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European Journal of Pharmacology 507 (2005) 301–310

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The herbal medicine Sho-Saiko-To selectively inhibits $CD8^+$ T-cell proliferation

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Received 12 November 2004; accepted 16 November 2004
Available online 6 January 2005

Abstract

Sho-saiko-to (SST), a Chinese/Japanese traditional herbal medicine, has been widely used to treat chronic hepatitis in Japan, and the immunomodulatory properties of SST are likely to mediate its beneficial effect. In the present study, we examined the effects of SST and its various ingredients on the count and proliferation of T-cell subsets in cultured splenocytes and hepatic mononuclear cells. SST, wogonin-7-*O*-glucuronoside (a major SST ingredient), and wogonin (an intestinal metabolite of wogonin-7-*O*-glucuronoside) increased CD4/CD8 ratio via a decrease of $CD8^+$ T-cell counts with no effect on $CD4^+$ T-cell counts. Flow cytometric analyses of viability, proliferation, and cell cycle revealed that wogonin suppressed $CD8^+$ T-cell proliferation without inducing cell death. SST and wogonin administered to mice increased the CD4/CD8 ratio in hepatic mononuclear cells but not in splenocytes. These findings suggest that SST may modulate the CD4/CD8 ratio via the selective inhibition of $CD8^+$ T-cell proliferation by the SST ingredient wogonin-7-*O*-glucuronoside or its metabolite wogonin.

Keywords: Sho-saiko-to, (SST); Wogonin; CD4/CD8 ratio; $CD8^+$ T-cell; Proliferation

1. Introduction

In Japan, certain traditional herbal medicines (Kampo-medicines), which have been used for more than 1000 years, are now being manufactured as approved ethical drugs with standardized quality and quantities of ingredients. One such medicine, Sho-saiko-to (SST), has been shown to have protective effects against hepatitis (Okita et al., 1993; Tajiri et al., 1991) and fibrosis (Sakaida et al., 1998; Shimizu et al., 1999) in various experimental and clinical studies, including a double-blind multicenter clinical trial for liver injury in patients with chronic activate hepatitis C virus (HCV) infection (Hirayama et al., 1989). SST, therefore, has been widely used for the treatment of chronic HCV hepatitis and various chronic inflammatory diseases. Several reports have noted that HCV patients often have various immunological abnormalities (Tajiri et al., 1991; Oka et al., 1995). SST has a wide variety of immunomodulating effects (Yamashiki et al., 1992, 1997a,b; Fujiwara et al., 1995), including the ability to stimulate the recovery of abnormal cytokine responses by peripheral blood mononuclear cells in HCV patients (Yamashiki et al., 1997b). Therefore, in addition to antioxidant properties (Sakaguchi et al., 1993; Shiota et al., 2002; Egashira et al., 1999) and antiproliferative activity against hepatic stellate cells in hepatic fibrosis (Shimizu, 2000; Inoue and Jackson, 1999; Kayano et al., 1998; Liu et al., 1998), SST may ameliorate chronic viral liver diseases via modulating the immune response to viral infection. We have been investigating SST's immunomodulating effect in mice and have previously reported that SST and its ingredients modulate mitogenic activity, cytokine production, and, in some cases, the immune cell

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doi:10.1016/j.ejphar.2004.11.037

populations of mononuclear cells of the spleen, lung, and liver both in vitro and in vivo (Ohtake et al., 2000, 2002).

The balance between $CD4^+$ and $CD8^+$ T-cells has been suggested to reflect the aberrant immunological status induced by inflammatory diseases and may also be involved in inflammatory disease pathogenesis. For example, several investigators have reported aberrant lymphocyte CD4/CD8 ratios in patients with various diseases, such as chronic viral hepatitis (Pham et al., 1994; Fei et al., 1999; Khakoo et al., 1997), alcoholic hepatitis (Sakai et al., 1993), human immunodeficiency virus (HIV) infection (Sharma et al., 1997), Paget's disease (Rapado et al., 1994), measles virus infection (Myou et al., 1993), and asthma (O'Sullivan et al., 2001). In the present study, to clarify whether SST affects the CD4/CD8 ratio, we investigated the modulatory effects of SST and its ingredients on cell count, cell cycle, and DNA synthesis of T-cell subsets, both in vitro and in vivo.

2. Materials and methods

2.1. Preparation and analysis of SST

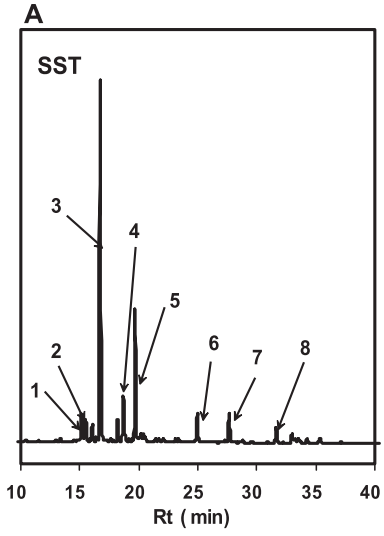
Spray-dried extract powder of SST was obtained from Tsumura & Co. (Tokyo, Japan). SST consisted of the hot water extracts from a mixture of 7.0 g of Radix of *Bupleurum falcatum* (Bupleurum Radix), 5.0 g of Tuber of *Pinellia ternate* (Pinelliae Tuber), 3.0 g of Radix of *Scutellaria baicalensis* (Scutellariae Radix), 3.0 g of Fructus of *Zizyphus jujube* (Zizyphi Fructus), 3.0 g of Radix of *Panax ginseng* (Ginseng Radix), 2.0 g of Radix of *Glycyrrhiza uralensis* (Glycyrrhizae Radix), and 1.0 g of Rhizoma of *Zingiber officinale* (Zingiberis Rhizoma). SST was analyzed by high-performance liquid chromatography (HPLC; Shimadzu, Tokyo, Japan). The representative major ingredients of SST detected at 254 nm are shown in Fig. 1A.

2.2. Animals

Male BALB/c mice (6–9 weeks old) were obtained from Charles River Japan (Yokohama, Japan), housed 4–6 per cage, and provided with standard mouse chow. All mice were fed under specific pathogen-free conditions. All animals were treated humanely, in accordance with the institution's guidelines for the care of animals.

