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Tanshinlactone A from *Salvia miltiorrhiza* modulates interleukin-2 and interferon-γ gene expression

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Abstract

Salvia miltiorrhiza Bunge (Tanshen), a traditional Chinese herbal medicine, is popularly used to treat cardiovascular disorders. In the present study, effects of tanshinlactone A ($C_{16}H_{12}O_4$; M.W. 268), newly discovered from *Salvia miltiorrhiza*, on phytohemagglutinin (PHA)-stimulated cell proliferation were investigated in human peripheral blood mononuclear cells (PBMC). The results indicated that tanshinlactone A inhibited PBMC proliferation activated with PHA with an IC_{50} of 15.6 ± 1.9 μM. Cell viability test indicated that inhibitory effects of tanshinlactone A on PBMC proliferation were not through direct cytotoxicity. Furthermore, tanshinlactone A significantly decreased the interleukin-2 (IL-2) and interferon-γ (IFN-γ) gene expression in PHA-activated PBMC. It reduced the phosphorylation of mitogen-activated protein kinases (MAPK) involving extracellular signal-regulated protein kinase (ERK), P38, and c-Jun NH_2-terminal kinase (JNK) in PHA-treated PBMC. We suggested that the inhibitory effects of tanshinlactone A on PHA-induced PBMC proliferation, appeared to be mediated, at least in part, through reduction of MAPK activation and IL-2 and IFN-γ production. Therefore, data demonstrate for the first time that tanshinlactone A is likely an immunomodulatory agent for PBMC.

Keywords: *Salvia miltiorrhiza*; Tanshinlactone A; PBMC; IL-2; IFN-γ; MAPK

1. Introduction

Tanshen, the *Salvia miltiorrhiza* Bunge, belongs to Lamiaceae family and is regarded as an effective traditional Chinese medicine for cardiovascular diseases (Da et al., 2005). Some species of *Salvia* have been demonstrated to produce natural products with various biological activities such as antibacterial, antitumor, and antimutagenic effects (Ryu et al., 1997). The active functions of *Salvia miltiorrhiza* include: (1) improvement of memory (Ren et al., 2004), (2) reduction of neuron damage (Wu et al., 1997), (3) suppression of ethanol withdrawal syndrome (Mostallino et al., 2004), (4) decrease of liver fibrosis and tumor growth (Wang et al., 2004; Lin et al., 2006), and (5) inhibition of proinflammatory cytokines production (Kang et al., 2000; Jang et al., 2003). There has been a relative scarcity of definitive evidence to prove its effect on T cell-mediated immune responses. In the present study, a new component, tanshinlactone A (TA) identified from *Salvia miltiorrhiza* (Sun et al., 2006) was selected for immunomodulatory activity determination.

The central event in generation of immune responses is the activation and clonal expansion of T cells (Goldsby et al., 2000). Interaction of T cells with antigens or phytohemagglutinin (PHA) initiates a cascade of biochemical events and genes expression that induces the resting T cells to enter the cell cycle then proliferating and differentiating (Ajchenbaum et al., 1993; Kuo et al., 2000). It has been demonstrated in many previous studies with T cells that a series of genes such as interleukin-2 (IL-2) and interferon-γ (IFN-γ) are pivotal in the growth of T lymphocytes induced by antigens or PHA (Robb, 1984; Young, 1996). In mammalian cells, mitogen-activated protein kinases (MAPK) compose of the extracellular signal-regulated protein

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kinase (ERK), P38, and c-Jun NH_2-terminal kinase (JNK) (Dong et al., 2002). The activation of ERK is involved in T cells proliferation (Arnaud et al., 2004). Inhibition of JNK and P38 exhibits deficiencies in IFN-γ production (Yang et al., 1998). Thus, growth modulators or other external events that affect the T cell proliferation are ultimately likely to act by controlling the MAPK signaling or the expression of these genes (Robb and Greene, 1983; Kuo et al., 2003).

Yet the immune responses to invasive organisms, if sufficiently intense, could paradoxically aggravate the injury or even causing death. The use of immunomodulatory medications must therefore be discreet. Regulation of T lymphocyte activation and proliferation and cytokine production is one of the action mechanisms (Arai et al., 1990; Liu et al., 2004). Traditional Chinese herbs are now widely acknowledged for their immunomodulatory, antiviral, and antitumor activities (Kuo et al., 1994, 2006; Liu et al., 2005). In a previous study, we found that the ethanolic extracts of *Salvia miltiorrhiza* inhibited proliferation in human peripheral blood mononuclear cells (PBMC) activated by PHA (Yang et al., 1999). In the present study, TA was purified from *Salvia miltiorrhiza* and PBMC were used as target cells. In order to elucidate the effects of TA on PBMC proliferation, the tritiated thymidine uptake method was utilized to detect total cellular DNA synthesis in the cultures. Furthermore, we analyzed the actions of TA on production and gene expression of IL-2 and IFN-γ and activation of MAPK in PBMC induced by PHA and examined their roles in regulation of PBMC proliferation.

