Original Research

Regulation of interferon-γ, interleukin-4 and interleukin-2 by *Schizonepeta tenuifolia* through differential effects on nuclear factor-κB, NFATc2 and STAT4/6

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Abstract

Contact with antigen on T-cells is made via the T-cell receptor/CD3 complex plus CD28, resulting in the production of cytokines including interleukin (IL)-2, IL-4 and interferon (IFN)-γ. In particular, dysregulation of IFN-γ and IL-4 accounts in part for organ-specific autoimmune diseases, allergic inflammation and other chronic inflammatory disorders. The dried above-ground parts of *Schizonepeta tenuifolia* Briq are used for the treatment of common cold and skin rashes observed in allergic dermatitis, psoriasis and other dermatological disorders in oriental medicine. In the present study, we investigated whether *S. tenuifolia* water extract (STE) may modulate systemic levels of IFN-γ, IL-4 and IL-2 in anti-CD3-stimulated mice and the production of those cytokines in anti-CD3-stimulated peripheral blood mononuclear cells (PBMCs). In addition, the effects of STE on anti-CD3-induced activation of several transcription factors were examined. Oral administration of STE significantly reduced the serum levels of IFN-γ and IL-4 from anti-CD3-treated mice but enhanced those of IL-2. Similar patterns were demonstrated in anti-CD3-stimulated splenocytes and PBMCs *in vitro*. Further analysis showed that STE enhanced the nuclear translocation of nuclear factor of activated T cells (NFAT)c2 but reduced that of the nuclear factor (NF)-κB. The downregulation of IFN-γ and IL-4 was not mediated by its effects on signal transducer and activator of transcription (STAT)4 and STAT6 activation. These results suggest that the differential regulation of STE on IFN-γ, IL-4 and IL-2 may be due to its suppression of NF-κB, concomitant with its enhancement of NFATc2. Further mechanistic work is required to investigate the role of STE on its modulation of anti-CD3-induced cytokines.

Keywords: *Schizonepeta tenuifolia*, NF-κB, NFATc2, IL-2, IL-4, IFN-γ

Experimental Biology and Medicine 2010; **235**: 230–236. DOI: 10.1258/ebm.2009.008357

Introduction

Contact with antigen on T-cells through the T-cell receptor (TCR)/CD3 complex plus co-stimulatory signals such as CD28 from the neighboring antigen-presenting cells results in proliferation of the responding cells and production of cytokines.[1] Among the cytokines produced by activated T-cells, interleukin (IL)-2 plays a critical role in proliferating T-cells while interferon (IFN)-γ and IL-4, representing the major Th1 and Th2 cytokines, are essential not only in characterizing the main effector functions of T-cells but also in determining the differentiation of Th1 and Th2 cells.[2] Stimulation of TCR/CD3 plus CD28 increases intracellular calcium levels and activates protein kinase Cθ (PKCθ), leading to the translocation of nuclear factor of activated T cells (NFAT) and the nuclear factor (NF)-κB and thus to the induction of various cytokine genes including IFN-γ, IL-2 and IL-4.[3–5] Within a few hours of antigenic stimulation, an early peak in IFN-γ and IL-4 transcription is reported to occur independently of signal transducer and activator of transcription (STAT) proteins, but after the peak, STAT4 and STAT6 are essential in the maintenance of IFN-γ and IL-4 transcription, respectively.[6] Thus, a single cytokine gene can be controlled by several signal transduction pathways.

The most common anti-inflammatory agents such as cyclosporin A (CsA), tacrolimus and glucocorticoids can suppress the production of an array of cytokines during T-cell activation. CsA blocks not only the translocation of NFAT but also that of NF-κB in activated T-cells.[7–9] Glucocorticoids are known to inhibit the expression of the cytokine genes by interfering with several transcription factors such as NF-κB, NFAT and some STAT proteins instead of directly inhibiting the promoter regions.[10,11] Because of their wide range of gene suppression, chronic

use of these pharmacological agents may impair some necessary processes during T-cell activation and cause unwanted side-effects.

