Acta Pharmacologica Sinica (2013) 34: 522–530
 1671-4083/13 $32.00
www.nature.com/aps

Original Article

Astragaloside II triggers T cell activation through regulation of CD45 protein tyrosine phosphatase activity

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Aim: To investigate the immunomodulating activity of astragalosides, the active compounds from a traditional tonic herb *Astragalus membranaceus* Bge, and to explore the molecular mechanisms underlying the actions, focusing on CD45 protein tyrosine phosphatase (CD45 PTPase), which plays a critical role in T lymphocyte activation.

Methods: Primary splenocytes and T cells were prepared from mice. CD45 PTPase activity was assessed using a colorimetric assay. Cell proliferation was measured using a [^{3}H]-thymidine incorporation assay. Cytokine proteins and mRNAs were examined with ELISA and RT-PCR, respectively. Activation markers, including CD25 and CD69, were analyzed using flow cytometry. Activation of LCK (Tyr505) was detected using Western blot analysis. Mice were injected with the immunosuppressant cyclophosphamide (CTX, 80 mg/kg), and administered astragaloside II (50 mg/kg).

Results: Astragaloside I, II, III, and IV concentration-dependently increased the CD45-mediated of pNPP/OMFP hydrolysis with the EC_{50} values ranged from 3.33 to 10.42 μg/mL. Astragaloside II (10 and 30 nmol/L) significantly enhanced the proliferation of primary splenocytes induced by ConA, alloantigen or anti-CD3. Astragaloside II (30 nmol/L) significantly increased IL-2 and IFN-γ secretion, upregulated the mRNA levels of IFN-γ and T-bet in primary splenocytes, and promoted CD25 and CD69 expression on primary $CD4^+$ T cells upon TCR stimulation. Furthermore, astragaloside II (100 nmol/L) promoted CD45-mediated dephosphorylation of LCK (Tyr505) in primary T cells, which could be blocked by a specific CD45 PTPase inhibitor. In CTX-induced immunosuppressed mice, oral administration of astragaloside II restored the proliferation of splenic T cells and the production of IFN-γ and IL-2. However, astragaloside II had no apparent effects on B cell proliferation.

Conclusion: Astragaloside II enhances T cell activation by regulating the activity of CD45 PTPase, which may explain why *Astragalus membranaceus* Bge is used as a tonic herb in treating immunosuppressive diseases.

Keywords: immunomodulating agent; *Astragalus membranaceus* Bge; astragaloside II; CD45 PTPase; LCK; T cell; splenocyte; Src-family kinase; IFN-γ; IL-2; cyclophosphamide

Acta Pharmacologica Sinica (2013) 34: 522–530; doi: 10.1038/aps.2012.208; published online 25 Mar 2013

Introduction

Astragalus membranaceus (Fisch) Bge (AM) is a well-known traditional Chinese herbal medicine that has been used as a tonic herb in various immuno-deficient diseases. Clinically, it is thought to act by boosting the body's general vitality and by strengthening resistance to exogenous pathogens. In recent decades, AM was reported to be composed primarily of polysaccharides and saponins[1, 2], which were considered the most important components for the immunomodulatory activities of AM. It has been reported that triterpene saponins, including astragaloside I, II, II, and IV, possess a prominent IL-2-inducing activity, which may have a contributing role in immune-stimulating and anticancer effects of these compounds[2]. Astragaloside IV, a key component of AM, has been shown to increase T and B lymphocyte proliferation and antibody production *in vivo* and *in vitro*, but inhibit the production of IL-1 and TNF-α from peritoneal macrophages *in vitro*[3]. Astragaloside IV can boost both cellular and humoral immune responses[4, 5]. However, comparatively little immu-

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Received 2012-10-23 Accepted 2012-12-26

nomodulating activity has been reported for other saponins. Additionally, the molecular mechanism of action of AM has yet to be fully elucidated.

The delicate balance between kinases and phosphatases is crucial for regulating lymphocyte signaling[6]. CD45 is a member of a family of transmembrane PTPs and is expressed exclusively on the surface of all hematopoietic cells and their precursors, except mature erythrocytes and platelets. The major intracellular targets of CD45 PTPase activity are the Src-family kinase (SFKs). In T cells, the predominant SFKs are p56LCK and p59Fyn (Fyn). LCK, in particular, is a primary initiator of signal transduction upon T cell receptor engagement[6]. CD45 is highly glycosylated and has been estimated to comprise up to 10% of the T lymphocyte surface area[7]. CD45 plays a critical role in T-cell receptor (TCR)-mediated signaling by regulating the phosphorylation and activation of protein tyrosine kinases and their substrates[7]. Given that the cytosolic portion of CD45 contains protein tyrosine phosphatase activity and is critical for TCR-mediated T cell activation, compounds specifically targeting this PTPase might be useful in treating immune disorders.