2. Materials and methods

2.1. The source of Salvia miltiorrhiza

The dried roots of *Salvia miltiorrhiza* were purchased from Chinese medicine shops in Taipei and identified by Mr. Jun-Chih Ou, a former research fellow of National Research Institute of Chinese Medicine (NRICM). A voucher specimen was deposited in the herbarium of NRICM.

2.2. TA purified from Salvia miltiorrhiza

The dried roots of *Salvia miltiorrhiza* (5.0 kg) were cut into small pieces before grinding. The ground roots were then extracted with ethanol (10 L × 3) at room temperature. The solvent was removed under reduced pressure and the residue was partitioned between H_2O and ethyl acetate (EtOAc). The concentrated EtOAc extracts (285 g) were subjected to chromatography over silica gel and eluted with *n*-hexane/EtOAc (4:1), *n*-hexane/EtOAc (1:1), and EtOAc, successively. The first fraction was rechromatographed on silica gel using mixtures of *n*-hexane and EtOAc under gradient condition (10:1–2:1). The subfraction was further purified by preparative TLC using *n*-hexane and EtOAc (3:1) as the mobile phase to give TA (10 mg; $C_{16}H_{12}O_4$; M.W. 268). The mass and NMR spectral data for this compound were identical with those previously reported by Sun et al. (2006). It was dissolved in dimethylsulfoxide (DMSO) to a concentration of 100 mM and then stored at 4 °C for use.

2.3. Human subjects

Ten healthy male subjects (20–30 years, mean age 25 years) were chosen for this investigation. The experimental protocol had been reviewed and approved by the institutional human experimentation committee. Written informed consent was obtained from each and every subject.

2.4. Preparation of PBMC

Heparinized human peripheral bloods (30 ml) were obtained from healthy donors. PBMC were isolated by the Ficoll–Hypaque gradient density method as described previously (Kuo et al., 2005). The peripheral blood was centrifuged at 850 × *g*, 4 °C for 10 min to remove the plasma. Blood cells were diluted with PBS buffer then centrifuged in a Ficoll–Hypaque discontinuous gradient (specific gravity 1.077) at 420 × *g* for 30 min. The PBMC layers were collected and washed with cold distilled water and 10× Hanks' buffer saline solution (HBSS) to remove red blood cells. The cells were resuspended to a concentration of 2×10^6 cells/ml in RPMI-1640 medium supplemented with 2% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.5. Lymphoproliferation test

The lymphoproliferation test was modified from previously described (Kuo et al., 2000). The density of PBMC was adjusted to 2×10^6 cells/ml before use. One hundred microlitres of cell suspension was applied into each well of a 96-well flat-bottomed plate (Nunc 167008, Nunclon, Raskilde, Denmark) with or without 5 μg/ml PHA (Sigma). Cyclosporin A, an immunosuppressor, was used as a reference drug (Schreiber and Crabtree, 1992). TA was added to the cells at varying concentrations (6.25–100 μM). The plates were incubated in 5% CO_2-air humidified atmosphere at 37 °C for 3 days. Subsequently, tritiated thymidine (1 μCi/well, NEN) was added into each well. After a 16-h incubation, the cells were harvested on glass fiber filters by an automatic harvester (Dynatech, Multimash 2000, Billingshurst, U.K.). Radioactivity in the filters was measured by a scintillation counting. The inhibitory activity of TA on PBMC proliferation was calculated with the following formula:

$$\text{Inhibitory activity}(\%) = \frac{\text{Control group(CPM)} - \text{Experiment group(CPM)}}{\text{Control group(CPM)}} \times 100$$

2.6. Determination of IL-2 and IFN-γ production

PBMC (2×10^5 cells/well) were cultured with PHA alone or in combination with varying concentrations of TA for 3 days. The cell supernatants were then collected and assayed for IL-2 and IFN-γ concentrations by the enzyme immunoassays (EIA; R&D systems, Minneapolis, USA). No detectable cross-reactivity with other cytokines has been reported for the EIA kits used.