The dried above-ground parts of *Schizonepeta tenuifolia* Briq are used for the treatment of common cold and skin rashes observed in allergic dermatitis, psoriasis and other dermatological disorders in oriental medicine.[12] It is not singularly prescribed but included as a major constituent of several herbal formulas for the applications described above. Our previous study demonstrated that administration of *S. tenuifolia* water extract (STE) to animals inhibited IFN-γ and IL-4 mRNA expression in response to intravenous anti-CD3 stimulation.[13] Also, STE inhibited the differentiation of mouse Th1 and Th2 cells and, at the same time, it strongly inhibited the transcription of T-bet while its effects on GATA-3 and c-Maf were minimal. In the present study, we investigated whether STE may modulate systemic levels of IFN-γ, IL-4 and IL-2 in anti-CD3-stimulated mice and the time course production of those cytokines in anti-CD3-stimulated peripheral blood mononuclear cells (PBMCs). In addition, the effects of STE on anti-CD3-induced activation of several transcription factors were examined. The results presented here show that despite its downregulatory effects on IFN-γ and IL-4, STE increased IL-2 involving suppressed NF-κB and enhanced NFATc2 and STAT4/6.

Materials and methods

Mice

Female BALB/c mice at eight weeks of age were purchased from Japan SCL and maintained with rodent chow and water *ad libitum* in a temperature- and humidity-controlled pathogen-free animal facility at Kyunghee University. Mice were cared for according to the guidelines issued by the US National Institute of Health (NIH publication # 85-23, 1985) and the protocol was approved by our institutional ethical committee for animal welfare.

Reagents

The reagents used in this study were as follows: RPMI was purchased from WelGENE (Daegu, Korea). Fetal bovine serum (FBS), antibiotic-antimycotic and SuperScript™ II Reverse Transcriptase were purchased from Invitrogen (Carlsbad, CA, USA). Recombinant IL-12 (rIL-12) and anti-mouse CD3e mAbs (145-2C11), anti-human CD3 (UCHT1), OptEIA set mouse IL-2, IL-4 and IFN-γ, human IL-2, IL-4 and IFN-γ were from BD Pharmingen (San Diego, CA, USA). Power SYBR Green PCR Master Mix was purchased from Applied Biosystems (Foster City, CA, USA). Dexamethasone (DEX) and CsA were purchased from Sigma (St Louis, MO, USA). Antibodies (Abs) to p65, NFATc2, β-actin, Lamin B, STAT4, STAT6, phospho-STAT4 and phospho-STAT6 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-rabbit and anti-mouse IgG and horseradish peroxidase (HRP) were purchased from Cell Signaling Technology (Beverly, CA, USA).

Isolation of PBMCs

Venous whole blood was obtained from healthy human volunteers. Subjects had given written informed consent approved by the Ethics Committee of Kyunghee Oriental Medical Center. PBMCs were separated using the Lymphoprep (Oslo, Norway) density gradient centrifugation. PBMCs were washed twice with a phosphate-buffered saline (PBS) and resuspended in a culture medium made of RPMI 1640 supplemented with 1% antibiotic-antimycotic and 10% heat-inactivated FBS.

Preparation of *S. tenuifolia* extract

The plant of *S. tenuifolia* was purchased from Kyunghee University Medical Center Medicinal Herbs (Seoul, Korea), and its identification was authenticated by Prof. Choi Ho-Young, Department of Herbology, Kyunghee University. A voucher herbarium specimen (ST-2007) was deposited at the Department of Oriental Medical Science. The plant sample was soaked in one volume of distilled water overnight, and further dissolved using sonication for 1 h. The extract was filtered and evaporated using a −70°C freeze dryer (Eyela, Tokyo, Japan). The yield of the extract was about 2.67%. The sample was dissolved in PBS and sterilized by passing through a 0.22 μm syringe filter.

In vivo anti-CD3 activation

Balb/c mice were divided into six groups ($n = 10$ in each group). (1) *Untreated group*: PBS was orally given and injected intravenously with PBS. (2) *Control group*: mice were intravenously injected with a single dose of anti-CD3 Ab (40 μg/mL in PBS).[14] (3) *Dexamethasone group*: DEX (5 mg/kg) was injected intraperitoneally 18 h before the injection of anti-CD3. (4–6) *STE group*: STE was given orally to mice at a dose of 100, 200 and 500 mg/kg for seven days and on day 8, mice were intravenously injected with anti-CD3. Serum was obtained 90 min after anti-CD3 injection.

Cell culture

Splenocytes from Balb/c mice were prepared by disrupting the spleen between glass slides in complete medium (RPMI 1640 with 10% FBS and 1% antimycotic-antibiotic). After centrifugation at 1000 rpm to separate cells from debris, cells were washed in medium, followed by the lysis of erythrocytes. Cells were counted and viability was determined by trypan blue exclusion. For cytokine measurement, splenocytes (2×10^6/mL) were activated with anti-CD3 Ab (2.5 μg/mL), and PBMCs (1×10^6/mL) were activated with immobilized 10 μg/mL anti-CD3 Ab. STE or DEX was added at the start of culture. For Western blot, splenocytes (2×10^7/2 mL) were treated with STE, CsA or DEX 1 h before stimulation with anti-CD3. For STAT4 activation, rIL-12 (1 ng/mL) was added.