2.3. Preparation of cells

Hepatic mononuclear cells were isolated as follows. Briefly, the liver was removed from mice sacrificed under anesthesia with sodium pentobarbital, pressed through a 100-μm nylon mesh, and then suspended in RPMI 1640 (Gibco BRL, Rockville, MD, USA) containing 1% penicillin/streptomycin (Sigma, St. Louis Missouri, USA), 10 mM HEPES (Sigma), 50 μM 2-mercaptoethanol (Wako, Tokyo, Japan), and 2% heat-inactivated fetal bovine serum (Dainippon pharmaceutical, Osaka, Japan). After washing twice with RPMI 1640/2% fetal bovine serum, hepatic mononuclear cells were isolated from both the hepatocytes and the nuclei of the hepatocytes by gradient centrifugation with 35% Percoll containing 100 IU/ml heparin (Amersham Biosciences, NJ, USA). The pellet was resuspended in an ACK lysis solution (155 mM NH_4Cl, 0.1 mM Na_2EDTA, 10 mM $KHCO_3$, pH 7.2) to remove red blood cells (RBC), and then washed twice in RPMI 1640/2% fetal bovine serum. Splenocytes were isolated as follows. Briefly, the harvested spleen was pressed through a 70-μm nylon mesh and then suspended in RPMI 1640/5% fetal bovine serum. After



B

Ingredients of SST	Content (μmol/g)	Aglycons
1 liquiritin apioside	7.7	liquiritigenin
2 liquiritin	8.5	liquiritigenin
3 baicalin	100.1	baicalein
4 oroxylin A-7-*O*-glucuronoside	5.5	oroxylin A
5 wogonin-7-*O*-glucuronoside	14.1	wogonin
6 glycyrrhizin	10.4	glycyrrhetic acid
7 baicalein	7.6	baicalein
8 saikosaponin b2	1.9	saikogenin D

Fig. 1. High-performance liquid chromatographic profile of SST. SST (1.51 mg) was applied to a column of TSK GEL ODS-80TS. (A) The chromatographic profile was obtained using a linear gradient of 0.05 M $AcONH_4$–AcOH buffer (pH 3.6) and 100% CH_3CN. The effluent from the column was analyzed at 254 nm. (B) Ingredients of SST and their metabolites formed by intestinal bacterial hydrolysis following the oral administration of SST. The contents of the ingredients in SST are shown.

washing once with RPMI 1640/5% fetal bovine serum, the cell pellet was resuspended in ACK lysis solution and then washed twice in RPMI 1640/5% fetal bovine serum. Hepatic mononuclear cells and splenocytes were washed once with RPMI 1640/10% fetal bovine serum.

T cells were prepared as follows. Splenocyte suspensions in a medium with 0.3% bovine serum albumin (BSA; Sigma) were incubated at 37 °C under humidified conditions with 5% CO_2 for 30 min, and then nonadherent cell suspensions were harvested. The nonadherent cells were incubated with saturating amounts of anti-I-A/I-H (M5/114.15.2), anti-CD19 (1D3), anti-CD24 (M1/69), anti-CD11b (M1/70), and anti-CD11c (HL3) monoclonal antibodies (mAbs; BD PharMingen, San Diego, CA) and were further treated with saturating amounts of microbead-conjugated anti-DX5 mAb and goat antimouse IgG antibody (Ab; Miltenyi Biotec, Bergisch Gladbach, Germany). T cells were negatively purified by magnetic separation by magnetic cell sorting (Miltenyi Biotec). The purity of the T-cell populations was 96% to 98% $CD3^+$ cells, as determined by flow cytometry.

B cells were prepared as follows. Splenocytes were treated with saturating amounts of FITC-conjugated anti-CD3 (145-2C11), anti-CD4 (GK1.5), anti-CD8a (53-6.7), anti-CD11b, and anti-CD11c mAbs (BD PharMingen) and were further treated with saturating amounts of microbead-conjugated anti-DX5 mAb and anti-FITC (Miltenyi Biotec). B cells were negatively purified by magnetic separation. The purity of the B-cell populations was 95% to 97% $CD19^+$ and 0.1% <$CD3^+$, as determined by flow cytometry.

Enriched macrophages and dendritic cells (EMDC) were prepared as follows. Splenocyte suspensions in ice-cold RPMI 1640/10% fetal bovine serum were incubated at 37 °C under 5% CO_2 for 2 h, and then nonadherent cell suspensions were removed. Adherent cells on the culture plate were twice washed with warm phosphate buffered saline (PBS) (pH 7.2). Adherent cells were harvested after incubation with PBS (pH 7.2) containing 10 mM Na_2EDTA for 10 min. The harvested cells were treated with saturating amounts of microbead-conjugated anti-CD90, anti-CD19, and anti-DX5 mAbs. EMDC were negatively purified by magnetic separation. The purity of the EMDC populations was 60% >$CD11b^+DX5^-$ and 30% >$CD11c^+$I-A/I-H^+, as determined by flow cytometry.

2.4. Cell culture

For in vitro assays, splenocytes or hepatic mononuclear cells were plated in flat-bottomed culture plates at a density of 2×10^6 cells/ml. The cells were stimulated with soluble anti-CD3 mAb (2 μg/ml) in RPMI 1640/10% fetal bovine serum and incubated at 37 °C under humidified conditions with 5% CO_2 for the indicated time periods. In T-cell monocultures, purified T cells were plated at a density of 5×10^5 cells/ml and stimulated with both plate-immobilized anti-CD3 mAb (5 μg/ml) and soluble anti-CD28 mAb (5 μg/ml) for 48 h. In the co-culture of T cells with either B cells or EMDC, T and B cells were plated at a density of 5×10^5 and 1×10^6 cells/ml, respectively, and T cells and EMDC were plated at a density of 5×10^5 and 1.25×10^5 cells/ml, respectively. Each co-culture was stimulated with soluble anti-CD3 mAb for 48 h. Cultured cells were harvested at indicated time periods and washed with PBS containing 2% fetal bovine serum (pH 7.2).

