

©2004, Acta Pharmacologica Sinica
Chinese Pharmacological Society
Shanghai Institute of Materia Medica
Chinese Academy of Sciences
<http://www.ChinaPhar.com>

Immunoenhancing activity of protopanaxatriol-type ginsenoside-F3 in murine spleen cells¹

Jun-li YU, De-qiang DOU², Xiao-hong CHEN, Hong-zhen YANG, Na GUO², Gui-fang CHENG³

Department of Pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing 100050; ²Department of Natural Products Chemistry, Shenyang Pharmaceutical University, Shenyang 110001, China

KEY WORDS ginsenoside F3; immunity; cytokines; transcription factors

ABSTRACT

AIM: To investigate the immunoenhancing activity of ginsenoside-F3 in murine spleen cells and explore its mechanism. **METHODS:** The enhancing effect of ginsenoside-F3 on murine spleen cell proliferation was studied using [³H] thymidine incorporation assay. Effects of ginsenoside-F3 on the production of type 1 cytokines IL-2, IFN- γ , and type 2 cytokines IL-4 and IL-10 from murine spleen cells were detected by ELISA method. Effects of ginsenoside-F3 on mRNA level of cytokines IL-4, IFN- γ , and transcription factors T-bet and GATA-3 were evaluated by RT-PCR analysis. Effect of ginsenoside-F3 on NF- κ B DNA binding activity in murine spleen cells was investigated by electrophoretic mobility shift assays (EMSA). **RESULTS:** Ginsenoside-F3 at 0.1-100 μ mol/L not only promoted the murine spleen cell proliferation, but also increased the production of IL-2 and IFN- γ , while decreased the production of IL-4 and IL-10 from murine spleen cells with the maximal effect at 10 μ mol/L. RT-PCR analysis displayed that ginsenoside-F3 enhanced the IFN- γ and T-bet gene expression and decreased IL-4 and GATA-3 gene expression. EMSA experiment showed that ginsenoside-F3 10 μ mol/L enhanced the NF- κ B DNA binding activity induced by ConA in murine spleen cells. **CONCLUSION:** Ginsenoside-F3 has immunoenhancing activity by regulating production and gene expression of type 1 cytokines and type 2 cytokines in murine spleen cells.

INTRODUCTION

Panax ginseng has been a valuable and important tonic medicine in many Asian countries such as Korea, China, and Japan. The main components of *Panax ginseng* are known to be the ginsenosides that are divided into two types by structural, protopanaxadiol ginsenosides (PPDGs) and protopanaxatriol ginsenosides (PPTGs). To date, it has been reported that many kinds of

ginsenosides played important anti-inflammatory and immunomodulatory roles by affecting cytokine production and lymphocyte proliferation^[1-4]. However, the immunomodulatory activity of ginsenoside-F3, an important component of PPTGs (an minor saponin in the leaves of *Panax ginseng*), has rarely been studied till now.

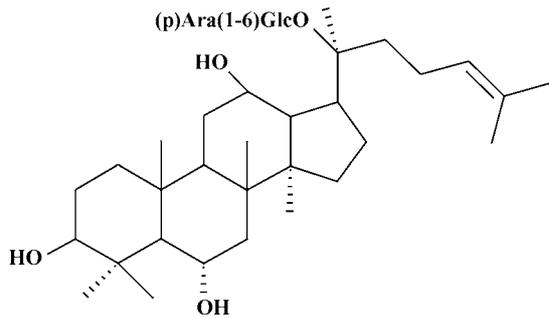
The immune response can be broadly categorized into cellular- or humoral-mediated responses. The two types of immune responses are respectively regulated by cytokines that control two general subsets of helper cells known as Th1 and Th2. IFN- γ and IL-2 are evaluated as representative type 1 cytokines mainly secreted from Th1 cells, while IL-4 and IL-10 as key type 2 cytokines mainly secreted from Th2 cells^[5]. Produc-

¹ Project supported by the National Natural Science Foundation of China (No 30171105).