Cytokine analysis

Supernatants or serum were appropriately diluted and analyzed for IFN-γ, IL-4 and IL-2 by enzyme-linked immunosorbent assay, as described by the manufacturer.

Realtime reverse transcriptase-polymerase chain reaction

Total RNA from splenocytes was isolated with an RNAqueous™ phenol-free total RNA isolation kit (Ambion, Austin, TX, USA) and cDNA was reverse-transcribed by a superscript α reverse transcriptase. The following forward and reward primer sequences were used: for IFN-γ, 5′ TCA AGT GGC ATA GAT GTG GAA-3′ and 5′ TGG CTC TGC AGG ATT TTC ATG 3′; for IL-4, 5′ ACA GGA GAA GGG ACG CCA T 3′ and 5′ GAA GCC CTA CAG ACG AGC TCA 3′; for IL-2, 5′ GCC CAA GCA GGC CAC AGA AT 3′ and 5′ GGG CTT GTT GAG ATG ATG CTT TGA 3′; and for GAPDH, 5′ GGC ATG GAC TGT GGT CAT GA 3′ and 5′ TTC ACC ACC ATG GAG AAG GC 3′. Quantitative real-time polymerase chain reaction (PCR) was performed in triplicate using a 96-well optic tray on an ABI PRISM 7300 sequence detector (Applied Biosystems, Foster City, CA, USA). After initial heat denaturation at 95°C for 10 min, the PCR conditions were set at 95°C for 15 s, 60°C for 1 min for 40 cycles. PCR efficiency was determined using serial dilutions of the template cDNA. For each PCR, a corresponding no-RT mRNA sample was included as a negative control. The quantification of each cDNA copy number was performed using control samples according to the manufacturer's protocol. GAPDH gene was used as an endogenous control to normalize the expression of IFN-γ, IL-4 and IL-2 mRNA.

Western blot

Total cell extracts were prepared by resuspending the cells in the lysis buffer (50 mmol/L Tris-Hcl [pH 7.5], 150 mmol/L NaCl, 1 mmol/L EDTA, 20 mmol/L NaF, 0.5% NP-40, 1% Triton X-100). Nuclear protein extracts were prepared using the Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA). Protein concentration was determined using the Bradford assay. Cell extracts were run on a 10% sodium dodecyl sulfate-polyacrylamide gel and were transferred to polyvinylidene fluoride. The membranes were blocked with 5% skim milk in TBST (Tris-buffered saline with 0.1% Tween 20) for 1 h and incubated with primary Abs (diluted 1/500 or 1/1000 in 5% skim milk in TBST) overnight at 4°C. The blots were washed with TBST and then incubated for 1 h with anti-rabbit or anti-mouse HRP-conjugated Ab (diluted 1:2000 in 5% skim milk in TBST). Immunoreactive bands were developed using the enhanced chemiluminescence system (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Statistical analysis

Data represent mean ± SEM. *In vivo* serum cytokine levels were compared using analysis of variance. *In vitro* splenocyte cytokine levels were compared using Student's unpaired *t*-test. Because cytokine levels from PBMCs did not follow a normal distribution, the Wilcoxon signed rank test was used to compare the cytokine production in control and STE or DEX groups. Calculations were carried out using the SPSS version 17. *P* values less than 0.05 were considered significant.

Results

Pretreatment with STE decreased serum levels of IFN-γ and IL-4, but increased IL-2 in anti-CD3-injected mice

Our previous work demonstrated that the *in vivo* administration of STE inhibited the expression of IFN-γ and IL-4 in the splenocytes from anti-CD3-stimulated mice at the transcription level. In the current work, we tried to evaluate whether STE treatment could affect the systemic production of IFN-γ, IL-4 and IL-2 from the *in vivo* anti-CD3 activation model. Balb/c mice were orally given STE (100, 200 and 500 mg/kg) for seven days and serum cytokines were measured