2.7. Determination of cell viability

Approximately 2×10^5 PBMC were cultured with medium, 0.1% DMSO, or 6.25–100 μM TA for 4 days. Total, viable, and non-viable cell numbers were counted under the microscope with the help of a hemocytometer following staining by trypan blue. The percentage of viable cells was calculated:

$$\text{Viability}(\%) = \frac{\text{Viable cell number}}{\text{Total cell number}} \times 100$$

2.8. Extraction of total cellular RNA

PBMC (5×10^6) were activated with or without PHA and cocultured with 25, 50, or 100 μM of TA for 18 h. PBMC were collected and lysed by RNA-Bee™ (Tel-Test Inc., Friendswood, USA). After centrifugation, the supernatants were extracted with a phenol–chloroform mixture. The extracted RNA was precipitated with 100% cold ethanol. The total cellular RNA was pelleted by centrifugation and redissolved in diethyl pyrocarbonate (DEPC)-treated H_2O. The concentration of RNA was calculated by measuring the optical density at 260 nm.

2.9. Reverse transcription-polymerase chain reaction (RT-PCR)

The RT-PCR was performed by a method described previously (Kuo et al., 2000). Aliquots of 1 μg of RNA were reverse-transcribed to cDNA using the Advantage™ RT-for-PCR kit from CLONTECH according to the manufacturer's instructions. Briefly, 10 μl of cDNA was mixed with 0.75 μM primers, 4 units of Taq polymerase, 10 μl of reaction buffer (2 mM Tris–HCl, pH 8.0; 0.01 mM ethylenediaminetetraacetate, EDTA; 0.1 mM dithiothreitol, DTT; 0.1% Triton X-100; 5% glycerol; 1.5 mM $MgCl_2$), and 25 μl of water in a total volume of 50 μl. All primer pairs for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), IL-2, and IFN-γ were designed from the published human cDNA sequence data (Kuo et al., 2002) and shown in Table 1. The PCR was done by the following setting of the air thermocycler: denaturing temperature of 94 °C for 1 min, annealing temperature of 60 °C for 1 min, and elongation temperature of 72 °C for 80 s for the first 35 cycles and finally elongation temperature of 72 °C for 10 min. Following the reaction, the amplified products were taken out of the tubes and run on 2% agarose gel.

Table 1
Nucleotide sequences of the primers used for amplification of IL-2 and IFN-γ in PBMC by RT-PCR

Cytokine	Sequence	Predicted size (bp)
IL-2	5′-GTC ACA AAC AGT GCA CCT AC-3′ 5′-GAA AGT GAA TTC TGG GTC CC-3′	262
IFN-γ	5′-GCA GAG CCA AAT TGT CTC CT-3′ 5′-ATG CTC TTC GAC CTC GAA AC-3′	320
GAPDH	5′-TGA AGG TCG GAG TCA ACG GAT TTG GT 3′-CAT GTG GGC CAT GAG GTC CAC CAC	983

2.10. Real-time quantitative PCR

The procedures for real-time PCR were modified as described previously (Chen et al., 2007). Extracted cellular RNA was reversed transcribed into cDNA by a commercial available kit (Applied Biosystems, Foster City, CA). The real-time PCR reactions were performed in special optical tubes format on an ABI PRISM 7700 Sequence Detector System (Applied Biosystems, Foster City, CA) and the Taq-Man Universal PCR Master Mix (PE Applied Biosystems). The pre-developed FAM (6-carboxyfluorescein)- and TAMRA (6-carboxytetramethylrhodamime)-labeled primers and probes were used to amplify human IL-2 (4327036F; PE Applied Biosystems), IFN-γ (4327052F; PE Applied Biosystems), and GAPDH (4326317E; PE Applied Biosystems). The reaction conditions were 50 °C for 2 min following by 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. ΔCycle of threshold (ΔC_T) was calculated by subtracting the C_T of GAPDH mRNA from the C_T of IL-2 or IFN-γ mRNAs.

2.11. Western blotting

Western blot analysis was performed by a method described previously (Kuo et al., 2002; Chen et al., 2007). PBMC (1×10^7 cells) were treated with PHA (5 μg/ml) in the presence or absence of TA (25, 50, 100 μM) for 1 h. Cells were harvested and lysed by a solution containing 20 mM Tris–HCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 5 mM EDTA, 0.5% Triton X-100, 1 mM DTT, 10 μg/ml leupetin, 5 μg/ml aprotinin, and 10 mM β-glycerophosphate. The 50 μg proteins were resolved by 10% SDS-PAGE and transferred to nitrocellulose filters. After blocking the filters with a solution containing 1% bovine serum albumin (BSA), the filters were incubated with mouse monoclonal Abs raised against human ERK, pERK, P38, pP38, JNK, or pJNK (BD Biosciences, San Diego, USA). Specific reactive proteins were detected by an enhanced chemiluminescence method, employing a rabbit anti-mouse Ig Ab linked to horseradish peroxidase (Pierce, Rockford, USA).