2.5. Flow cytometric analysis of cells

Cells (4×10^5) were first preincubated with anti-CD16/32 (2.4G2) mAb, to avoid the nonspecific binding of Abs to FcγR, and then incubated with saturating amounts of PE-labeled anti-CD4 (GK1.5) and FITC-labeled anti-CD8a (53-6.7) mAbs for 20 min on ice in the dark. After washing twice with PBS/2% fetal bovine serum, the double-stained cells (live gated on the basis of forward and side scatter profiles and propidium iodide exclusion) were analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA), and the data were processed using Cell Quest software (Becton Dickinson).

2.6. Analysis of cell proliferation

The analysis of the effect of wogonin on T-cell subset proliferation was measured by 5-bromo-2′-deoxyuridine (BrdU) incorporation in cultured splenocytes and hepatic mononuclear cells. The analysis of BrdU incorporation was measured using a BrdU Flow Kit (BD PharMingen) as follows. Briefly, splenocytes or hepatic mononuclear cells were cultured for 24 or 26 h, respectively, and then for an additional 2 h, with or without the addition of 10 μM of BrdU. At 26 or 28 h, the cultured cells were harvested and stained with optimal amounts of both PE-labeled anti-CD4 and Cy-Chrome-labeled CD8a mAbs. The fixation, permeability, and measurement of BrdU incorporation of the stained cells were determined using the BrdU Flow Kit according to the manufacturer's instructions. The triple-fluorescence labeled cells were analyzed with a flow cytometer, and the data were processed using Cell Quest software. Cultured cells without BrdU were used as nonspecific binding controls for the FITC-labeled anti-BrdU mAb.

2.7. Analysis of cell cycle, viability, and apoptosis

The analysis of cell cycle was done as follows. Briefly, splenocytes cultured for 36 h were treated with a saturating amount of microbead-conjugated anti-CD4 or anti-CD8a mAb. Enriched $CD4^+$ or $CD8^+$ cells were positively partial purified by magnetic separation. The enriched $CD4^+$ or $CD8^+$ fraction was labeled with an optimal amount of FITC-labeled anti-CD4 or anti-CD8a mAb, respectively. The FITC-labeled enriched $CD4^+$ or $CD8^+$ cells were permeabilized and stained with propidium iodide using Cycle

TEST™ PLUS DNA Reagent Kit (Becton Dickinson). The double-fluorescence-labeled T cells (FITC-fluorescent cells gated on the basis of forward, FITC-fluorescence, and side scatter profiles) were analyzed by flow cytometry using a doublet discrimination model to include only single cells in the cell cycle analysis, and data were processed using Cell Quest software.

To investigate the effect of wogonin on cell viability, the mitochondrial membrane potential ($\Delta\psi_m$) of the T-cell subsets in cultured splenocytes with or without wogonin was measured by flow cytometry. At 48 h after anti-CD3 mAb-stimulated splenocytes, the cells were incubated for 30 min at 37 °C with 5 μg/ml rhodamine 123 (Molecular Probes, Eugene, OR). After washing three times with PBS containing 2% fetal bovine serum, the rhodamine-123-incorporated cells were further stained with optimal amounts of either FITC-labeled anti-CD4 or anti-CD8a mAb for 20 min on ice in the dark, and then washed three times. The fluorescence-labeled T cells (FITC-fluorescent cells gated on the basis of forward, FITC-fluorescence, and side scatter profiles) were analyzed using a FACScan flow cytometer, and the data were processed using Cell Quest software.

In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane; therefore, the ability of wogonin to induce cell apoptosis was investigated by the measurement of exposed PS on the surface of the T-cell subset in cultured splenocytes treated with or without wogonin. T-cell subset apoptosis was analyzed as follows. Briefly, splenocytes cultured for 36 h were stained with optimal amounts of PE-labeled annexin V (BD PharMingen), which has a high affinity for PS, and either FITC-labeled anti-CD4 or anti-CD8a mAb for 20 min on ice in the dark. After washing three times with PBS/2% fetal bovine serum, the double-fluorescence-labeled T-cell subset (FL-3 negative-gated on the basis of forward and side scatter profiles and propidium iodide exclusion) were analyzed using a FACScan flow cytometer.

2.8. Drug administration

SST was mixed with a sterile MF diet (Oriental Bioservices Co. Ltd., Tokyo, Japan), and either the SST-containing diet or sterile MF diet (Control) was provided to mice ad libitum for 8 weeks. The concentration of SST was adjusted to 0.5% in the sterile MF diet. The amount of SST-containing diet consumed was equal to the amount of sterile diet consumed by the control group. SST was administered at a dose of about 0.7 g/kg/day by regulating its concentration in response to the amount of sterile diet consumption. Although the dose of SST tested in the experiment was much higher (roughly eightfold) than that used clinically, the sensitivity of animals to certain drugs differs among species, and various biological effects of SST have been shown at doses of 0.5–3 g/kg in rodent models. The diets were replaced every 7 days. Mice were injected subcutaneously with 10 mg/kg/day of wogonin or vehicle (PBS containing 10% dimethyl sulfoxide and 10% polyethylene glycol) for 14 days.

2.9. Statistical analysis

Data were expressed as mean±S.E.M. Group differences were analyzed by one-way analysis of variance with Dunnet's test or Bonferroni/Dunn's test for multiple comparisons. For comparisons between two groups, Student's *t* test or Mann–Whitney *U*-test (for unequal variances) were used. In all tests, statistical significance was defined as $P<0.05$.