In the present study, we performed a high-throughput screen by utilizing the cytosolic portion of CD45 and *pNPP*/OMFP as the substrates to screen our in-house compound library. The results showed that astragaloside I, II, III and IV significantly improved CD45-mediated *pNPP*/OMFP hydrolysis. Consequently, we demonstrated that astragaloside II was the most promising compound for regulating immune activities *in vitro*. Astragaloside II enhanced T lymphocyte proliferation in response to ConA, alloantigen and anti-CD3 stimulation. It also increased IL-2 and IFN-γ production, upregulated mRNA expression of IFN-γ and T-bet, and enhanced the expression of CD25 and CD69 on $CD4^+$ T cells. In immunosuppressed mice, oral administration of astragaloside II (50 mg/kg, *po*) promoted the recovery of splenic T cell proliferation and the production of IFN-γ and IL-2. However, astragaloside II had no apparent effects on B cell proliferation *in vitro* or *in vivo*. Compounds with a thiophene core can bind to PTP receptors with high affinity and have demonstrated extensive cellular activities[8]. We hypothesized that astragaloside II might also interact directly with T lymphocyte surface proteins, which are critical components of the TCR signaling pathway. In this case, a specific CD45 PTPase inhibitor was used. The results showed that astragaloside II significantly promoted T cell proliferation and IFN-γ production, and the immunomodulatory effects were blocked by a CD45 PTPase inhibitor.

This observation implied that astragaloside II could directly affect T cell function by regulating the activity of CD45 PTPase. It has been confirmed that LCK (Tyr505) is a physiologically relevant target of CD45 activity *in vivo* and that the dephosphorylation of CD45 enables LCK to be active[9]. Astragaloside II significantly promoted the CD45 PTPase-dependent dephosphorylation of LCK (Tyr505), which was blocked by a specific CD45 PTPase inhibitor. Based on these findings, we proposed a new mechanism for the immunomodulating activity of astragaloside.

Materials and methods

Chemicals and reagents

Astragaloside I, II, III, and IV were extracted and purified from AM. The purity of the astragalosides was over 98%, as ascertained using HPLC analysis.

Concanavalin A (ConA), lipopolysaccharide (LPS, *Escherichia coli* O55:B5), and 3, 3′,5,5′-tetrametylbenzidine (TMB) were purchased from Sigma (St Louis, MO, USA). RPMI (Roswell Park Memorial Institute)-1640 medium was purchased from Gibco BRL, Life Technologies (Carisbad, CA, USA). Fetal bovine serum (FBS) was purchased from Hyclone Laboratories (Logan, UT, USA). Mouse cytokine (IL-2, IFN-γ)-detecting ELISA kits were from BD Biosciences (San Diego, CA, USA). [^{3}H]-thymidine (1 mCi/mL) was purchased from the Shanghai Institute of Atomic Energy. CD45 inhibitor N-(9,10-dioxo-9,10-dihydro-phenanthren-2-yl)-2,2-dimethyl-propionamide was obtained from Calbiochem (La Jolla, CA, USA). A polyclonal antibody against LCK (phospho-Tyr505) was obtained from Biolabs (San Diego, CA, USA). Rat-anti-mouse CD16/CD32 (clone 2.4G2) was purified in-house. Anti-CD3 (145-2C11) monoclonal antibody, PE-conjugated anti-CD4, FITC-conjugated anti-CD25, biotin-conjugated anti-CD69 and cytochrome-conjugated streptavidin were purchased from BD Bioscience. Cyclophosphamide was purchased from Jiangsu Hengri Pharmaceutical Company.

Enzyme-based assay for CD45 phosphatase activity

A colorimetric assay to measure the activity of CD45 was performed in a 96-well plate. PNPP (50 mmol/L)/OMFP (0.5 mmol/L) and amylopectin (0.9 μg/μL) were used to determine the phosphatase activity of Hs-laforin, Cm-laforin, and SEX4. Assays were performed as previously described[10]. Briefly, reactions were carried out in buffer containing 0.1 mol/L sodium acetate, 0.05 mol/L bis-Tris, 0.05 mol/L Tris-HCl, 2 mmol/L dithiothreitol, pH 6.0 for the pNPP assay or in a buffer (0.1 mol/L Tris-HCl pH 8, 40 mmol/L NaCl, 2 mmol/L DTT) for the OMFP assay. The absorbance of the product was measured at 410 nm for the pNPP assay and at 490 nm for the OMFP assay. The reaction was terminated by the addition of 0.1 mol/L N-ethylmaleimide prior to the addition of malachite green reagent. The activity of the compound was continuously monitored, and the initial rate of dephosphorylation was determined by using the early linear region of the enzymatic reaction kinetic curve.

Experimental animals

Female BALB/c and C57BL/6 mice (6–8 weeks old) were obtained from Shanghai Laboratory Animal Center of the Chinese Academy of Sciences, and the mice were housed in controlled, pathogen-free conditions (12-h light/12-h dark photoperiod, 22±1 °C, 55%±5% relative humidity). All mice were allowed to acclimate in our facility for 1 week before the experiments began. All experiments were performed according to the Institutional Ethical Guidelines on Animal Care and

approved by the Institute Animal Care and Use Committee at Shanghai Institute of Materia Medica.

Cell preparation

The mice were sacrificed, and the spleens were removed aseptically. A splenocyte suspension was prepared as previously described[11] and resuspended in RPMI-1640 media (containing 10% FBS) supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL).

Purified T cells were prepared by using immuno-magnetic negative selection to deplete B cells and I-A^+APC, as described previously[12]. Briefly, lymphocytes were allowed to react with an anti- I-$A^{d/b}$ monoclonal antibody (mAb) and then incubated with magnetic beads bound to goat anti-mouse Ig. The purity of the resulting T cell populations was examined by flow cytometry and was consistently >95%.