2.12. Statistical analysis

Data were presented as mean ± S.D., and the differences between groups were assessed with Student's *t*-test at a significant level of $p < 0.05$.

3. Results

3.1. Inhibitory effects of TA on PBMC proliferation induced by PHA

To study the effects on PBMC proliferaiton, resting cells or cells activated with PHA were treated with various concentration of TA isolated from *Salvia miltiorrhiza* and cell proliferation was determined by tritiated thymidine uptake. As

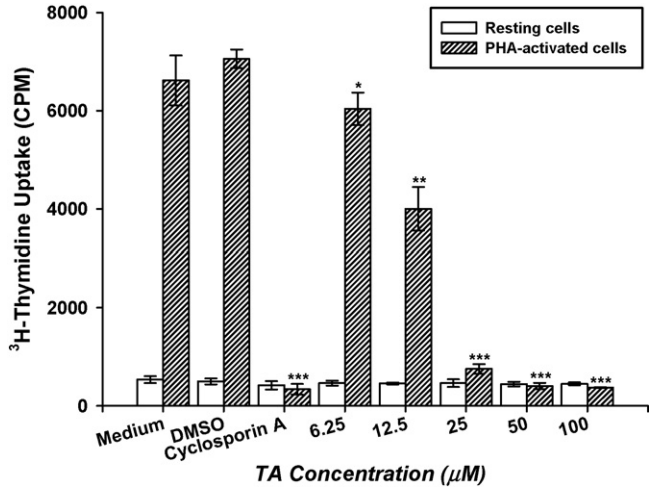

Fig. 1. Effects of TA on PBMC proliferation induced by PHA. PBMC (2×10^5/well) were treated by indicated concentration of TA with or without PHA (5 μg/ml) for 3 days. The proliferation of cells was measured as described under Section 2. Each bar represents the mean ± S.D. of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$ vs. the cells treated with DMSO and PHA.

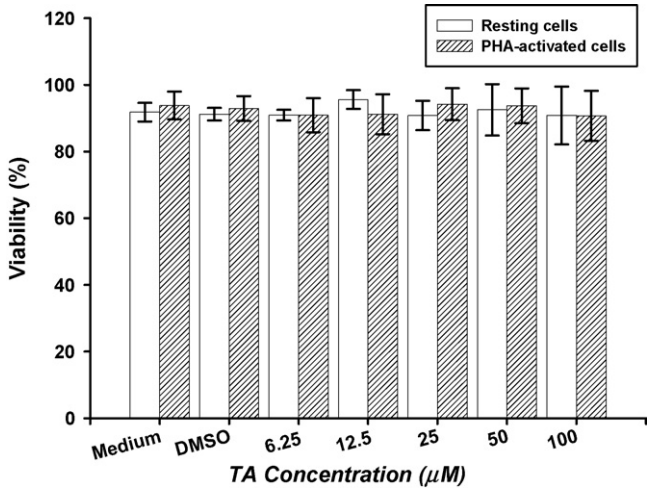

Fig. 2. The viability of PBMC treated with TA. PBMC (2×10^5) were stimulated with or without PHA (5 μg/ml) and treated with medium, 0.1%DMSO, or the indicated concentration of TA for 4 days. Numbers of total, viable, and nonviable cells were counted after trypan blue staining.

shown in Fig. 1, treatment with PHA for 3 days, stimulated cell proliferation by about 14-fold, as reflected by the increase in tritiated thymidine uptake. Neither the tritiated thymidine uptake in the resting nor stimulated states were affected by DMSO treatment. By contrast, cyclosporin A suppressed PBMC proliferation induced by PHA. Although TA had little effect on tritiated thymidine uptake in resting PBMC, the enhanced uptake observable in activated cells was significantly suppressed by TA. In addition, the inhibitory effect of TA on the activated cells was in a dosage-dependent fashion. At 12.5 μM, the inhibitory percentage of TA was 43.3 ± 6.3% on PBMC proliferation activated with PHA. The corresponding degree of inhibition for 25 μM was 89.3 ± 1.4%, while that for 100 μM was 94.7 ± 0.1%. The IC_{50} of TA on activated PBMC proliferation is 15.6 ± 1.9 μM.