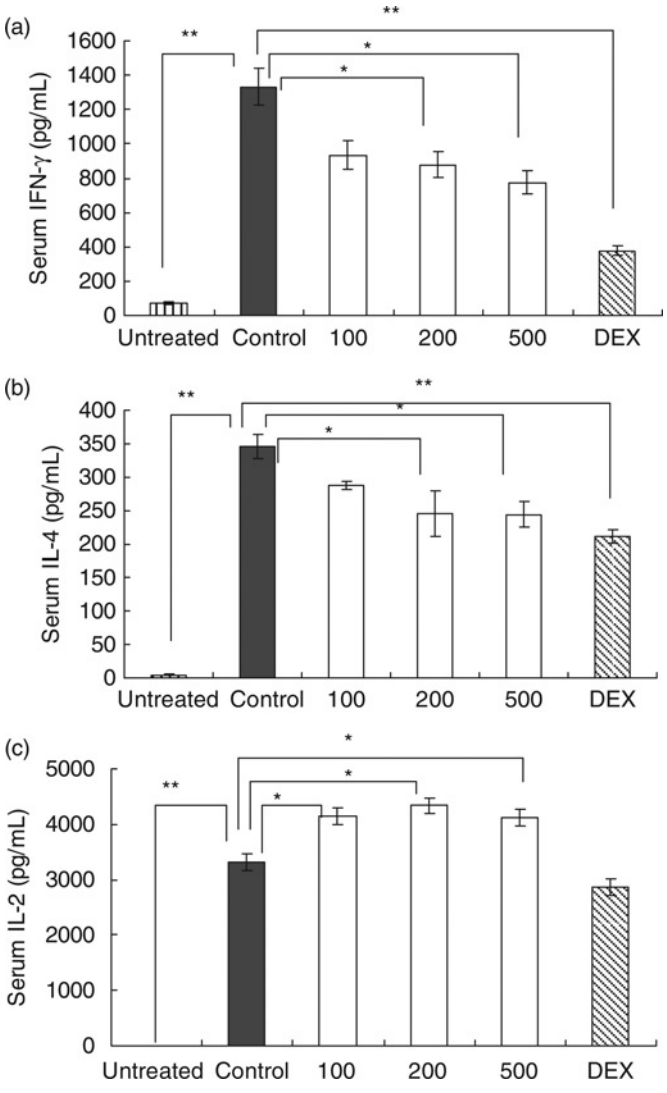

Figure 1 Effects of STE on serum IFN-γ, IL-4 and IL-2 in anti-CD3-injected mice. STE (100, 200 and 500 mg/kg) was orally administered to Balb/c mice for seven days. Untreated, control and DEX groups were given the equal volume of PBS. Each group consisted of 10 animals. On day 8, control, STE and DEX groups were intravenously injected with 4 μg anti-CD3 Ab. Ninety minutes after injection, serum was obtained and cytokine levels (a, b, c) were measured by enzyme-linked immunosorbent assay. Data are the mean ± SEM of four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.005$; significantly different from control group (analysis of variance). STE, *Schizonepeta tenuifolia* water extract; IFN, interferon; DEX, dexamethasone; IL, interleukin