Proliferation assay

The proliferation of splenocytes in response to ConA, LPS or alloantigen was determined by [^{3}H]-thymidine as described previously[13]. Briefly, BALB/c splenocyte suspensions (4×10^5 cells/well) were cultured in a 96-well flat-bottom plate (Costar) with ConA-treated (1 μg/mL), LPS-treated (10 μg/mL) or 30 Gy (Gammacell 3000, Canada)-irradiated C57BL/6 splenocytes (4×10^5 cells/well). The cultures were incubated for 48 h and 96 h for ConA, LPS and alloantigen-induced proliferation. The cultures were then pulsed with 0.5 μCi [^{3}H]-thymidine for 8 h (ConA- and LPS-induced proliferation) or 24 h (alloantigen-induced proliferation) prior to the termination of the cultures. The cultured cells were harvested onto glass fiber filters. The radioactivity was determined with a Beta Scintillation Counter (MicroBeta Trilux, MA, USA).

Anti-CD3 mediated splenic T lymphocyte activation

BALB/c splenocytes (4×10^5 cells/well) were cultured in 96-well flat-bottom plates that were coated with anti-CD3 (5 μg/mL), in the absence or presence of astragaloside II, at the indicated concentrations. The culture supernatants were harvested after 36 h to measure IL-2 and IFN-γ levels by ELISA following the manufacturer's instruction.

To investigate IL-2, IFN-γ and T-bet mRNA expression, total RNA was isolated 16 h after stimulation with anti-CD3 (5 μg/mL) by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), reverse transcribed, and amplified by polymerase chain reaction using specific primers. RT-PCR products were visualized by electrophoresis through 1.2% agarose gels containing ethidium bromide. Gene-specific primers are as follows:

IL-2, (sense) 5′-TGAGCAGGATGGAGAATTACAGG-3′,
(anti-sense) 5′-GTCCAAGTTCATCTTCTAGGCAC-3′;
IFN-γ, (sense) 5′-ATGAACGCTACACACTGCATC-3′,
(anti-sense) 5′-CCATCCTTTTGCCAGTTCCTC-3′;
T-bet, (sense) 5′-CCAGGAAGTTTCATTTGGGAAGC-3′',
(anti-sense) 5′-ACGTGTTTAGAAGCACTG-3′;
β-actin, (sense) 5′-GGCTGTATTCCCCTCCATCG-3′,
(anti-sense) 5′-CCAGTTGGTAACAATGCCATGT-3′.

To detect the expression of an activated marker, splenocytes were stimulated with anti-CD3 (5 μg/mL) in the absence or presence of astragaloside II (30 nmol/L) for 36 h. Next, the cells were collected and blocked with rat-anti-mouse CD16/CD32 and stained with phycoerythrin-conjugated anti-CD4, fluorescein isothiocyanate-conjugated anti-CD25 and biotin-conjugated anti-CD69 plus Cytochrome-conjugated streptavidin. The expression levels of CD69 and CD25 were analyzed on a FACS Calibur (BD Biosciences, San Jose, CA, USA).

Induction of immunosuppression and treatment protocols

Mice received intraperitoneal injections (ip) of CTX (80 mg/kg) on d 1 and d 4, and were then randomly divided into two groups: a vehicle control group and an astragaloside II group. Astragaloside II (50 mg/kg) was orally administered daily from d 1 for 8 d. Twenty-four hours after the last drug administration, the mice were sacrificed, and splenic lymphocytes were prepared.

T cell activation and Western blotting assay

Purified primary T cells (5×10^6/mL) were pretreated with astragaloside II at the indicated concentrations for 2 h in the presence or absence of the CD45 PTPase inhibitor before stimulation with anti-CD28 (2 μg/mL). The cells were then added to anti-CD3-coated plates (5 μg/mL) for 30 min. After the incubation, the cells were harvested and lysed in SDS sample buffer (62.5 mmol/L Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.02% bromophenol blue) and boiled for 10 min at 100 °C. The proteins were separated by running them through 10% SDS-polyacrylamide gel electrophoresis (PAGE) gels and transferring them to a nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The blots were blocked with 5% BSA-TBST buffer (TBS containing 0.1% Tween-20) for 1 h at room temperature. The membranes were incubated overnight at 4 °C with 1:500 or 1:5000 dilutions of the polyclonal antibodies against phospho-LCK (Tyr505). The blots were rinsed three times with TBST buffer for 15 min each time. The washed blots were incubated with a 1:4000 dilution of horseradish peroxidase conjugated-secondary antibody for 1 h and then washed three times with TBST buffer. The transferred proteins were visualized with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

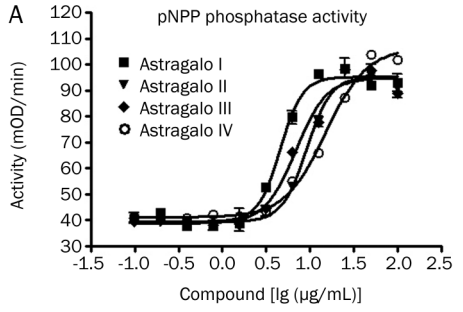
Statistical analysis

Data are expressed as the mean±SEM of each indicated experiment. Student's *t* test was used to determine significance between two groups, where appropriate. $P<0.05$ was considered statistically significant.