3.2. The viability of PBMC treated with various concentration of TA

To delineate whether the inhibitory activity of TA on PBMC proliferation was related to cytotoxicity, we examined the viability of PBMC after treated with 6.25, 12.5, 25, 50, and 100 μM for 4 days. The results indicated that TA had no cytotoxicity because the viabilities of resting or activated cells were not significantly decreased after treated with the various concentration of TA for 4 days (Fig. 2). Comparison with medium treated, neither the viability of resting or PHA-activated PBMC was reduced by DMSO.

3.3. Effects of TA on IL-2 and IFN-γ production in activated PBMC

Production of IL-2 and IFN-γ is a hallmark of PBMC (Carter et al., 1998). To investigate whether TA suppressed PHA-induced PBMC proliferation was related to IL-2 and IFN-γ production, the cells were stimulated with PHA in the presence or absence of varying concentrations of TA (6.25–100 μM) for 3 days. Supernatants were then collected, and the production of IL-2 and IFN-γ was assayed by EIA. As shown in Fig. 3, treatment with 5 μg/ml of PHA for 3 days, stimulated IL-2 and IFN-γ production in PBMC by about 140-fold and 700-fold, respectively. By contrast, the stimulated production of IL-2 and IFN-γ in activated PBMC was significantly suppressed by TA. The inhibitory activity of TA was concentration dependent. The IC_{50S} of TA on IL-2 and IFN-γ prodcution in PBMC activated with PHA were 21.3 ± 3.7 and 18.8 ± 2.9 μM.

3.4. Inhibitory effects of TA on IL-2 and IFN-γ gene expression in PBMC

To determine whether TA reduced IL-2 and IFN-γ production was related to gene expression, total cellular RNA was extracted from activated PBMC in the presence or absence of TA and available for RT-PCR. The results of RT-PCR analyses are shown in Fig. 4. The mRNA for GAPDH was detectable in the samples treated with medium (Lane 1), DMSO (0.1%; Lane 2), TA (100 μM; Lane 3), PHA (5 μg/ml; Lane 4), DMSO and PHA (Lane 5), or TA and PHA (Lanes 6–8). The results indicated that the levels of IL-2 and IFN-γ mRNA in PBMC were significantly induced by PHA. By contrast, PCR products for both cytokines amplified from activated PBMC RNA preparations were reduced by TA. The laser densitometry analysis demonstrated that the ratios of IL-2 and IFN-γ to GAPDH mRNAs in PHA-activated PBMC were significantly decreased by TA. The inhibitory actions of TA on IL-2 and IFN-γ mRNAs expression were also analyzed with the real-time PCR (Table 2). Comparison with the PHA-treated group, TA reversed the ΔC_T values of IL-2 and IFN-γ in activated PBMC. Thus, these data are consistent with inhibition of IL-2 and IFN-γ production by TA.