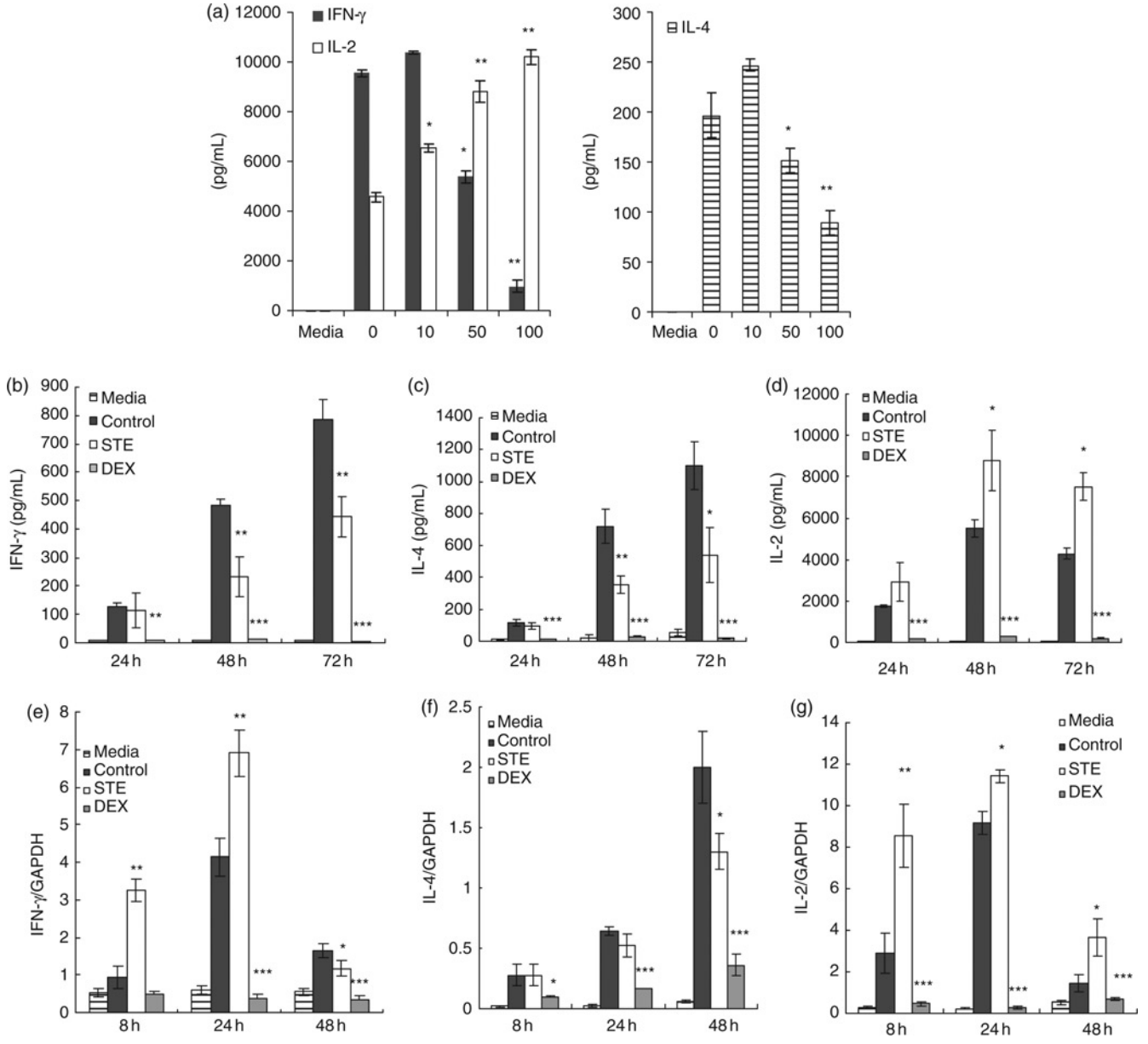

Figure 2 Effect of STE on the expression of IFN-γ, IL-4 and IL-2 in anti-CD3-activated splenocytes *in vitro*. Splenocytes (2×10^6/mL) were stimulated with anti-CD3 (2.5 μg/mL) and STE was added at the beginning of culture. (a) Various concentrations of STE (10, 50 and 100 μg/mL) were added and cytokine levels from the supernatants were measured by the enzyme-linked immunosorbent assay. (b–d) Time course of cytokine production was illustrated. STE (100 μg/mL) or DEX (1 μmol/L) was added at the beginning of culture and supernatants were collected at 24, 48 and 72 h. (e–g) RNA was collected at 8, 24 and 48 h. The relative mRNA levels were calculated by real-time reverse transcriptase-polymerase chain reaction and adjusted to the expression of the housekeeping gene GAPDH. Data are the mean ± SEM of four independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$; significantly different from control cells (0 μg/mL) (Student's non-paired *t*-test). STE, *Schizonepeta tenuifolia* water extract; IFN, interferon; DEX, dexamethasone; IL, interleukin

90 min after stimulation with intravenous anti-CD3. DEX was used as a reference drug. Both STE and DEX significantly reduced the serum levels of IFN-γ and IL-4 (Figures 1a and b). Interestingly, serum IL-2 levels were enhanced in STE groups tested at all doses, whereas those in DEX groups were unaffected (Figure 1c).

Dose and time kinetic measurement of anti-CD3-induced cytokines by STE treatment *in vitro*

STE, up to a concentration of 100 μg/mL, was not cytotoxic and rather induced 1.5 − 2.5-fold mitogenic activity in splenocytes using the MTS assay (data not shown). In addition, STE alone did not induce any appreciable levels of IFN-γ, IL-4 and IL-2 (data not shown). As shown in Figure 2a, high doses of STE reduced the levels of IFN-γ and IL-4, but an increase in IL-2 was observed in a dose-dependent manner. Subsequently, the time kinetics of cytokine expression in STE treatment was carried out at the secreted protein and mRNA levels. The decreases of IFN-γ and IL-4 secretion were observed at 48 and 72 h while the increase of IL-2 was detected as early as at 24 h and lasted throughout the entire culture period (Figures 2b–d). DEX completely inhibited all these cytokines. Since the expression of mRNA precedes that of protein, each mRNA

was obtained at earlier time points. A significant reduction in IFN-γ and IL-4 transcription by STE did not occur until late at 48 h, while the IL-2 increase was maintained during the entire culture period (Figures 2e–g). The reductions of secreted IFN-γ and IL-4 by STE were greater than those of each mRNA, suggesting that the inhibitions by STE were more potent at the post-transcriptional level.