Results

Astragalosides significantly increased CD45 phosphatase activity

A colorimetric assay was used to examine the effects of compounds on CD45 phosphatase activity. As shown in Figure 1, astragalosideI, II, III, and IV significantly enhanced CD45-mediated *p*NPP/OMFP hydrolysis in a dose-dependent manner. Additionally, the EC_{50} value and activation ratio were pre-



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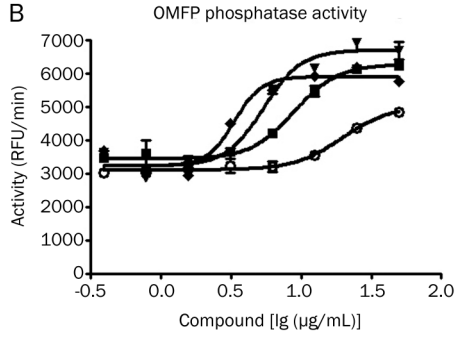
Compound	pNPP		OMFP	
	EC_{50} (μg/mL)	Activation ratio	EC_{50} (μg/mL)	Activation ratio
As I	4.57	2.41	8.88	1.83
As II	9.23	2.39	5.37	2.08
As III	6.86	2.44	3.33	1.83
As IV	15.15	2.58	19.41	1.62



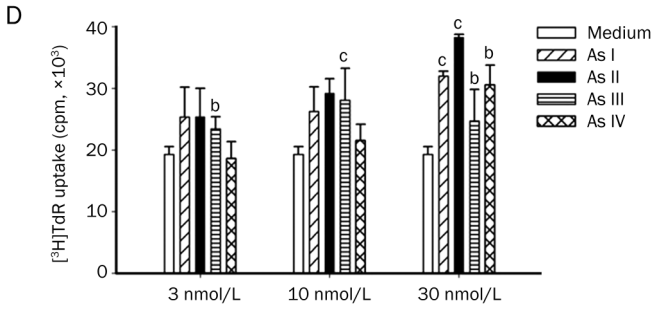


Figure 1. Astragalosidel, II, III, and IV significantly enhanced CD45 phosphatase activity towards the substrates pNPP (A) and OMFP (B). CD45 activity was detected by monitoring the dephosphorylation of pNPP and OMFP, and the EC_{50} value (C) was also analyzed. The lymphocyte proliferation assay was used to investigate anti-CD3-stimulated splenocyte proliferation (D). The results are expressed as the mean±SEM of three independent experiments. $^{b}P<0.05$, $^{c}P<0.01$ *vs* the medium group. The EC_{50} is the concentration of compound required to increase enzyme activity by 50%. The activation ratio was calculated as Top Value/Bottom Value.

sented in Figure 1C. Then, we investigated the proliferation of anti-CD3-stimulated splenocytes. The results indicated that astragaloside I, II, III, and IV significantly increased anti-CD3 induced splenocyte proliferation. Astragaloside II possessed the strongest proliferation-enhancing effect (Figure 1D). Thus, astragaloside II was chosen as the representative compound for further investigation of immunomodulating activity and exploration of the underlying mechanism of AM.

Astragaloside II enhanced ConA-induced murine splenocyte proliferation and mixed lymphocyte reaction (MLR)

ConA and LPS have been considered T and B cell mitogens, respectively. MLR is a model of T cell response to an alloantigenic peptide complex with major histocompatibility (MHC) protein on the APC. The *in vitro* immunomodulatory activity of astragaloside I, II, III, and IV were investigated. As shown in Figure 2, astragaloside II promoted ConA- and alloantigen-induced T cell proliferation. However, astragaloside II exerted no obvious effects on LPS-induced B cell proliferation (Figure 2B), suggesting that it was the T cell population that was mainly influenced by astragaloside II.

Astragaloside II elevated the immune response in CTX-treated mice

Astragaloside II showed significant immunomodulatory activity *in vitro*, as described above. To further explore the immunoregulatory properties of astragaloside II, the *in vivo* immunological activity was investigated in CTX-induced immunocompromised mice. The proliferative responses of splenocytes upon ConA stimulation were markedly impaired in CTX-treated mice as compared with the normal mice. Administration of astragaloside II (50 mg/kg, *po*) significantly rescued the splenic T cell proliferation induced by ConA (Figure 3A). ConA-induced production of IL-2 and IFN-γ was also consistently and markedly decreased in CTX-treated mice, but the production of these proteins increased after the administration of astragaloside II (Figure 3B and 3C).

Astragaloside II significantly promoted anti-CD3-stimulated splenocyte activation

Because T cell populations have been considered an important target in the immunomodulatory activity of astragaloside II, both *in vitro* and *in vivo*, we further studied the effect of astragaloside II on anti-CD3-mediated splenocyte activation. The results indicated that astragaloside II significantly promoted cell proliferation (Figure 4A) and the secretion of IL-2 (Figure 4B) and IFN-γ (Figure 4C) in a dose-dependent manner in anti-CD3-stimulated splenocytes.