³ Correspondence to Prof Gui-fang CHENG.
Phn 86-10-6316-5192. Fax 86-10-6301-7757.
E-mail chenggf@imm.ac.cn

Received 2004-05-24

Accepted 2004-08-07



Chemical structure of ginsenoside-F3, $M_r=780$

tion and expression of type 1 cytokines and type 2 cytokines are strictly regulated. During this process, T-box expressed in T cells (T-bet) and GATA-binding protein 3 (GATA-3) play crucial roles. T-bet, a Th1-specific transcription factor, is thought to initiate Th1 development but inhibit Th2 cell differentiation^[6,7]. GATA-3 is a member of the GATA family of zinc finger proteins (so-called because they bind to consensus DNA sequence, A/T; GATA A/G), which plays a pivotal role in the development of the Th2 phenotype while inhibiting Th1 cells^[8]. Thus, T-bet and GATA-3 are generally thought to represent type 1 or type 2 immune response and the ratio of expression these transcription factors may reflect the Th1 and Th2 status in mixed populations^[9].

Since cytokines are pivotal for regulation of immune responses, compounds that modulate expression of key cytokines that regulate immune responses would have clinical utility in treating patients with characteristically decreased cell-mediated immune responses or patients with chronic inflammatory and autoimmune disease. The present study was conducted to investigate the regulatory effects of ginsenoside-F3 on type 1 and type 2 cytokines that are involved in immune responses.

MATERIALS AND METHODS

Reagents Concanavalin A (Con A) was purchased from Sigma. TRIzol reagent and M-MLV reverse transcriptase were from GIBCO-BRL. *Taq* DNA polymerase was from TaKaRa and T4 polynucleotide kinase was from Pharmacia. NF- κ B probe was from Promega. ELISA kits for murine recombinant IL-2, interferon- γ (IFN- γ), IL-4, and IL-10 were from R&D systems.

Test compound Ginsenoside-F3 was isolated from leaves of *P. ginseng* as described previously^[10,11], the

purity of which was more than 95 % at HPLC analysis. Ginsenoside-F3 was prepared in stock solution 0.1 mol/L with Me₂SO and stored at -20 °C. Before being used, the stock solution was diluted to appropriate concentrations in RPMI-1640.

Animals Male BALB/c mice (H-2^d, 17±1 g, 6-7 weeks old) were from the Experimental Animal Center, Chinese Academy of Medical Sciences & Peking Union Medical College (SPF, certificate No SCXK 11-00-0006). All animals were housed in groups under 12 h regime (lights on from 7:00 AM to 19:00 PM) at 23±2 °C prior to the experiments, and were given standard laboratory chow and tap water *ad libitum*.

Preparation of spleen cells Mice were sacrificed by cervical dislocation, and spleens were removed aseptically. Spleens were placed in cold Hank's solution and teased apart with a pair of forceps and a needle. A single cell suspension from the teased tissue was obtained by passing it through a 20-gauge needle and hemolysed by buffer containing 1 mmol/L Tris-HCl and 1 % NH₄Cl. Cells were washed twice with RPMI-1640 medium and subsequently suspended in complete RPMI-1640 culture medium. Cell viability was determined by trypan blue dye exclusion.

[³H]thymidine incorporation assay To determine the effect of ginsenoside-F3 on the proliferation of spleen cells, 2×10⁹ cells/L spleen cells treated with T lymphocyte mitogen ConA (1 mg/L) and different concentrations of ginsenoside-F3 were cultured in flat bottom 96 well plates in a total volume of 200 μL/well. After an incubation for 3 d at 37 °C in 95 % humidity and 5 % CO₂, cultures were harvested with an automatic cell harvester using distilled water. The amount of radioactivity incorporated into DNA was determined in a liquid scintillation spectrometer.

ELISA kits for cytokine determination Spleen cells (2×10⁹ cells/L) were treated with ginsenoside-F3 in presence of Con A (1 mg/L) for 48 h or 72 h, and cell supernatants were collected and levels of IL-2 (48 h), IFN- γ (72 h), IL-4 (48 h), and IL-10 (48 h) were measured by ELISA kits.