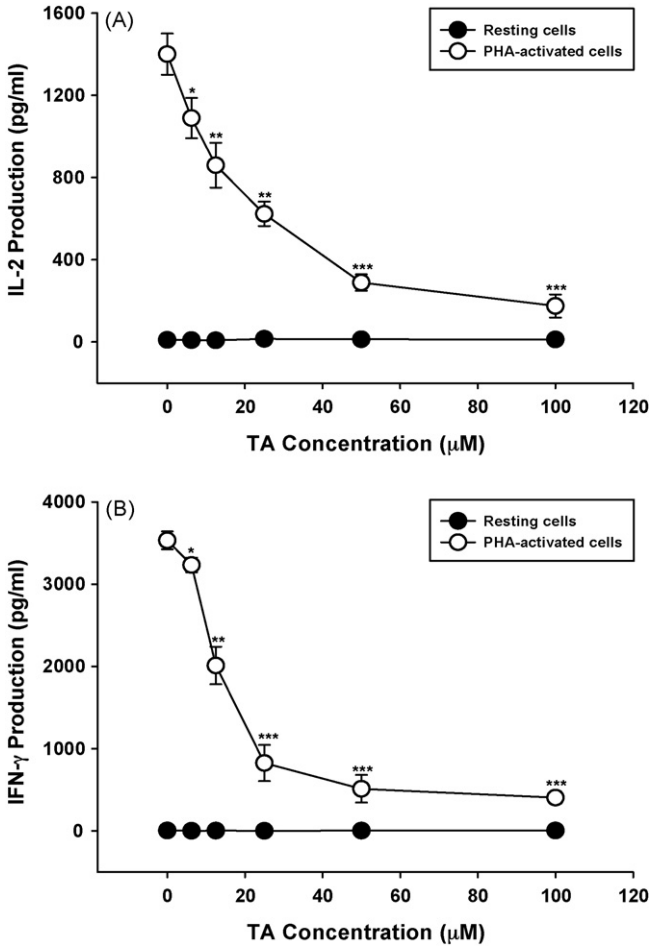

Fig. 3. IL-2 and IFN-γ production in PBMC treated with TA. PBMC (2×10^5/well) were treated by 6.25–100 μM of TA with or without PHA (5 μg/ml) for 3 days. Then IL-2 and IFN-γ productions in the cell supernatants were were determined by EIA. Each point is the mean ± S.D. of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$ vs. the cells treated with DMSO and PHA.

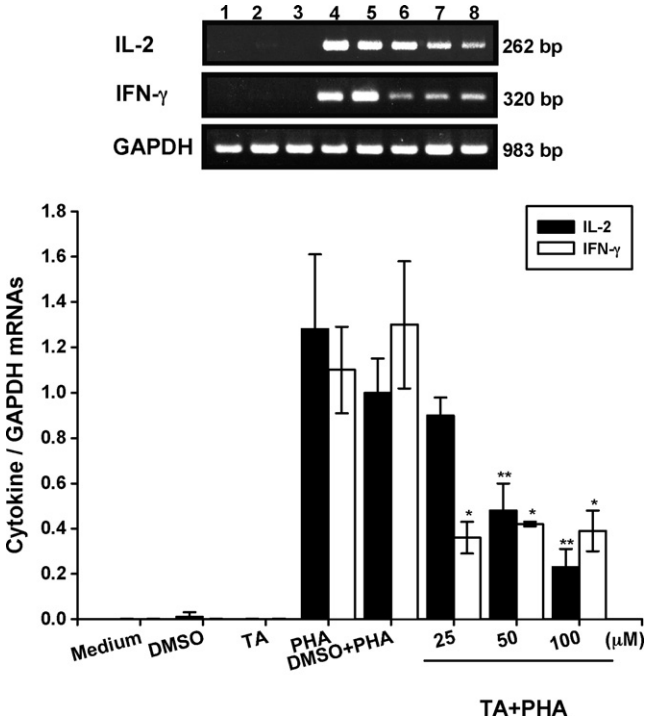

Fig. 4. Effects of TA on IL-2 and IFN-γ transcrips in PBMC induced by PHA. Resting or PHA-activated PBMC (5×10^6) were treated with 25, 50. or 100 μM TA for 18 h. The total cellular RNA was isolated from PBMC treated with medium (Lane 1), DMSO (0.1%; Lane 2), TA (100 μM; Lane 3), PHA (5 μg/ml; Lane 4), DMSO and PHA (Lane 5), or TA (25, 50, 100 μM) and PHA (Lanes 6–8), respectively. The RT-PCR was done as described under Section 2. Each band was quantitated using laser-scanning densitometer SLR-2D/1D (Biomed Instruments Inc., Fullerton, CA). The ratio of each cytokine to GAPDH mRNAs was calculated. Each bar is the mean ± S.D. of three independent experiments. *$p < 0.05$, **$p < 0.01$ vs. the cells treated with DMSO and PHA.

3.5. Effects of TA on MAPK activation in PBMC stimulated by PHA

To delineat whether TA affected PBMC signaling, total cellular proteins were extracted from PBMC, phosphorylation of ERK, P38, and JNK was determined by Western blotting. As shown in Fig. 5, the phosphorylation of ERK, P38, and JNK in resting PBMC was not affected by DMSO (0.1%; Lane 2) or TA (100 μM; Lane 3) treated alone. After treatment with PHA for 1 h, the phosphorylation of ERK, P38, and JNK was detected in PBMC (Lane 4). While the presence of DMSO did not affect ERK, P38, and JNK activation (Lane 5), the phosphorylation of MAPK was significantly impaired by TA with