The transcription factor T-bet controls the polarization of Th1 cells, which preferentially produce IFN-γ. Additionally, expression of T-bet is the hallmark of Th1 cells[14, 15]. After anti-CD3 stimulation, the expression of Th1-related cytokines and transcription factors at the mRNA level was examined (Figure 4D). The results showed that astragaloside II significantly

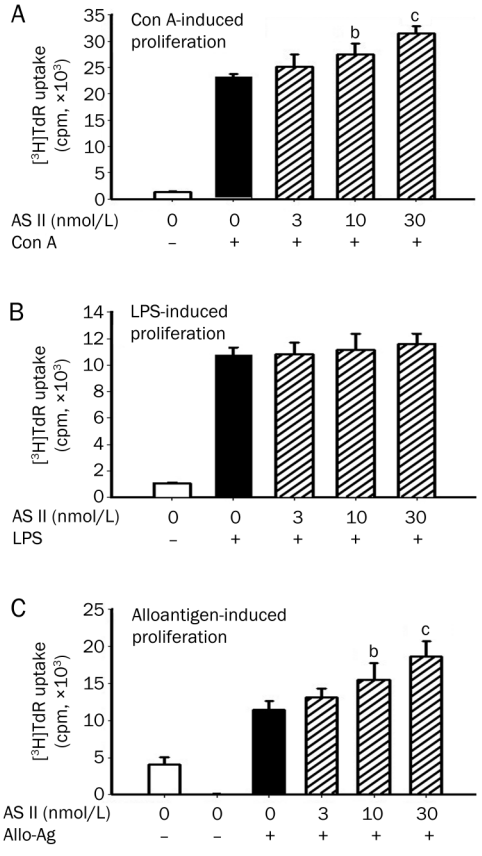


Figure 2. Astragaloside II enhanced the splenocyte proliferation induced by ConA, LPS, and alloantigen treatment. BALB/c mouse splenocytes (4×10^5 cells/well) were stimulated with ConA (1 μg/mL) (A), LPS (5 μg/mL) (B), or irradiated C57BL/6 splenocytes (1:1) (C) for 48 h or 96 h, in the absence or presence of astragaloside II. Cells were plated in triplicate in a 96-well plate. The results are presented as the mean±SEM. n=3. $^bP<0.05$, $^cP<0.01$ *vs* the control group.

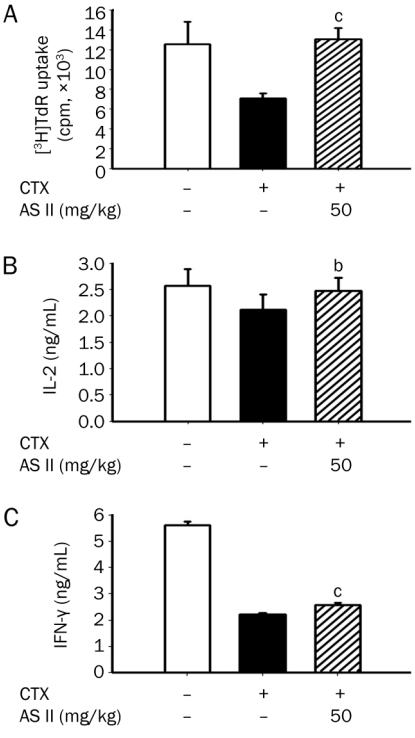


Figure 3. Administration of astragaloside II promoted recovery of splenic T cell function in immunosuppressed mice. BALB/c mice received and intraperitoneal injection (ip) of CTX (80 mg/kg) on d 1 and d 4; astragaloside II (50 mg/kg) was given orally for 8 d (5 mice per group). Splenocytes (4×10^5 cells/well) from each group were incubated with ConA (1 μg/mL) in triplicate for 24 h. (A) A proliferation assay. An ELISA was used to detect IL-2 (B) and IFN-γ (C) in supernatants. Results presented are the mean±SEM. n=3. $^bP<0.05$, $^cP<0.01$ *vs* CTX-treated mice orally administered saline vehicle.

upregulated mRNA expression of both IFN-γ and T-bet after anti-CD3 stimulation.

Astragaloside II markedly enhanced the expression of activation markers on the cell surface

CD25 is as IL-2 receptor α that expressed on $CD4^+$ T cells during an early period of the immune response, and this type of CD25 expression indicates T cell activation[16]. CD69 is generally thought to be the earliest cell surface activation marker on T cells, which may act as a co-stimulatory molecule for T cell activation and proliferation[17, 18]. To examine activated cell surface marker expression, splenocytes were stimulated with anti-CD3 (5 μg/mL) for 36 h. The cells were stained for CD4, CD25 and CD69 and then analyzed by flow cytometry. The results indicated that anti-CD3 stimulation up-regulated the expression of CD25 and CD69 on primary $CD4^+$ T cells, an effect that was largely enhanced in the presence of astragaloside II (30 nmol/L) (Figure 5).

Astragaloside II triggered T cell activation via regulating the activity of CD45 PTPase

As the most important transmembrane protein tyrosine phosphatase in T lymphocytes, CD45 plays a pivotal role in regulating T cell activation. To reveal the correlation between astragaloside-triggered T cell activation and CD45 phosphatase activity, a specific CD45 PTPase inhibitor, we used N-(9,10-dioxo-9,10-dihydro-phenanthren-2-yl)-2,2-dimethyl-propionamid. Not surprisingly, the immuno-enhancement activity of astragaloside II, including the proliferative response (Figure 6A) and increased IFN-γ production (Figure 6C) upon anti-CD3 stimulation, was blocked in presence of the specific CD45 PTPase inhibitor. These results confirmed that astragaloside II triggered T cell activation through the regulation of CD45 PTPase activity. There was no significant change in IL-2 production (Figure 6B) in astragaloside II-treated splenocytes. Because IL-2 is necessary for T cell survival, proliferation and differentiation, we deduced that may due to the specific CD45 PTPase inhibitor reduced T cell proliferation in this *in vitro* culture system.

