the pentapeptide YGSRS on the proliferation, cytokine production and CD4+ CD25+ Foxp3+ Treg cell-frequency in human PBMCs activated by T-cell mitogen *in vitro*. YGSRS did not significantly affect the mitogen-activated proliferation of PBMCs, though the effects were somewhat promotive. YGSRS at a concentration range of 1–1000 ng/ml (1.56–1560 nM) tended to promote the production of Th1/Th2/Th17 cytokines from the activated PBMCs, and the effects on IL-6 and IL-10 production were statistically significant ( $p < 0.05$ ). YGSRS did not significantly affect Treg cell-frequency in the activated PBMCs. These observations suggest that the pentapeptide YGSRS affects the production of several types of cytokines from activated human peripheral T cells, which may modulate Th2 type immunity.

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## Declaration of interest

The authors declare that there are no conflicts of interest in this study.

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