Table 2
ΔC_T for IL-2 and IFN-γ at TA-treated PBMC

	C_T		ΔC_T	C_T		ΔC_T
	IL-2	GAPDH		IFN-γ	GAPDH	
Medium	31.89 ± 0.04	20.22 ± 0.10	11.67 ± 0.07	32.78 ± 0.13	19.85 ± 0.08	12.93 ± 0.18
Medium + PHA	23.18 ± 0.09	18.55 ± 0.02	4.63 ± 0.06	24.77 ± 0.08	22.01 ± 0.06	2.76 ± 0.10
DMSO	32.14 ± 0.06	20.51 ± 0.03	11.63 ± 0.09	33.47 ± 0.04	20.44 ± 0.05	13.03 ± 0.08
DMSO + PHA	22.79 ± 0.08	18.13 ± 0.10	4.66 ± 0.01$^{\#}$	24.02 ± 0.10	21.36 ± 0.05	2.66 ± 0.05$^{\#}$
TA (100 μM)	32.88 ± 0.11	20.97 ± 0.02	11.91 ± 0.07	33.15 ± 0.01	20.02 ± 0.01	13.13 ± 0.01
TA(25 μM) + PHA	23.64 ± 0.03	19.13 ± 0.04	4.52 ± 0.06*	26.16 ± 0.02	20.56 ± 0.11	5.60 ± 0.13**
TA(50 μM) + PHA	26.88 ± 0.05	20.45 ± 0.03	6.43 ± 0.04**	25.12 ± 0.04	19.77 ± 0.01	5.35 ± 0.04**
TA(100 μM) + PHA	29.33 ± 0.02	19.79 ± 0.05	9.54 ± 0.04**	25.89 ± 0.23	20.87 ± 0.20	5.02 ± 0.20**

PBMC (5×10^6 cells) were cultured with 25, 50, or 100 μM TA in the presence or absence of PHA for 18 h. The cDNA was reverse-transcribed from cellular RNA and amplified by TaqMan PCR assay with an ABI prism 7700 sequence detection system. Each ΔC_T was calculated by subtracting the C_T of GAPDH mRNA from the C_T of IL-2 or IFN-γ mRNAs, respectively. The representative data from three independent experiments are shown. $^{\#}p < 0.0001$, as compared with DMSO group; *$p < 0.05$; **$p < 0.001$, as compared with the DMSO + PHA group.

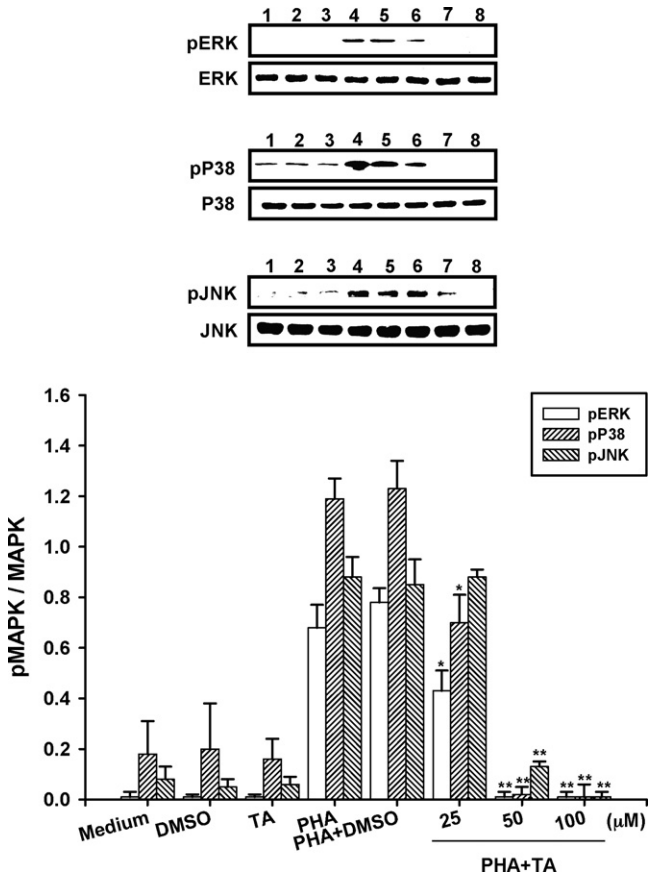

Fig. 5. Effects of TA on the phosphorylation of ERK, p38, and JNK in PBMC. PBMC (1×10^7 cells) were treated with medium (Lane 1), 0.1% DMSO (Lane 2), 100 μM TA (Lane 3), 5 μg/ml PHA (Lane 4), DMSO and PHA (Lane 5), or TA (25, 50, 100 μM) and PHA (Lanes 6–8), for 60 min. The total cellular proteins (50 μg) were run on a 10% SDS-PAGE gel and analyzed by immunoblotting with anti-pMAPK or MAPK Abs. Each band was quantitated by laser-scanning densitometer SLR-2D/1D (Biomed Instruments Inc., Fullerton, USA) and the ratio of pMAPK to MAPK was calculated. Each bar is the mean ± S.D. of more than three independent experiments. $*p < 0.05$, $**p < 0.01$ vs. the cells treated with DMSO and PHA.

a dosage-dependent manner (Lanes 6–8). The laser densitometry analysis demonstrated that the ratios of pMAPK to MAPK in PHA-activated PBMC were significantly decreased by TA. Taken together, these results indicate that TA affects the activation of MAPK in PBMC induced by PHA.