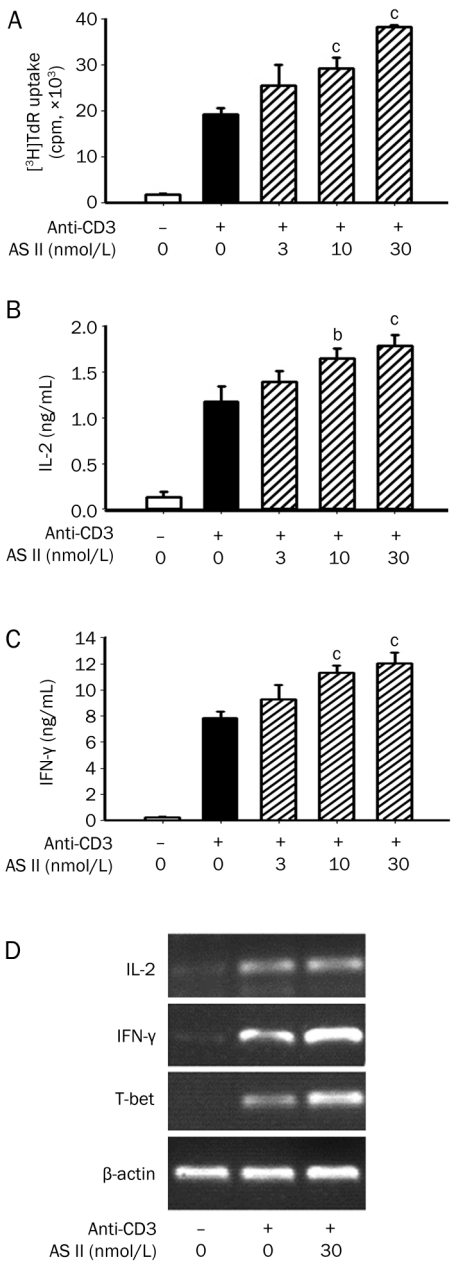


Figure 4. Astragaloside II significantly promoted anti-CD3-stimulated splenocyte activation. (A) For the proliferation assay, BALB/c splenocytes (4×10^5 cells/well) were cultured in a plate that was coated with anti-CD3 (5 μg/mL) in the absence or presence of astragaloside II for 48 h. For cytokine measurements, the culture supernatants were harvested at 36 h to measure the IL-2 (B) and IFN-γ (C) levels by ELISA. IL-2, IFN-γ, and T-bet expression in splenocytes at the mRNA level were examined by RT-PCR after 16 h of stimulation (D). The results are expressed as the mean±SEM of three independent experiments. $^bP<0.05$, $^cP<0.01$ *vs* control group.

Astragaloside II facilitated CD45-mediated LCK dephosphorylation

The major intracellular target of CD45 phosphatase activity is the p56LCK tyrosine kinase in T cells, which belongs to the Src-family of kinases. CD45 is capable of activating Src family tyrosine kinases by dephosphorylating the negative regulator p56LCK (Tyr505)[7]. To better understand the contribution of CD45 to astragaloside II-induced T cell activation, we examined CD45-mediated dephosphorylation of LCK (Tyr505). The endogenous levels of LCK at tyrosine 505 were detected by Western blot. The results presented in Figure 7 demonstrate that astragaloside II significantly promoted the dephosphorylation of LCK (Tyr505); however, this effect was blocked by a specific CD45 PTPase inhibitor. Therefore, concluded that astragaloside II exerted an immunomodulatory effect by initiating the activation cascade via CD45-mediated dephosphorylation of the Lck tyrosine kinase.

Discussion

Signal transduction events depend on the subtle balance between protein tyrosine kinases and protein tyrosine phosphatases, which control phosphorylation and dephosphorylation events, respectively. CD45 is a member of a family of transmembrane PTPs that are expressed exclusively on the surface of all nucleated hematopoietic cells and their precursors[7]. CD45 plays a critical role in TCR-mediated signaling by regulating the phosphorylation of protein tyrosine kinases and their substrates. It has been observed that primary $CD4^+$ T cells that have been pretreated with anti-CD45 mAb become unresponsive to anti-TCR cross-linking *in vitro*[7]. A mAb against CD45 was reported to inhibit IL-2 secretion, Ca^{2+} mobilization, and tyrosine phosphorylation in response to anti-CD3 stimulation[19]. Additionally, T cells from CD45 knockout mice fail to respond to antigen challenge[20]. Several studies have also demonstrated that a mAb specific for the CD45 RB isoform is a potent immunomodulator that prolongs allograft survival in several murine transplantation models and induces long-term engraftment and donor-specific tolerance in murine renal and islet allograft models[21]. Given that the cytoplasmic domain of CD45 contains PTPase activity and plays a role in TCR-mediated signaling, it is reasonable to hypothesize that specifically targeting the PTPase may be useful in the treatment of immune disorders. Advances have been made in recent decades in the development of potent small-molecule inhibitors for CD45 PTPase. A series of CD45 inhibitors have been discovered and used for the treatment of variety of inflammatory and immune disorders[22–24]. However, there have been fewer reports showing the immunomodulating activity of small-molecule CD45 PTPase activators for the treatment of primary immune deficiencies and for anticancer immunotherapy[25]. In the present study, astragaloside I, II, III, and IV, as bioactive components from Chinese herb AM, were identified as CD45 activators. Among these saponins, astragaloside II exhibited significant immunomodulatory effects, including a remarkable enhancement of T lymphocyte proliferation upon ConA and alloantigen stimulation. Additionally, it demonstrated a significant increase in IL-2 and IFN-γ production and increased the expression of CD25 and CD69 on $CD4^+$ T cells. In CTX-induced immunocompromised mice, oral administration of astragaloside II (50 mg/kg, *po*) promoted splenic T cell proliferation and produc-