3. Results

3.1. Effects of SST and SST ingredients and metabolites on the CD4/CD8 ratio in splenocytes

Splenocytes were cultured with various concentrations of SST in the presence of anti-CD3 mAb for 48 h, and the total live cell count and the numbers of $CD4^+$, $CD8^+$, and $B220^+$ cells were measured by cell counting and flow cytometry. SST dose dependently elevated the CD4/CD8 ratio (Fig. 2A-1). SST, at doses of 62.5 and 125 μg/ml, decreased $CD8^+$ cell counts, with minor effects on $CD4^+$ cell counts (Fig. 2A-2 and A-3). At 250 μg/ml, SST decreased not only $CD8^+$ cell counts but also $CD4^+$ cell counts (Fig. 2A-2 and A-3), as well as the total cell counts (data not shown). The number of $B220^+$ cells showed no changes; therefore, the change in total cell count appears to reflect mainly changes in T lymphocytes. To gain insight into the principal active ingredient responsible for the changes in T-cell subsets induced by SST, splenocytes were cultured with 10 μM of various major SST ingredients or their aglycons (Fig. 1), which are absorbed as metabolites when SST is administered orally. Among the 14 tested ingredients, wogonin-7-*O*-glucuronoside, a characteristic flavonoid of Scutellariae Radix, and wogonin, an aglycon of wogonin-7-*O*-glucuronoside, decreased $CD8^+$ cell populations without decreasing $CD4^+$ cell populations, and both compounds increased the CD4/CD8 ratio (Table 1). The efficacy for increasing CD4/CD8 ratio was the same for wogonin and wogonin-7-*O*-glucuronoside at concentrations of 1 to 10 μM (data not shown). In accordance with previous reports (Chang et al., 2002), baicalein, an aglycon of baicalin, which is the most abundant low molecular weight ingredient in SST, markedly decreased the populations of both $CD4^+$ and $CD8^+$ cells, although baicalin had no effect on either cell type. Other SST ingredients and their aglycons had no effect (Table 1). Roughly, 1.8 μmol of wogonin-7-*O*-glucuronoside, 12.5 μmol of baicalin, and 1.0 μmol of baicalein were found

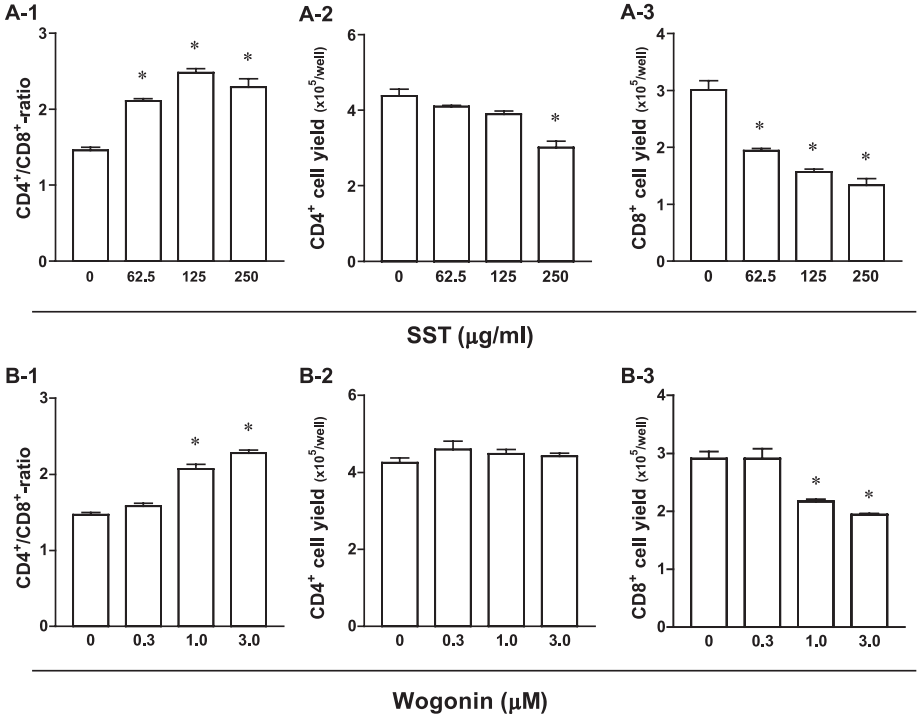


Fig. 2. SST and wogonin selectively decrease $CD8^+$ cell count and elevate the CD4/CD8 ratio in anti-CD3 mAb-stimulated splenocytes. Splenocytes were cultured with various concentrations of SST or wogonin in the presence of anti-CD3 mAb. At 48 h, cells were harvested, and total cell recovery was determined using a Coulter counter. The cells were stained with PE-labeled anti-CD4 and FITC-labeled anti-CD8a mAbs. The count of $CD4^+$, $CD8^+$ cells or CD4/CD8 ratio was calculated as follows: $CD4^+$ or $CD8^+$ cell count=the total cell count×%population analyzed with triple-color flow cytometry; and CD4/CD8 ratio=$CD4^+$ cell count/$CD8^+$ cell count. (A-1) CD4/CD8 ratio, (A-2) $CD4^+$ cell count, and (A-3) $CD8^+$ cell count in the cultured splenocytes in the presence of SST. (B-1) CD4/CD8 ratio, (B-2) $CD4^+$ cell count, and (B-3) $CD8^+$ cell count in the cultured splenocytes in the presence of wogonin. Data are expressed as mean±S.E.M. of four determinations. *Significantly different from vehicle-treated group (vehicle: PBS containing 2% fetal bovine serum and 0.1% dimethyl sulfoxide; $P<0.01$). Shown are results for one experiment repeated twice, with similar results.

to be contained in 125 μg/ml of SST by quantitative analysis with HPLC. Wogonin was detectable in trace amounts. Since 12.5 μM baicalin and 1.0 μM baicalein had no effect on the $CD4^+$ and $CD8^+$ populations (data not shown), wogonin-7-*O*-glucuronoside appeared to be responsible for the decrease in $CD8^+$ cell count induced by the addition of 62.5 or 125 μg/ml SST.

3.2. Effects of wogonin on splenic T-cell proliferation

When SST is administered orally, wogonin-7-*O*-glucuronoside is thought to be hydrolyzed to its aglycon, wogonin, by intestinal bacterial metabolism and then absorbed as wogonin in the blood (Zuo et al., 2002; Li et al., 1998; Lai et al., 2003). Therefore, we used wogonin, rather than

Table 1
Effects of SST ingredients and their aglycons on T-cell subset populations in anti-CD3 monoclonal antibody (mAb)-stimulated splenocytes[a]

Glycoside	None	WGG	BA	OAG	LN	GL	Sb_2
CD4/CD8 ratio	1.22±0.02[b]	2.35±0.05*	1.30±0.04	1.28±0.03	1.22±0.06	1.25±0.04	1.24±0.03
$CD4^+$ (%)	33.0±0.9[c]	36.1±0.8	34.3±0.8	34.8±0.6	33.2±1.2	34.7±0.6	33.4±0.7
$CD8a^+$ (%)	27.1±0.4	15.4±0.2*	26.4±0.8	27.1±0.3	27.3±0.4	27.8±0.8	26.9±0.5

Aglycon	None	WG	BE	OA	LG	GA	SD
CD4/CD8 ratio	1.22±0.06	2.16±0.11*	1.40±0.01	1.35±0.02	1.21±0.02	1.15±0.02	1.14±0.03
$CD4^+$ (%)	32.8±0.9	32.5±0.8	23.9±1.2*	32.0±0.1	31.4±1.4	31.5±0.8	30.8±0.4
$CD8a^+$ (%)	27.1±1.8	15.1±0.7*	17.1±0.8*	23.7±0.3	26.1±1.1	27.4±1.1	27.0±0.7

Each value is expressed as the mean±S.E.M. of four determinations.