RT-PCR for cytokine and transcription factor gene expression The total RNA was extracted from 5×10⁶ spleen cells stimulated by different concentrations of ginsenoside-F3 and ConA (1 mg/L) for 10 h. Cultured spleen cells were washed and the RNA was extracted with the TRIzol reagent according to the recommendation of the manufacturer. First strain cDNA was synthesized from equal amount of total RNA with

M-MLV reverse transcriptase and random hexamer. Genes were amplified by PCR using sense and anti-sense primers of IFN- γ , T-bet, IL-4, GATA-3 as described before^[9] with some modifications. T-bet primers were designed corresponding to the mouse T-bet gene sequence outside of the T box domain to maintain its specificity for T cells. GATA-3, IFN- γ , and IL-4 PCR primers were designed according to the corresponding structures of mouse genes. Primers were as follows: T-bet: sense: 5'-AACCAGTATCTGTCCAGC-3', anti-sense: 5'-TGTCGCCACTGGAAGGATAG-3'; GATA-3: sense: 5'-CTCCTTTTTGCTCTC CTTTC-3', anti-sense: 5'-AAGAGATGAGGACTGGAGTG-3'; IFN- γ : sense: 5'-CGTCTTGGTTTTGCAGCTC-3', anti-sense: 5'-ACTCCTTTTCTTCTTCTTA -3'; IL-4: sense: 5'-ACGGCACAGAGCTATTGATG-3', anti-sense: 5'-ATGGTGGC CAGTACTACGA-3'; GAPDH: sense: 5'-CATCACCATCTTCCAGGAGCG-3'; anti-sense 5'-GAGGGGCCATCCACAGTCTTC-3'. PCR annealing temperature: T-bet: 58 °C; GATA-3: 60 °C; IFN- γ : 53 °C; IL-4: 65 °C; GAPDH: 58 °C. Semi-quantitative RT-PCR was performed using GAPDH as an internal control to normalize gene expression for the PCR templates. The PCR products were studied on a 1 % agarose gel and the amplified bands were visualized after staining with ethidium bromide. The size of the amplified fragments was determined by comparison with a standard DNA marker.

Preparation of nuclear protein fractions and electrophoretic mobility shift assay (EMSA) for DNA binding activity Nuclear extracts of spleen cells were prepared as described previously^[12] with some modifications. Spleen cells were washed twice with D-Hank's balanced salt solution, resuspended in Buffer A (HEPES 10 mmol/L, MgCl₂ 1.5 mmol/L, KCl 10 mmol/L, DTT 0.5 mmol/L, Triton X-100 0.05 %) and then disrupted in Pestle B. After centrifugation, nuclei were resuspended in Buffer C (HEPES 20 mmol/L, glycerol 25 %, NaCl 420 mmol/L, MgCl₂ 1.5 mmol/L, EDTA 0.2 mmol/L, PMSF 0.5 mmol/L, DTT 0.5 mmol/L) and lysed on ice. Supernatants were collected, diluted with Buffer W (HEPES 20 mmol/L, KCl 20 mmol/L, MgCl₂ 1 mmol/L, DTT 2 mmol/L, PMSF 1 mmol/L, glycerol 17 %). Oligonucleotides used for the gel shift analysis were as follows: NF- κ B (5'-AGTTGAGGGGACTTTCCCA-AGGC-3', 5'-GCCTTGGGAAAGTACCCTCAACT-3'). Mut-NF- κ B (5'-AGTTGAGGCGACTTTCCCAAGGC-3', 5'-GCCTTGGGAAAGTCCCCTCAACT-3'). Nuclear extracts were incubated with ³²P-labeled oligonucleotides,

solved on 8 % nondenaturing polyacrylamide gels and analyzed by autoradiography.

Statistical analysis All values expressed as mean \pm SD were obtained from at least 3 separate observations performed in triplicate. Statistical analysis was carried out using one-way ANOVA, followed by multiple comparisons by Dunnett's test using SPSS 11.5 for windows. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of ginsenoside-F3 on proliferation of ConA-induced murine spleen cells Lymphocytes played an important role in immune functions as they acted both as effectors and regulators. To assess the effect of ginsenoside-F3 on the proliferation of murine spleen cells, ³H-TdR incorporation assay was performed. As the results, ginsenoside-F3 significantly increased ($P < 0.01$) the proliferation of ConA-induced murine spleen cells at the concentrations of 0.1-100 μ mol/L with the maximal increase at 10 μ mol/L by 87.2 % (Fig 1).