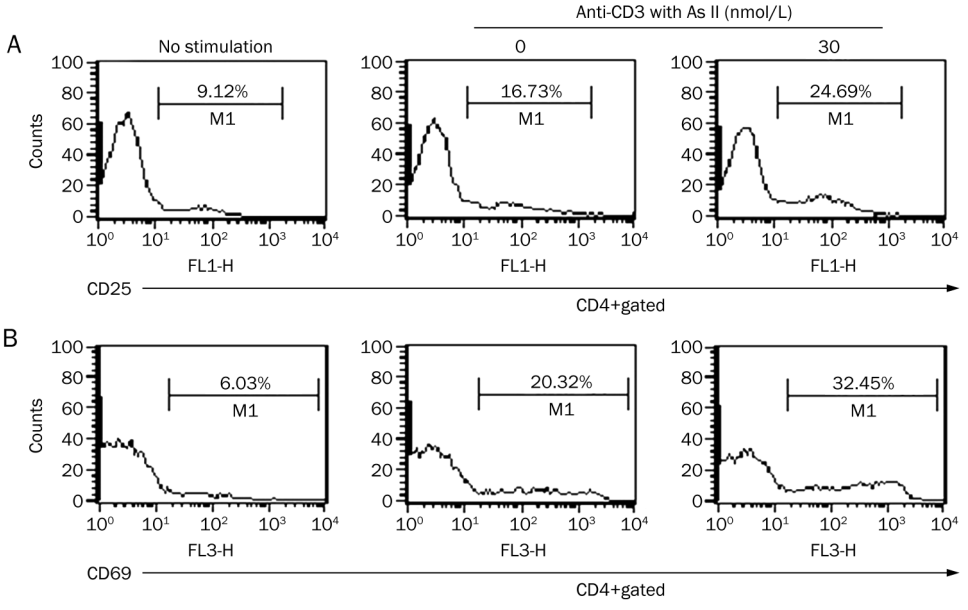


Figure 5. Astragaloside II enhanced the expression of activation markers on $CD4^+$ T cells. BALB/c splenocytes (4×10^6 cell/well) were cultured in 24-well flat-bottom plates that were coated with anti-CD3 (5 μg/mL) in the absence or presence of astragaloside II (30 nmol/L) for 36 h. Subsequently, CD25 (A) and CD69 (B) expression on $CD4^+$ T cells were analyzed by flow cytometry. The result presented here is representative of three individual experiments.

tion of immunostimulatory cytokines. To investigate the role of CD45 PTPase activation in astragaloside II-triggered T cell responses, a specific CD45 PTPase inhibitor was used. We demonstrated that astragaloside II significantly promoted T cell proliferation and IFN-γ production, and the effect was markedly blocked by a CD45 PTPase inhibitor, which suggests that astragaloside II triggered T cell activation by regulating the activity of CD45 PTPase.

The positive role of CD45 in T cell activation has largely been attributed to Src family kinase activity through the dephosphorylation of the negative regulator, carboxy-terminal tyrosine p56LCK (pTyr505), thus maintaining Lck in an open, active configuration[26]. Recent studies have demonstrated that CD45 is the only known phosphatase that dephosphorylates p56LCK at Tyr505 site[27]. Further studies have shown that astragaloside II reduces the expression of phosphorylated forms of Lck in anti-CD3/CD28-stimulated T cells. However, this effect is blocked by a specific CD45 PTPase inhibitor. These data support the assertion that astragaloside II significantly improves CD45-mediated LCK dephosphorylation .

Although CD45 is also expressed on B cells and associated with B cell receptor signaling, astragaloside II had no obvious effects on B cell function. Several reports have demonstrated that the extracellular domain regulates CD45 function, perhaps by binding to a ligand or by mediating CD45 dimerization, which induces the inhibition of its phosphatase activity[23]. Thus, CD45 can be regulated by the differential homo-dimerization of isoforms; the expression of different CD45 isoforms is cell type specific and depends on the state of the activation and differentiation of hematopoietic cells[7]. B lymphocytes express the high molecular weight isoform, 220 kDa (also termed B220), which includes all alternatively spliced CD45 exons (CD45RABC). A potential explanation for the lack of efficacy on B cells is that astragaloside II interacts with CD45 RO, which is expressed on T cells, but with the high molecular weight isoforms, which are expressed on B cells. Further studies should be carried out to verify this hypothesis.