[a] Splenocytes (2×10^6/ml) were cultured with 10 μM of various SST ingredients [wogonin-7-*O*-glucuronoside (WGG), baicalin (BA), oroxylin A-7-*O*-glucuronoside (OAG), liquiritin (LN), glycyrrhizin (GL), or saikosaponin b_2 (Sb_2)], 10 μM of the corresponding aglycons (metabolites), wogonin (WG), baicalein (BE), oroxylin A (OA), liquiritigenin (LG), glycyrrhetic acid (GA), saikogenin D (SD), or without addition (vehicle; PBS containing 2% fetal bovine serum and 0.1% dimethyl sulfoxide) in the presence of soluble anti-CD3 mAb (2 μg/ml) for 48 h.

[b] CD4/CD8 ratio in anti-CD3 mAb-stimulated splenocytes was calculated as the ratio of $CD4^+$ cell population/$CD8a^+$ cell population.

[c] $CD4^+$ and $CD8a^+$ cell populations were determined as in Materials and methods.

* $P<0.01$ compared with no addition (vehicle). The experiment was repeated twice, with similar results.

Table 2
Wogonin dose not induce necrotic nor apoptotic T-cell death in anti-CD3 mAb-stimulated splenocytes[a]

T-cell subset	Treatment	PI^+ cells (%)[b]	Annexin V^+ cells (%)[c]	Rhodamine 123 fluorescence mean[d]
$CD4^+T$	CCCP[e]			3.68±0.10*,[e]
	Vehicle	2.37±0.15	4.12±0.37	24.56±1.51
	Wogonin	1.92±0.06	3.20±0.11	23.20±4.96
$CD8^+T$	CCCP[e]			3.45±0.12*
	Vehicle	1.47±0.11	2.94±0.09	11.73±0.27
	Wogonin	1.54±0.07	1.97±0.07	12.37±0.18

[a] Splenocytes were cultured with 3 μM wogonin or vehicle, PBS containing 2% fetal bovine serum and 0.1% dimethyl sulfoxide, in the presence of anti-CD3 mAb, and harvested at the indicated time periods.
[b] At 48 h, the cells were stained with PE-labeled anti-CD4, FITC-labeled anti-CD8 mAbs, and propidium iodide.
[c] At 36 h, the cells were stained with PE-labeled annexin V and either FITC-labeled anti-CD4 or anti-CD8 mAb. T cells in freshly isolated splenocytes (>95% viability by trypan blue exclusion test) and dexamethasone-treated thymocytes were used as technical controls, indicating viable and dead cells (data not shown).
[d] At 48 h, the cells were incubated for 30 min at 37 °C with the addition of 5 μg/ml rhodamine 123, and then the cells were stained with either FITC-labeled anti-CD4 or anti-CD8 mAb.
[e] A negative control with decayed $\Delta\psi_m$ [which was treated with 50 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP)] is shown. The fluorescence-labeled T cells were analyzed using a FACScan flow cytometer. Data are expressed as mean±S.E.M. (n=3–4).
* Significantly different from vehicle treatment group (P<0.01).

wogonin-7-*O*-glucuronoside, to further characterize the inhibitory effect on $CD8^+$ T-cell proliferation. In cultured splenocytes, in the presence of anti-CD3 mAb for 48 h, wogonin elevated the CD4/CD8 ratio (Fig. 2B-1) and decreased the $CD8^+$ cell count (Fig. 2B-3) in a concentration-dependent manner, while the compound had no effect on the $CD4^+$ cell count (Fig. 2B-2). The selective decrease of $CD8^+$ T-cell count by wogonin was observed at 48 h in the time-course studies of T-cell subset counts. The increase in the number of propidium iodide-positive necrotic cells in wogonin-treated cultures was marginal and did not differ from that of control cultures (Table 2). Furthermore, rhodamine 123 incorporation in $CD4^+$ and $CD8^+$ T-cells treated for 48 h with wogonin did not differ from that of each control T-cell subset (Table 2). Cell apoptosis, estimated by annexin V staining, of $CD4^+$ and $CD8^+$ T-cells treated for 36 h with wogonin did not differ from that of control (Table 2). The cell cycle analysis (Fig. 3) of $CD4^+$ and $CD8^+$ T-cells treated for 36 h with wogonin showed a decrease in $CD8^+$ T-cells at the S/G2/M phase and an increase in cells at G0/G1. BrdU incorporation, a DNA synthesis analysis method, of $CD8^+$ T-cells also decreased both at 3 and 10 μM, while that of $CD4^+$ T-cells did not decrease when splenocytes were cultured with wogonin and anti-CD3 mAb for 26 h (Fig. 4). Thus, the inhibitory effect on $CD8^+$ T-cell proliferation was found to be due not to the induction of cell death but to the inhibition of DNA synthesis and/or cell cycle progression.