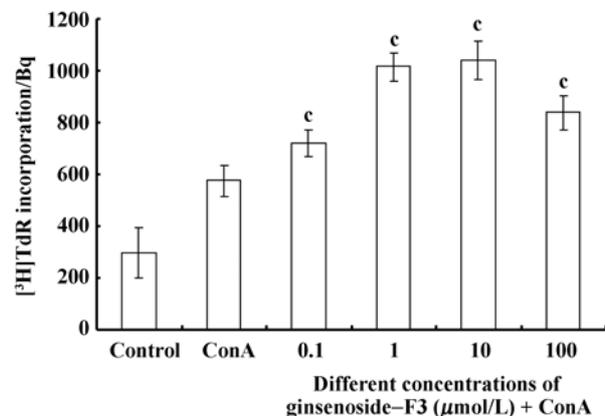


Fig 1. Effect of ginsenoside-F3 on the proliferation of ConA-induced murine spleen cells. Murine spleen cells were treated with different concentrations of ginsenoside-F3 in the presence of ConA 1 mg/L for 72 h. $n=5$. Mean \pm SD. $^cP < 0.01$ vs ConA group.

Effect of ginsenoside-F3 on cytokines production from murine spleen cells To assess the effects of ginsenoside-F3 on the production of type 1 and type 2 cytokines, murine spleen cells were treated with ConA and various concentrations of ginsenoside-F3 for 48 h or 72 h. The levels of IL-2, IFN- γ (type 1 cytokine) and IL-4, IL-10 (type 2 cytokine) were analyzed by ELISA method. Ginsenoside-F3 was found to increase type 1 cytokines production at the concentrations of

0.1-100 $\mu\text{mol/L}$ compared with ConA alone with the maximal increase at 10 $\mu\text{mol/L}$, while decreased type 2 cytokines production at the same concentration range (Tab 1).

Effect of ginsenoside-F3 on cytokines and transcription factors gene expression To determine if effects of ginsenoside-F3 on type 1 and type 2 cytokines were transcriptionally regulated, mRNA level of type 1-specific cytokine IFN- γ , type 2-specific cytokine IL-4 and transcription factors T-bet and GATA-3 were measured using RT-PCR. Ginsenoside-F3 at the concentrations of 0.1-100 $\mu\text{mol/L}$ increased ConA-induced expression level of IFN- γ and T-bet, while decreased that of IL-4 and GATA-3 (Fig 2).

Effect of ginsenoside-F3 on NF- κB activation in murine spleen cells Ginsenoside-F3 displayed maximal effects at the concentration of 10 $\mu\text{mol/L}$, so we measured the activation of NF- κB DNA binding activity in murine spleen cells treated with ginsenoside-F3 10 $\mu\text{mol/L}$ and Con A 1 mg/L for 1 h to investigate whether effects of ginsenoside-F3 resulted from their interference of NF- κB activation. The results from the study showed that ginsenoside-F3 at the concentration of 10 $\mu\text{mol/L}$ enhanced the NF- κB DNA binding activity induced by ConA in murine spleen cells (Fig 3).

DISCUSSION

Cellular- and humoral-mediated responses are two important aspects of immune response. The production of IL-2, IFN- γ leads to a Th1-type cellular response, while production of IL-4 and IL-10 leads to Th2-type humoral immunity. It has been reported^[13,14] that levels of Th2 cytokines were higher than that of Th1 cytokines in various diseases including cerebral infarction (CI), allergy and asthma. In the present study, we showed that ginsenoside-F3 not only increased the proliferation

of spleen cells but also strongly increased the production of type 1 cytokines IL-2, and IFN- γ , while decreased the production of type 2 cytokines IL-4, IL-10 with the maximal effects at 10 $\mu\text{mol/L}$. These results suggested ginsenoside-F3 might be a desirable agent for the correction of Th2 dominant pathological disorders.