The T helper lymphocyte is responsible for orchestrating the appropriate immune response to a wide variety of pathogens. Upon T cell activation, IL-2 and IFN-γ are two essential cytokines that contribute to adaptive immunity, and they protect against cancer progression and various intracellular infectious diseases[28]. IFN-γ can promote the differentiation of T cells and can activate macrophages, which results in increased phagocytosis, MHC class I and II expression, and the induction of IL-12, nitro oxide, and superoxide production, which contribute to the elimination of intracellular pathogens[14, 29]. Based on its induction of IFN-γ, T-bet was proposed to be the master switch for Th1 development[30]. Our experiments demonstrated that astragaloside II significantly upregulated mRNA expression of IFN-γ and T-bet in anti-CD3-stimulated T lymphocytes. However, astragaloside II could not enhance IL-2 transcription. Our data show that astragaloside II enhances IL-2 protein production, as found by ELISA. The accumulation of IL-2 in the supernatant should not be the consequence of diminished consumption of IL-2 by proliferating T cells because astragaloside II enhanced the proliferation consistently. Although there was a report that showed that IL-2 accumulation in the supernatant will suppress the transcription of IL-2 itself, this issue should not be the case in our system because we detected IL-2 mRNA expression as early as

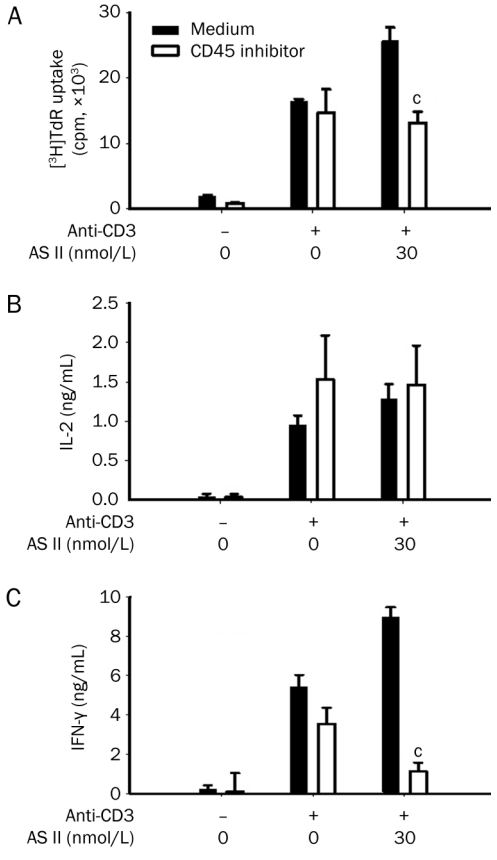


Figure 6. Astragaloside II triggered T cell activation by regulating the activity of CD45 PTPase. Splenocytes (4×10^5 cells/well) were stimulated with anti-CD3 (5 μg/mL) in the absence or presence of the CD45 PTPase inhibitor (1.5 μmol/L). The proliferative response (A) and cytokine level of IL-2 (B) and IFN-γ (C) were detected after 48 h or 36 h. The results are expressed as the mean±SEM of three independent experiments. $^cP<0.01$ *vs* medium control.

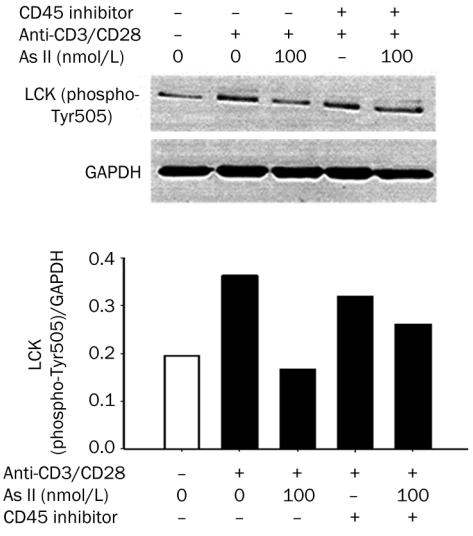


Figure 7. Astragaloside II facilitated CD45-mediated LCK dephosphorylation. Purified primary T cells were pretreated with astragaloside II (100 nmol/L) in the absence or presence of CD45 PTPase inhibitor (1.5 μmol/L) for 2 h, followed by anti-CD3 stimulation (5 μg/mL) and anti-CD28 (2 μg/mL) for 30 min. The cells were lysed and assayed for LCK (phospho-Tyr505) phosphorylation and GAPDH by western blotting assay. The result presented here is representative of three individual experiments.

16 h after stimulation[31]. Thus, we propose that astragaloside II might affect the transcription of IL-2, but not the mRNA expression, in T cells upon the TCR stimulation, which needs to be verified by further investigations.

In conclusion, the results from this study establish that astragaloside, and bioactive components from TCM, exerted immunomodulatory functions by regulating the activity of CD45 PTPase. We believe that these data warrant further evaluation of astragaloside as a tonic herb in the treatment of immune diseases.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (81072652 and 81273524), and National Science & Technology Major Project "New Drug Creation and Manufacturing Program" China (2012ZX09102-101-006).

Author contribution

Jian-ping ZUO, Jia LI and Jian-min YUE designed the research; Chun-ping WAN performed biological research, analyzed data and wrote the paper; Li-xin GAO performed *in vitro* studies of enzyme activity; Li-fei HOU, Xiao-qian YANG, Pei-lan HE and Yi-fu YANG performed biological research; Wei TANG and Jian-ping ZUO analyzed data and revised the paper.

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