It is well known that optimal activation of antigen (Ag)-specific T cells requires costimulatory signals provided by

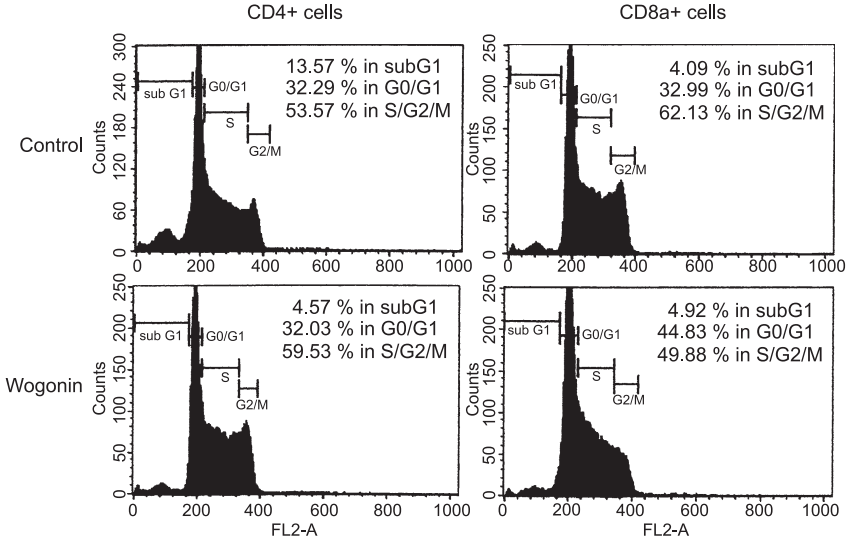


Fig. 3. Wogonin selectively decreases the proportion of $CD8^+$ cells that are in the S/G2/M of the cell cycle in anti-CD3 mAb-stimulated splenocytes. Cultured splenocytes with 10 μM wogonin or vehicle (Cont) in the presence of soluble anti-CD3 mAb for 36 h were refined to enrich $CD4^+$ or $CD8^+$ cells, and purified cells were stained with FITC-labeled anti-CD4 or anti-CD8a mAb, respectively. Labeled cells were fixed, permeabilized, and stained with propidium iodide. Cell cycle of T-cell subsets was measured by flow cytometry. Similar results were obtained from three independent experiments.

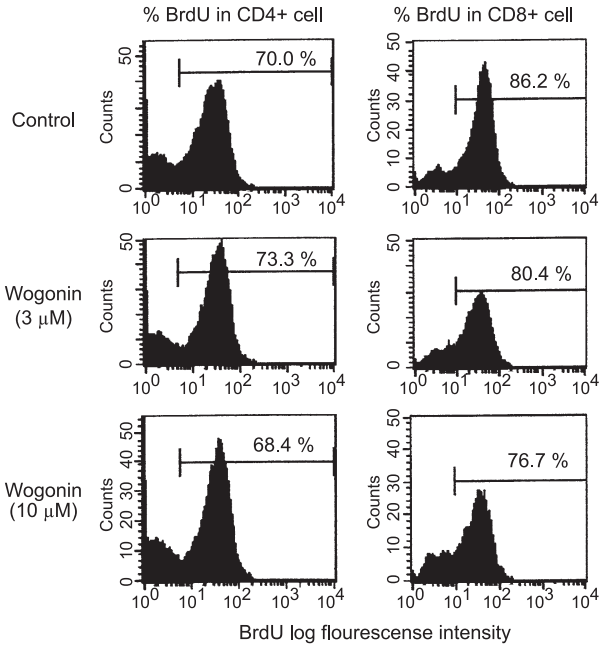


Fig. 4. Wogonin selectively inhibits $CD8^+$ cell proliferation in anti-CD3 mAb-stimulated splenocytes. Cultured splenocytes with wogonin (3 or 10 μM) or vehicle (Control) in the presence of soluble anti-CD3 mAb were pulsed for the final 2 h of a 26-h culture period with bromodeoxyuridine (BrdU). Cells were triple-stained with PE-labeled anti-CD4 mAb, Cy-Chrom-labeled anti-CD8a mAb, and FITC-labeled anti-BrdU. BrdU incorporation of T-cell subset in the fluorescence-stained cells was measured by flow cytometry. Similar results were obtained from three independent experiments.

antigen presenting cells (APC), in addition to the engagement of the T-cell receptor (TCR) with Ag/MHC. Therefore, to elucidate the mechanisms underlying the inhibitory effect of wogonin on $CD8^+$ T-cell proliferation, we examined whether APC are involved in the effect of wogonin. In the presence of various concentrations of wogonin, T cells were cultured for 48 h with or without APC (i.e., B cells or EMDC). Co-cultures of T cells and APC were stimulated with soluble anti-CD3 mAb, and cultures of T cells without APC were stimulated with plate-immobilized anti-CD3 mAb and soluble anti-CD28 mAb. Wogonin had no effect on the CD4/CD8 ratio of T-cell cultures without APC (Fig. 5A). However, in co-cultures of T cells and APC, irrespective of the presence of B cells or EMDC, wogonin elevated the CD4/CD8 ratio in a concentration-dependent manner (Fig. 5B and C).

Table 3
Wogonin or SST administered to mice increases CD4/CD8 ratio in hepatic mononuclear cells[a]

	CD4/CD8 ratio	
	Splenocytes	Hepatic mononuclear cells
Conrol	2.23±0.03	2.49±0.14
Wogonin	2.13±0.07	3.25±0.21*
Control	2.36±0.10	2.79±0.04
SST	2.40±0.09	3.39±0.17*

[a] For wogonin administration, mice were treated with 10 mg/kg/day wogonin or its vehicle (Control) by subcutaneous injection for 14 days. For SST administration, the mice were provided with a normal diet (Control) or a diet containing 0.5% SST (SST) ad libitum for 8 weeks. Splenocytes and hepatic mononuclear cells were prepared as described in Materials and methods. The cells were stained with both PE-labeled anti-CD4 and FITC-labeled anti-CD8a mAbs. T-cell subset count was calculated from the total count of prepared or cultured cells followed by double-color flow cytometry analysis. Data are expressed as mean±S.E.M. (n=6).

* Significantly different from Control mice (P<0.05). The experiments were repeated twice, with similar results.