In order to further elucidate the mechanism responsible for the changes in the amounts of type 1 and type 2 cytokines, we utilized RT-PCR to analysis the mRNA expression of IFN- γ , the archetypal Th1 cytokine, and IL-4, the signature Th2 cytokine. Furthermore, cytokines mRNA levels were for the most part transcriptionally regulated, and selective expression of T-bet and GATA-3 correlated with cytokine gene and protein expression^[9], so we measured T-bet and GATA-3 gene expression in murine spleen cells cultured with ginsenoside-F3 and ConA. Ginsenoside-F3 was shown to enhance the expression of IFN- γ and T-bet mRNA and decrease that of IL-4 and GATA-3 mRNA at the concentrations of 0.1-100 $\mu\text{mol/L}$, so the effects of ginsenoside-F3 on the production of type 1 and type 2 cytokines may result, at least in part, from regulation mRNA expression of these cytokine themselves and transcription factors T-bet and GATA-3.

Activation and nuclear translocation of transcription factor NF- κB are important for expression of genes involved in the development of immune and inflammatory responses and spleen cell proliferation^[15]. Aronica *et al* reported^[16] that NF- κB /Rel signaling had a preferential role in type 1 but not type 2 T cell-dependent immune response. Our results showed that ginsenoside-F3 increased ConA-induced NF- κB DNA binding activity in murine spleen cells, which could be, at least in part, responsible for the enhancement effect of ginsenoside-F3 on ConA-induced spleen cell proliferation and type 1 cytokines production and expression.

Tab 1. Effects of ginsenoside-F3 on cytokines secretion from ConA-induced murine spleen cells. $n=3$. Mean \pm SD. ^b $P<0.05$, ^c $P<0.01$ vs ConA group.

Groups	IL-2/ng·L ⁻¹	IFN- γ /ng·L ⁻¹	IL-4/ng·L ⁻¹	IL-10/ng·L ⁻¹
ConA	1613 \pm 37	865 \pm 34	425 \pm 14	887 \pm 22
ConA+F3 (0.1 $\mu\text{mol/L}$)	1948 \pm 98 ^c	1077 \pm 64 ^b	365 \pm 30 ^b	736 \pm 7 ^b
ConA+F3 (1 $\mu\text{mol/L}$)	2564 \pm 137 ^c	1248 \pm 121 ^c	288 \pm 11 ^c	609 \pm 99 ^c
ConA+F3 (10 $\mu\text{mol/L}$)	3624 \pm 34 ^c	1590 \pm 94 ^c	198 \pm 9 ^c	512 \pm 27 ^c
ConA+F3 (100 $\mu\text{mol/L}$)	3011 \pm 135 ^c	1181 \pm 66 ^c	296 \pm 20 ^c	619 \pm 27 ^c