Effects of STE on immobilized anti-CD3-activated PBMCs

In order to examine whether the same effect might occur in human lymphocytes in response to STE, PBMCs from six healthy donors were cultured in immobilized anti-CD3 for 24, 48 and 72 h in the presence of STE or DEX. Similar to the results observed with mouse splenocytes, the differences in control and in the STE or DEX group were statistically significant for all these cytokines at all time points (Figure 3). STE treatment reduced the secretion of IFN-γ and IL-4 by PBMCs but enhanced that of IL-2.

Effects of STE on the nuclear translocation of NFATc2 and NF-κB and the activation of STAT4/6

In this assay, we investigated the influence of STE on the nuclear localization of NFATc2 and NF-κB in anti-CD3-stimulated splenocytes. Nuclear extracts were isolated at 15, 30 and 60 min and the localization of NFATc2 and NF-κB was analyzed using Western blot. STE treatment resulted in an enhanced translocation of NFATc2 and a reduced presence of NF-κB whereas CsA and DEX inhibited the translocation of these proteins at 60 min (Figure 4a). These data indicate that the differential influences of STE on the T-cell cytokines may be in part attributed to its opposing effects on NFATc2 and NF-κB.

STAT4 and STAT6 mediate cytokine-induced IFN-γ or IL-4 expression. We also tested the effect of STE on the activation of STAT4 and STAT6. In order to induce the activation of STAT4 and STAT6, splenocytes were stimulated with anti-CD3 plus IL-12 (for the phosphorylation of STAT4) or anti-CD3 alone (for phosphorylation of STAT6), as previously described.[15] The results presented here show that STE treatment increased the phosphorylation of both STAT4 and STAT6 (Figure 4b). CsA and DEX did not inhibit STAT4 activation but completely inhibited STAT6 phosphorylation. Our findings indicate that the downregulation of IFN-γ and IL-4 by STE does not occur at the level of STAT4 and STAT6 activation.