3.3. Effects of wogonin and SST administration on liver T-cell subsets in mice

To examine whether wogonin or its parent drug, SST, affects the CD4/CD8 ratio in vivo, we treated mice with wogonin by subcutaneous injection for 2 weeks or SST orally for 8 weeks. The CD4/CD8 ratio in hepatic mononuclear cells isolated from wogonin-treated mice was significantly higher than that of control mice (Table 3). The

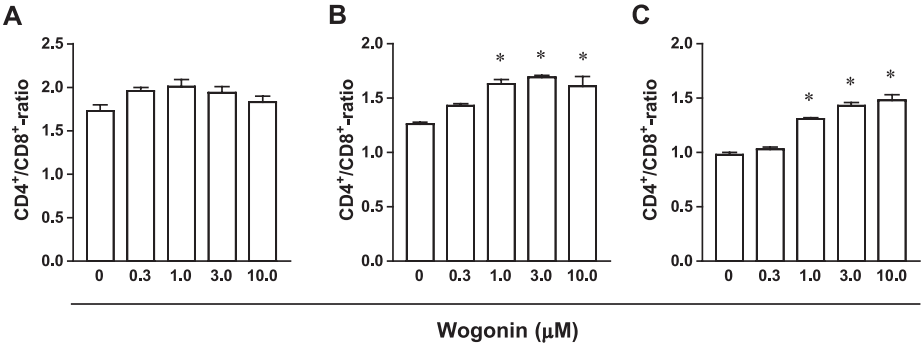


Fig. 5. Involvement of antigen-presenting cells (APC) in the inhibitory effect of $CD8^+$ T-cell proliferation by wogonin. Splenic T cells were cultured in plate-immobilized anti-CD3 mAb in the presence of soluble anti-CD28 mAb (A), or were co-cultured with either B cells (B) or enriched cells of macrophages and dendritic cells (EMDC) (C) in the presence of anti-CD3 mAb. Various concentrations of wogonin were added to each culture. Cells were harvested at 48 h, and total cell recovery was determined using a Coulter counter. The cells were stained with PE-labeled anti-CD4 and FITC-labeled anti-CD8a mAbs. The CD4/CD8 ratio was calculated as follows: CD4/CD8 ratio=$CD4^+$ cell population/$CD8^+$ cell population; each population was analyzed with triple-color flow cytometry. (A) T-cell-only culture in the presence of anti-CD28 mAb; (B) co-culture of T and B cells; and (C) co-culture of T cells and EMDC. Data are expressed as mean±S.E.M. of four determinations. *Significantly different from the vehicle-treated group (vehicle: PBS containing 2% fetal bovine serum and 0.1% dimethyl sulfoxide; P<0.01). Shown are the results of one experiment repeated twice, with similar results.

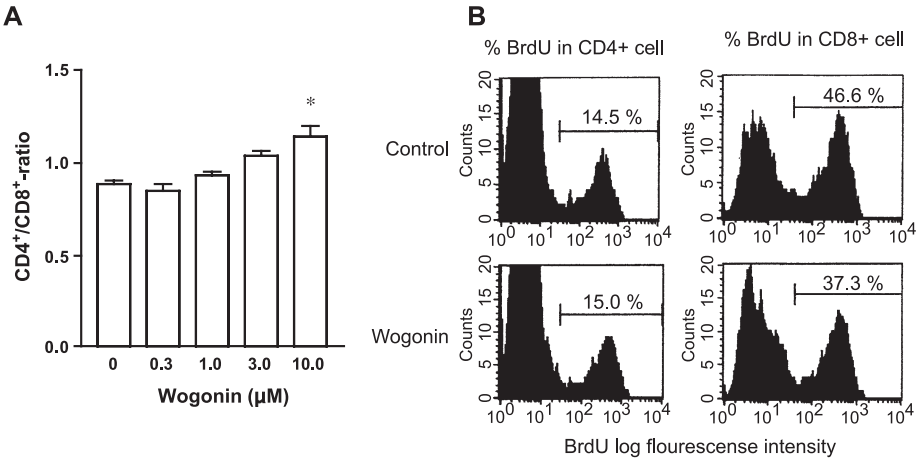


Fig. 6. Wogonin elevates the CD4/CD8 ratio and selectively inhibits $CD8^+$ cell proliferation in anti-CD3 mAb-stimulated hepatic mononuclear cells. Hepatic mononuclear cells were cultured with various concentrations of wogonin in the presence of anti-CD3 mAb and harvested at the indicated time periods. At 48 h, the cells were harvested and stained with both PE-labeled anti-CD4 and FITC-labeled anti-CD8a mAbs. The T-cell subset population of the fluorescence-stained cells was measured by triple-color flow cytometry analysis. CD4/CD8 ratio (A) was calculated from the proportion of $CD4^+$ to $CD8^+$ cell populations. Data are expressed as mean±S.E.M. (n=4). *Significantly different from vehicle-treated group (vehicle: 0.1% DMSO/2% fetal bovine serum–PBS; P<0.01). The experiments were repeated twice, with similar results. For T-cell subset proliferation, cultured hepatic mononuclear cells with 10 μM concentration of wogonin or vehicle (Cont) was pulsed for the final 2 h of a 28-h culture period with BrdU. Cells were triple stained with PE-labeled anti-CD4 mAb, Cy-Chrom-labeled anti-CD8a mAb, and FITC-labeled anti-BrdU. BrdU incorporation of T-cell subsets (B) in the fluorescence-stained cells was measured by triple-color flow cytometry analysis. Similar results were obtained from three independent experiments.

CD4/CD8 ratio in splenocytes, however, showed no difference between the wogonin-treated and untreated groups (Table 3). SST administration also gave similar results (Table 3). These data suggested the possibility that T lymphocytes in the liver are more sensitive to the effects of wogonin; therefore, we examined the effect of wogonin on hepatic mononuclear cells in vitro. Wogonin increased the CD4/CD8 ratio of hepatic mononuclear cells, as shown in Fig. 6, although the effect appeared to be weaker on hepatic mononuclear cells than on splenocytes. Selective suppression of $CD8^+$ T-cell proliferation in hepatic mononuclear cells was also confirmed by analysis of BrdU incorporation (Fig. 6).

4. Discussion

In the course of our investigation into the effects of SST on immune cells using various in vitro and in vivo assays, SST, wogonin-7-*O*-glucuronoside, and wogonin were found to selectively decrease the $CD8^+$ T-cell count and to elevate the CD4/CD8 ratio in splenocytes or hepatic mononuclear cells. In the present study, we focused our investigation on wogonin, and the results of the analysis of cell count, BrdU incorporation, cell cycle, and cell viability test of T-cell subsets clearly demonstrated that wogonin selectively inhibited $CD8^+$ T-cell proliferation, with minor effects on $CD4^+$ T-cells, without the inducement of necrotic nor apoptotic T-cell death.