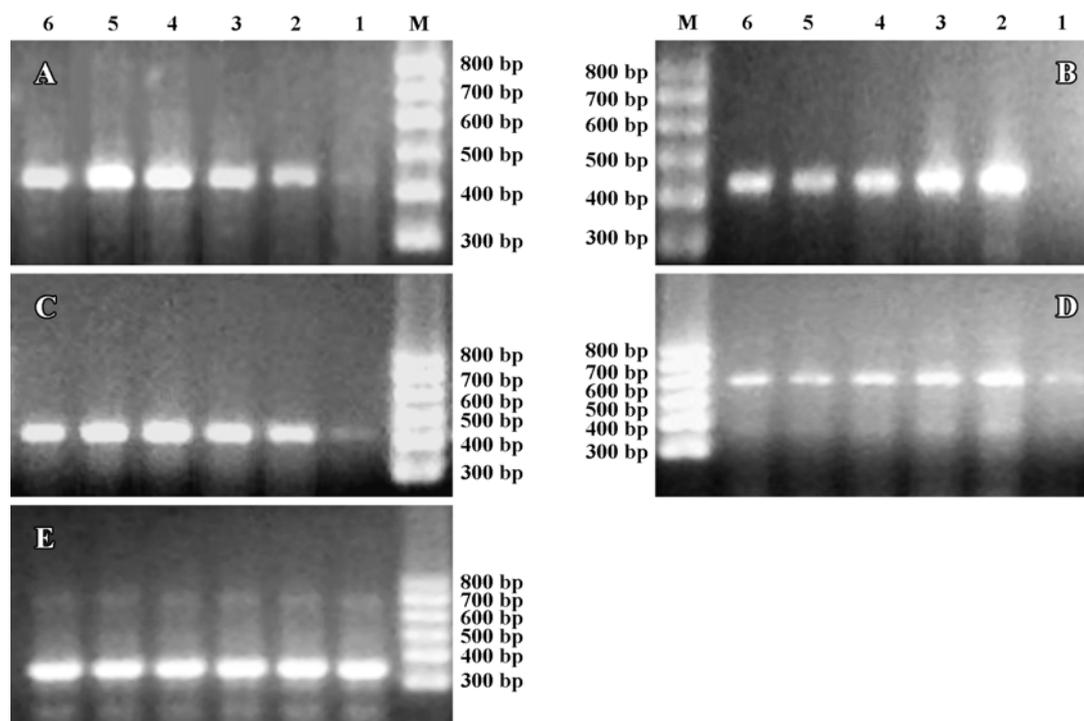


Fig 2. mRNA level of IFN- γ , IL-4, T-bet, GATA-3, GAPDH in mouse spleen cells treated with ginsenoside-F3 and ConA 1 mg/L. Mouse spleen cells were incubated with ginsenoside-F3 at the concentrations of 0.1-100 $\mu\text{mol/L}$ and ConA for 10 h. mRNA level of IFN- γ (A), IL-4 (B), T-bet (C), GATA-3 (D), GAPDH (E) were detected by PCR using specific primers. The amplified cDNA were resolved on 1 % (w/v) agarose gel and visualized by ethidium bromide. Lane M: DNA marker; Lane 1: untreated cell control; Lane 2: ConA; Lane 3: ginsenoside-F3 0.1 $\mu\text{mol/L}$ +ConA; Lane 4: ginsenoside-F3 1 $\mu\text{mol/L}$ +ConA; lane 5: ginsenoside-F3 10 $\mu\text{mol/L}$ +ConA; Lane 6: ginsenoside-F3 100 $\mu\text{mol/L}$ +ConA.

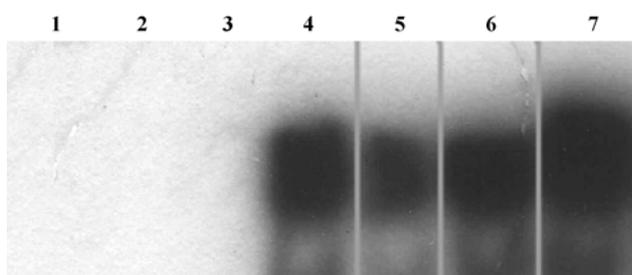


Fig 3. Effects of ginsenoside-F3 on activation of NF- κB in murine spleen cells. Nuclear extracts were prepared from murine spleen cells treated with ConA 1mg/L and ginsenoside-F3 10 $\mu\text{mol/L}$ and incubated with ^{32}P -labeled oligonucleotides encompassing NF- κB or mutational (mut) consensus motifs followed by analysis with EMSA. In lane 3 a 100-fold molar excess of unlabeled specific oligonucleotide was added to the binding reactions. Lane 1: Probe alone; Lane 2: NF- κB mut probe; Lane 3: NF- κB + competitive non ^{32}P -labeled probe; Lane 4: NF- κB positive control; Lane 5: control (unstimulated spleen cells) Lane 6: ConA; lane 7: ConA+ginsenoside-F3. Similar results were obtained in three experiments.