4. Discussion and conclusions

In the present study, we have shown for the first time that TA identified from *Salvia miltiorrhiza* has a profound inhibitory effect on the proliferation of human PBMC stimulated with PHA. The proliferation-suppressive actions of TA were not explained by a drug-induced reduction in cell viability. We observed that TA decreased production of IL-2 and IFN-γ and phosphorylation of MAPK in activated PBMC. We predict that an immunomodulatory agent is present in *Salvia miltiorrhiza*.

TA is a new diterpenoid tanshinone compound from *Salvia miltiorrhiza* (Sun et al., 2006). Diterpenoid tanshinones have a variety of pharmacological effects including (1) antibacterial activities (Lee et al., 1999; Jang et al., 2003), (2) improvement coronary heart diseases (Chen, 1984; Chang et al., 1990), (3) antiinflammatory activities (Kim et al., 2002), (4) antiallergic activities (Choi and Kim, 2004), (5) antioxidative effects (Zhang et al., 1990), (6) protective effects on injured hepatocytes (Liu et al., 2003), (7) antitumor activities (Mosaddik, 2003), and (8) antiplatelet aggregation activities (Lee et al., 1987). Additionally, we proved that TA suppressed proliferation, IL-2 and IFN-γ production, and MAPK activation in human PBMC stimulated with PHA. It is unlikely that TA suppressed PBMC proliferation through cytotoxic effects, because the data indicated that cell viabilities of PBMC treated with or without TA for 4 days had no difference. PBMC proliferation and viability were not changed by DMSO. Therefore, the inhibitory function of TA was unlikely related to DMSO.

PHA stimulates T cells to proliferate and produce cytokines (Goldsby et al., 2000). In the present study, T cells were major proliferating cells in PBMC activated with PHA. Thus, inhibitory effect of TA on PHA-activated PBMC proliferation could be suggested as suppression on T cells proliferation. As we know interaction of T cells with antigens or mitogens initiates IL-2 and IFN-γ mRNA expressions that induce resting T cells to proliferate (Janeway et al., 1997). Our results showed TA inhibited IL-2 and IFN-γ production in PBMC stimulated with PHA. The impairments of IL-2 and IFN-γ production were related to TA suppressing their mRNA transcriptions determined by RT-PCR and real-time quantitative PCR. Comparison with the conventional semi-RT-PCR, the fluorescence-based real-time quantitative PCR can combine high sensitivity with reliable specificity (Bustin et al., 2005). It may explain why IL-2 and IFN-γ mRNAs in medium, DMSO, or TA-treated PBMC were detectable using real-time quantitative PCR, but not by semi-RT-PCR. Since T lymphocyte proliferation is primarily mediated by IL-2, inhibition of IL-2 production is a central mechanism of action of several immunosuppressants including cyclosporin A. (Schreiber and Crabtree, 1992). It may be expected that failure to produce IL-2 and IFN-γ in TA-treated PBMC is the reason that PBMC do not proliferate.

Recently, the ERK, P38, or JNK inhibitors were added into PBMC and the cell proliferation and cytokines production induced by PHA were determined. The results indicate that these inhibitors decrease the proliferation and IL-2 and IFN-γ mRNAs expression in PBMC (Chen et al., 2007). ERK activation affects IL-2 and IFN-γ gene expression and T cell growth (Arnaud et al., 2004). Inhibition of JNK and p38 signaling causes the down regulation of IL-2 and IFN-γ production (D'Alimonte et al., 2007). In the present study, TA reduced the ERK, P38, and JNK phosphorylation. Thus, we predicted that the inhibitory effects of TA on PBMC proliferation and cytokines production were related to interruption of MAPK activation. It is believed that overexpression of inflammation will cause diseases such as nephritis, arthritis, and allergy (Kaliner and Lemanske, 1992; Janeway et al., 1997; Kuo et al., 1998; Linton and Morgan, 1999). If PBMC activation and proliferation and cytokines production were attenuated, the clinical symptoms associated with these diseases will be released (Lin et al., 1999). Our finding indicated that TA suppressed cell proliferation and gene expression

of IL-2 and IFN-γ in PBMC. These results may partly explain Tanshen's antiinflammatory effect and correlate with its putative pharmacological activities.

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