The differential effects of certain chemicals on $CD4^+$ or $CD8^+$ T-cells have not been widely reported.

In addition to the inhibition of cyclooxygenase (COX)-2 activity and down-regulation of the expression of COX-2 (Chen et al., 2001), wogonin has been suggested to have a possible inhibitory effect on arachidonic acid liberation (Nakahata et al., 1998; Park et al., 2004). Wogonin has also been demonstrated to have antioxidant activity by several investigators (Gao et al., 1999; Shieh et al., 2000; Cho and Lee, 2004). Arachidonic acid (Boilard and Surette, 2001) and oxidant radicals have been reported to be involved in T-lymphocyte proliferation (Nindl et al., 2004). Therefore, it is possible that the modulation of arachidonic acid and/or oxidative stress by wogonin is involved in the $CD8^+$ T-cell-selective inhibition of proliferation observed in the present study. However, because the differential effect of these mediators among T-cell subsets has not been previously reported, further investigation is necessary to clarify this point.

It has been reported that the depletion of intracellular glutathione (GSH) by buthionine sulfoximine (BSO) decreases the proportion of $CD8^+$ T-cells and elevates the CD4/CD8 ratio (Gmunder and Droge, 1991), although whether BSO prevents activation/proliferation or induces cell death in $CD8^+$ T-cells has not been elucidated. Wogonin and wogonin-7-*O*-glucuronoside had no effect on intracellular GSH content (estimated using a GSH assay kit; GSH-400, Oxis International, Inc. Portland, OR) in anti-CD3 mAb-stimulated splenic T cells (e.g., T cells treated with or without wogonin; data not shown). Furthermore, in contrast with the effect of BSO, the addition of 3 mM GSH to splenocytes cultured with wogonin (3 μM) has been found to have no effect on $CD8^+$ T-cell-selective suppres-

sion or the CD4/CD8 ratio (data not shown). Therefore, other mechanisms must be responsible for the selective inhibition of $CD8^+$ T-cell proliferation by wogonin.

The activation/proliferation of T lymphocytes has been investigated in a number of studies; however, these studies have not addressed the possibility of differential effects on $CD4^+$ and $CD8^+$ T-cells. In recent studies using DNA microarrays, the pattern of gene expression in response to TCR stimulation was shown to differ between $CD4^+$ and $CD8^+$ T-cells (Chtanova et al., 2001). Furthermore, several researchers have reported that the activation of the costimulatory signals of the CD27–CD70 and 4-1BB-4-1BBL pathways selectively activates $CD8^+$ T-cells rather than $CD4^+$ T-cells (Vinay and Kwon, 1998; Lens et al., 1998). These findings suggest that the activation and proliferation of $CD4^+$ T and $CD8^+$ T-cells involve different signal pathways, and that the CD4/CD8 balance and the immune responses of $CD4^+$ and $CD8^+$ T-cells are possibly modulated by the regulation of these signal pathways. Thus, the present study suggests the possibility of pharmacological interventions directed towards immunological aberration, based on the specific regulation of $CD4^+$ and $CD8^+$ T-cell activation, and therefore, CD4/CD8 balance. In fact, the aberration of CD4/CD8 balance has been reported in various disorders, although the physiological and pathological significances are not adequately understood. Among them, in viral hepatitis, such as HBV and HCV, $CD8^+$ cytotoxic T lymphocytes (CTL) are believed to initiate hepatic injury with pathologies that range from fulminant hepatitis to chronic hepatitis. The inhibition of the activation of aggressive CTL by anti-CD8 antibody treatment has been shown to be one possible therapeutic strategy against chronic hepatitis (Kiefersauer et al., 1997). It would be very interesting to determine whether the selective inhibition of $CD8^+$ T-cell proliferation by wogonin participates in the beneficial effect of SST on various viral and inflammatory diseases, including chronic hepatitis with HCV.

In the present study, the in vivo modulation of the CD4/CD8 ratio by SST or wogonin was observed only in hepatic mononuclear cells, and not in splenocytes. The selective inhibition of $CD8^+$ T-cell proliferation in hepatic mononuclear cells by wogonin was also observed in vitro; however, the effect was weaker on hepatic mononuclear cells than on splenocytes. In the case of oral administration of SST, it is possible that the concentration of wogonin (and wogonin-7-*O*-glucuronoside) in the liver was higher than that in the peripheral blood, because wogonin is first absorbed enterohepatically via the portal vein. However, this would not explain why subcutaneous injection of wogonin gave similar results. To answer this question, additional studies on the pharmacokinetics and bioavailability of wogonin are needed. Another possible explanation for the differential effect of wogonin on hepatic mononuclear cells and splenocytes was suggested by the data in Fig. 5. Namely, the results from studies of T cells cultured with or without APC clearly indicated that the modulatory effect of wogonin on the CD4/CD8 ratio requires APCs, such as B cells, macrophages, or dendritic cells. Kupffer cells are abundant in the liver and have biological functions similar to macrophages, and the hepatic mononuclear cells preparation method used in the present study could not effectively retrieve Kupffer cells. Therefore, it is possible that the effect of wogonin in hepatic mononuclear cells was more prominent in vivo, because abundant Kupffer cells may have functioned as potent APC. In any case, the questions of whether the observed effect of SST can be attributed to wogonin, and why the effect of these agents is prominent in hepatic mononuclear cells, remain to be answered.

In summary, SST, wogonin-7-*O*-glucuronoside, and wogonin selectively suppressed the proliferation of $CD8^+$ T-cells and elevated the CD4/CD8 ratio in vitro. The effects of SST and wogonin on CD4/CD8 ratio were also observed in vivo in hepatic mononuclear cells. To our knowledge, this is the first report of an agent that selectively suppresses $CD8^+$ T-cells without inducing cell death. Although the precise mechanism of action and medical and biological implications await further investigation, the present data enlarge the scope for the development of new therapeutic strategies for various infectious and inflammatory diseases via the selective modification of $CD4^+$ and/or $CD8^+$ T-cell-mediated immune responses.

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