It is well known that *P. ginseng* can stimulate the immune system, but the exact components that stimulate immune system in *P. ginseng* and the exact mechanisms were not clear and needed to be studied further. Our study demonstrated that ginsenoside-F3 not only enhanced ConA-induced murine spleen cells proliferation, but also increased type 1 cytokines production, and decreased type 2 cytokines production by modulation cytokines and transcription factors gene expression and regulation NF- κB DNA binding activity in murine spleen cells. Thus, ginsenoside-F3 has immunodulatory activity and potential role in improving cell-mediated immune response.

REFERENCES

- 1 Yu SC, Li XY. Effect of ginsenoside on IL-1 beta and IL-6 mRNA expression in hippocampal neurons in chronic inflammation model of aged rats. *Acta Pharmacol Sin* 2000; 21: 915-8.
- 2 Zhou DL, Kitts DD. Peripheral blood mononuclear cell production of TNF-alpha in response to North American ginseng stimulation. *Can J Physiol Pharmacol* 2002; 80: 1030-3.

- 3 Cho JY, Kim AR, Yoo ES, Baik KU, Park MH. Ginsenosides from *Panax ginseng* differentially regulate lymphocyte proliferation. *Planta Med* 2002; 68: 497-500.
- 4 Liu YH, Liu YF, Guo XX. Current studies on anti-endotoxic chemical components of traditional Chinese medicine in China. *Acta Pharmacol Sin* 2001; 22: 1071-7.
- 5 Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. Development of TH1 CD4+ T cells through IL-12 produced by *Listeria*-induced macrophages. *Science* 1993; 260: 547-9.
- 6 Shier P, Hofstra CL, Ma XJ, Wu Y, Ngo K, Fung-Leung WP. Tbet-1, a new T-box transcription factor induced in activated Th1 and CD8 + T cells. *Immunogenetics* 2000; 51: 771-8.
- 7 Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 2000; 100: 655-69.
- 8 Zhang DH, Cohn L, Ray P, Bottomly K, Ray A. Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene. *J Biol Chem* 1997; 272: 21597-603.
- 9 Chakir H, Wang H, Lefebvre DE, Webb J, Scott FW. T-bet/GATA-3 ratio as a measure of the Th1/Th2 cytokine profile in mixed cell populations: predominant role of GATA-3. *J Immunol Methods* 2003; 278: 157-69.
- 10 Dou DQ, Chen YJ, Liang LH, Pang FG, Shimizu N, Takeda T. Six new dammarane-type triterpene saponins from the leaves of *Panax ginseng*. *Chem Pharm Bull* 2001; 49: 442-6.
- 11 Dou DQ, Hou WB, Chen YJ. Studies on the characteristic constituents of Chinese ginseng and American ginseng. *Planta Med* 1998; 64: 585-6.
- 12 Li LC, Shen F, Hou Q, Cheng GF. Inhibitory effect and mechanism of action of sanggenon C on human polymorphonuclear leukocyte adhesion to human synovial cells. *Acta Pharmacol Sin* 2002; 23: 138-42.
- 13 Kim HM, Shin HY, Jeong HJ, An HJ, Kim NS, Chae HJ, *et al*. Reduced IL-2 but elevated IL-4, IL-6, and IgE serum levels in patients with cerebral infarction during the acute stage. *J Mol Neurosci* 2000; 14: 191-6.
- 14 Jeong HJ, Kim BS, Kim KS, Kim HM. Regulatory effect of cytokine production in asthma patients by SOOJI CHIM (Koryo Hand Acupuncture Therapy). *Immunopharmacol Immunotoxicol* 2002; 24: 265-74.
- 15 Ferreira V, Sidenius N, Tarantino N, Hubert P, Chatenoud L, Blasi F, *et al*. *In vivo* inhibition of NF-kappa B in T-lineage cells leads to a dramatic decrease in cell proliferation and cytokine production and to increased cell apoptosis in response to mitogenic stimuli, but not to abnormal thymopoiesis. *J Immunol* 1999; 162: 6442-50.
- 16 Aronica MA, Mora AL, Mitchell DB, Finn PW, Johnson JE, Sheller JR, *et al*. Preferential role for NF-kappa B/Rel signaling in the type 1 but not type 2 T cell-dependent immune response *in vivo*. *J Immunol* 1999; 163: 5116-24.