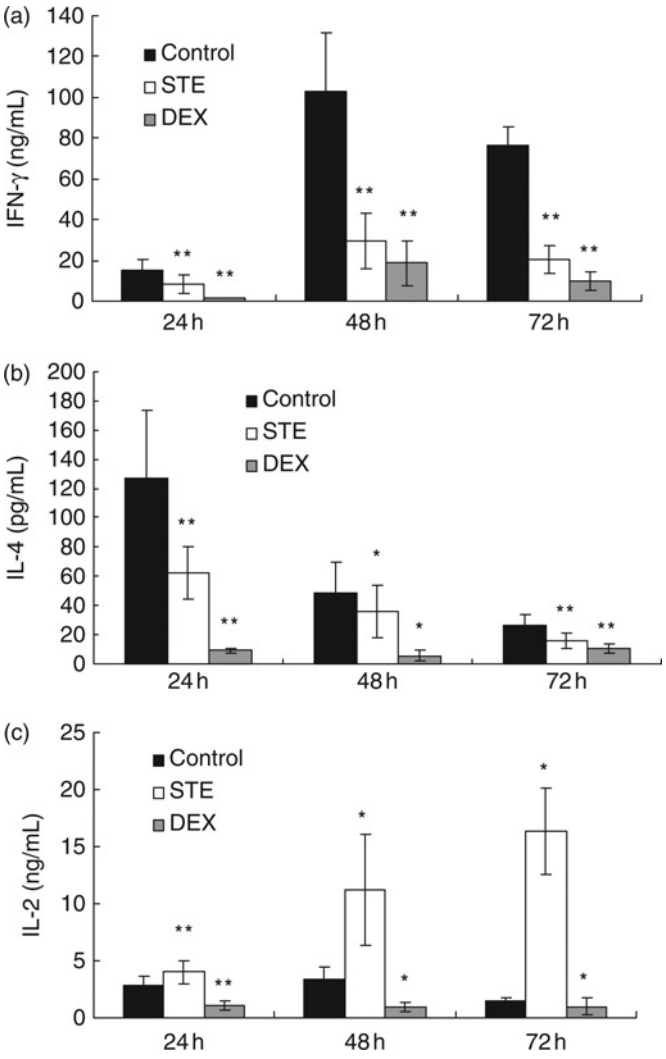

Figure 3 Effect of *Schizonepeta tenuifolia* water extract (STE) on the production of IFN-γ, IL-4 and IL-2 in immobilized anti-CD3-activated peripheral blood mononuclear cells (PBMCs). PBMCs were seeded in immobilized anti-CD3 (10 μg/mL), and STE (100 μg/mL) or DEX (1 μmol/L) was added at the beginning of culture. Supernatants were collected at 24, 48 and 72 h. Data are the mean ± SEM of six independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$; significantly different from control cells (Wilcoxon signed rank test). STE, *Schizonepeta tenuifolia* water extract; IFN, interferon; DEX, dexamethasone; IL, interleukin

Discussion

Over the last decades, much attention has been paid to the use of traditional herbal medicines owing to their safety. In this study, the immunomodulatory effects of STE on T-cell cytokine production were evaluated using anti-CD3-injected mouse *in vivo* and anti-CD3-activated splenocytes and PBMCs *in vitro*. Oral administration of STE significantly reduced the serum releases of IFN-γ and IL-4 in response to anti-CD3, whereas the serum IL-2 levels were enhanced. Similar patterns were demonstrated in splenocytes and PBMCs culture. Given the fact that many of the candidate anti-inflammatory agents tend to suppress all these cytokines, the action of STE to modulate excessive production of IFN-γ and IL-4 without losing the mitogenic capacity to stimulate IL-2 production should be noted.[16–20]

NFAT proteins consist of NFATc1, NFATc2, NFATc3, NFATc4 and other proteins, and among them NFATc2 accounts for 80–90% of the total family.[8] The control of NFAT activity is mainly determined at the level of nuclear entry, as the cytosolic NFAT is capable of DNA binding and transcription.[21] Its role in T-cell activation has been well documented in a number of studies in which the use of the NFAT inhibitor, CsA, completely inhibits the production of IL-2, IFN-γ and IL-4.[22–24] Therefore, the increase

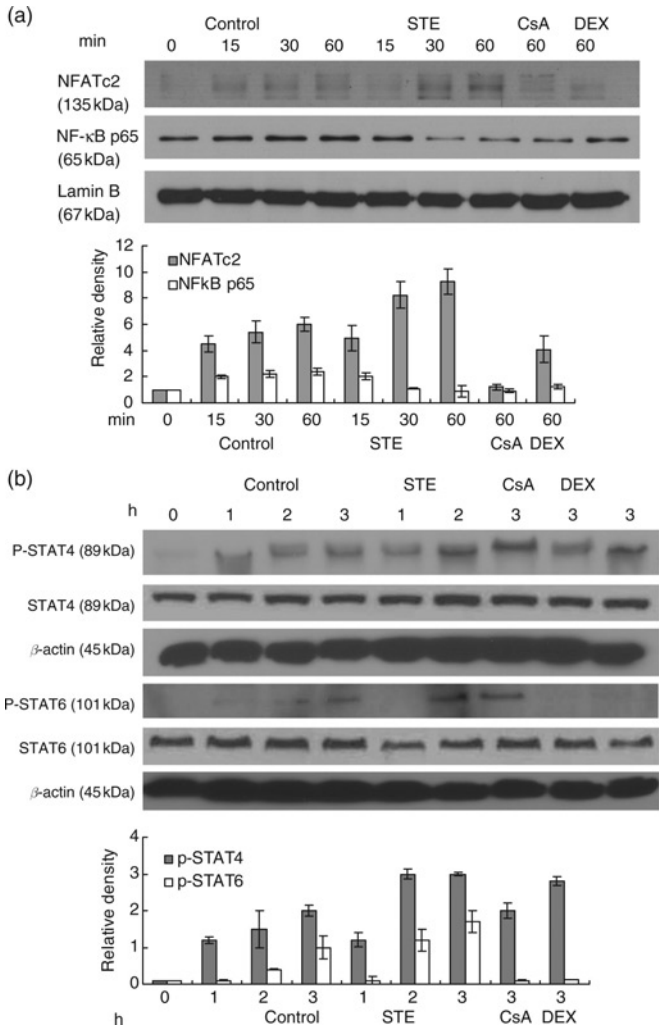

Figure 4 Effects of STE on NFATc2, NF-κB and STAT4/6. (a) Splenocytes were pre-treated with STE (100 μg/mL), CsA (1 μg/mL) or DEX (1 μmol/L) for 1 h before stimulation with anti-CD3 (2.5 μg/mL) and nuclear protein was harvested at the indicated time points. NFATc2 and NF-κB were assessed by Western blot. Lamin B was used as an internal control. (b) Splenocytes were stimulated with anti-CD3 (2.5 μg/mL) plus IL-12 (1 ng/mL) for STAT4 activation or with anti-CD3 alone for STAT6 activation in the presence of STE, CsA or DEX. Whole cell extracts were obtained at the indicated time points and each protein was analyzed by Western blot. β-Actin was used as an internal control. Quantification of the immunoblots was carried out by densitometry. Bars (percentage of NFATc2 or NF-κB/Lamin B or phosphoprotein/total protein) represent the mean ± S.E.M band intensity relative to unstimulated cells (0 min or o hour). One of the five experiments was shown. STE, *Schizonepeta tenuifolia* water extract; IFN, interferon; DEX, dexamethasone; IL, interleukin; CsA, cyclosporin A

of nuclear NFATc2 by STE appears to mediate its increase of IL-2 but not its inhibition of IFN-γ and IL-4.

It seems that full activation of IFN-γ and IL-4 by NFAT requires the co-operative activity of NF-κB, but that of IL-2 does not. NF-κB, co-operatively with NFAT, AP-1 or NF-IL6, is implicated in the maximal production of IFN-γ and IL-4.[25,26] Previous studies demonstrated that an impaired NF-κB signaling pathway *in vivo* decreased IFN-γ and IL-4, but not IL-2.[27] Similar to this, *in vitro* treatment with sulfasalazine, an NF-κB inhibitor, reduced the production of IFN-γ and IL-4 by anti-CD3-stimulated splenocytes without affecting IL-2 production (data not shown). Moreover, CD28, not TCR/CD3, is directly responsible for NF-κB activation through PI3K and the PKCθ–CARMA–Bcl10 complex, but it also uses an NF-κB-independent pathway to induce IL-2 mRNA stability.[28,29] This may account for normal IL-2 production despite defective NF-κB signaling. Thus the inhibition of STE on NF-κB signaling may be partly responsible for its differential effects on IL-2, IFN-γ and IL-4. PKCθ receives signals from both TCR/CD3 and CD28, and its multiple signaling includes activation of NF-κB and NFAT.[30] It is presumed that STE may negatively affect the PKCθ-mediated NF-κB pathway but enhance the PKCθ-mediated NFAT pathway. Further mechanistic study is required to investigate these possibilities.

Binding of IL-I2 to IL-12 receptor activates STAT1, STAT3 and STAT4 (in mice, only STAT4 is phosphorylated), and phosphorylated STAT4, in particular, migrates to the nucleus and binds with other transcription factors such as CREB binding protein or NF-κB to enhance IFN-γ expression.[31–33] Our results indicated that the IFN-γ inhibition by STE is unlikely to involve the STAT4 activation. Perhaps such inhibition may occur at the level of some interaction between STAT4 and other transcription factors, possibly NF-κB. STAT6, once stimulated by cytokines, is phosphorylated by Janus kinases to form homodimers and migrates to the nucleus, where it binds to STAT-6-responsive elements in the IL-4 promoter and other target genes.[34] STAT6 alone is a weak IL-4 inducer and direct interaction with NF-κB is required for its full activation of the cytokine.[35] In addition, signals other than IL-4, such as IL-2 and CD28, were reported to be able to activate STAT6 without affecting IL-4 mRNA.[36] Therefore, the enhanced STAT6 activation by STE may respond to the increased IL-2 secretion from the cellular environment. Or possibly the NF-κB inhibition of STE may have negative influences on STAT6-mediated IL-4 expression.

STE, like other natural products, consists of multiple ingredients, most of which are unknown. It seems that some of the STE constituents act as growth signals, as shown by its mitogenic activity and IL-2 upregulation. Supposedly, differential effects of STE might be exerted by its various components. Future study should include the fractionation of STE in order to identify its active components.

Taken together, our study demonstrated that STE reduced IFN-γ and IL-4, but maintained the capacity to stimulate IL-2 *in vivo* and *in vitro*. Importantly, the *in vitro* studies showed that STE decreased the anti-CD3-induced expression of NF-κB but enhanced that of NFATc2 and STAT4/6. Further mechanistic study is required to support the immunomodulatory effect of STE.

ACKNOWLEDGEMENTS

We thank Prof. Ki-Tae Rhie, Department of Biology, Kyunghee University, for helping in the statistical analysis.

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(Received December 9, 2008, Accepted September 24, 2009)