Immunomodulatory Effect of Astragali Radix Extract on Murine Th1/Th2 Cell Lineage Development

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Astragali Radix (AR), is a popular herbal medicine used to treat allergic diseases in Korea, Japan and China. Our study examined the effect of an AR ethanol extract on both in vitro and in vivo murine CD4 T cells’ differentiation into Th1 and Th2 subsets. CD4 T cells from Balb/c mice were activated with anti-CD3/anti-CD28 mAb in the presence of AR for 2 d. AR treated cells showed an elevated level of IL-4 but a reduced level of IFN-γ secretion. In addition, in vitro Th1/Th2 polarization experiments revealed that AR enhanced the levels of IL-4 in Th2 cells but reduced the levels of IFN-γ in Th1 cells. To elucidate the effects of AR in Th1/Th2 lineage development during the in vivo condition, AR was administrated orally to BALB/c mice. The results demonstrated that AR administration significantly increased IL-4 production in both the serum and supernatant of splenocyte culture, while IFN-γ secretion was diminished upon in vivo activation with anti-CD3 antibody. Our data clearly indicates that AR selectively alters Th1/Th2 cytokine secretion patterns and provides the pharmacological basis for AR’s clinical applications.

Key words Astragali Radix; CD4 T cell; IL-4; IFN-γ

MATERIALS AND METHODS

Mice Female Balb/c mice at 8 weeks of age were purchased from Takonic Korea 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 Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, D.C.). All experiments were approved by our institutional ethical committee for animal welfare.

Preparation of Astragali Radix Extract The dry root of 6-year-old Astragali membranaceus Bunge was purchased from the Korean Association of Crude Medicinal Herbs. The root was powdered to 785.35 g and extracted with 90% ethanol using sonication for 10 min. The total supernatant after extraction was concentrated at 60°C and evaporated using a freeze dryer. A total amount of 22.201 g (retrieval rate of 2.22%) of extract powder was obtained. The sample was dissolved in PBS and sterilized by passing through a 0.22 μm syringe filter. The endotoxin level (in EU/mg) of the sample was tested through use of the Limulus ameabocyte lysate assay (Charles River Endosafe, U.S.A.). The level was below 0.3 EU/mg.

The HPLC Analysis of Standard Materials to AR Extract Two-hundred forty milligrams of dried ethanol extract of AR was dissolved in 4 ml of methanol (HPLC reagent, J. T. Baker Co. Ltd., U.S.A.) and ultra-pure distilled water (with a resistivity greater than 18 MΩ) and filtered through 0.45 μm syringe filter (PVDF, Milford, U.S.A.).

The Standard material used for the quantitative analysis of AR was formononetin (Fluka, Germany), which is currently recommended for quality control by the Ministry of Health and Welfare, Korea. Ten milligrams of the standard was dissolved to give serial concentrations (0.2, 0.1, and 0.02 mg/ml, respectively) and the standard HPLC chromatogram was obtained. The relationship between the con-
centration and the peak-area was measured by the minimum square method \( (R^2 \text{ value}). \)

The HPLC apparatus was a Waters Breeze System equipped with a 717+ Autosampler, a 2487 dual \( \lambda \) absorbance detector, and a 1252 binary HPLC Pump (Waters, U.S.A.). A Waters Breeze System (Ver. 3.20, Waters Co., Milford, U.S.A.) was also used for data acquisition and integration. A \( 4.6 \times 150 \text{mm} \) I.D. Symmetry C\(_{18}\) reversed-phase column with 5-\( \mu \text{m} \) particles (Waters, U.S.A.) was operated. The chromatographic separation was carried out using two isocratic solvents with acetonitrile (HPLC grade, J. T. Baker Co. Ltd., U.S.A.)–water in the ratio of 30 : 70 (v/v). The chromatography was performed at room temperature with a flow-rate of 1.0 ml/min, and 10-\( \mu \text{l} \) volume was analyzed. The quantity of standard material was the amount (mg) of standard materials =the quantitative amount (mg) of standard materials\( \times AT/AS/n \) (\( n=3 \)) (AT: the peak-area of the test sample containing standard materials, AS: the peak-area of standard material).

**Cytokines and Antibodies** Recombinant IL-12 (rIL-12), rIL-4, anti-IL-12, anti-IL-4, anti-CD3e, and anti-CD28 mAbs were all purchased from BD Pharmingen (San Diego, CA, U.S.A.). rIL-2 was purchased from Sigma (U.S.A.). IL-4 ELISA Abs set and IFN-\( \gamma \) ELISA Abs set were purchased from BD Pharmingen (San Diego, CA, U.S.A.). Magnetic cell sorting CD4 (L3T4) microbeads were purchased from Miltenyi Biotec (Germany).

**Splenocytes Preparation and CD4 T Cell Isolation** Splenocytes were prepared by disrupting the spleen between glass slides in complete medium (RPMI 1640 with 10% Fetal bovine serum, 1% penicillin–streptomycin). After a 10 min centrifugation at 1000 rpm to separate cells from debris, the cells were washed in RPMI medium, followed by lysis of erythrocytes using ammonium chloride reagent (BD Pharmingen, CA, U.S.A.). CD4 T cells were isolated from splenocytes by positive selection using MACS CD4 (L3T4) microbeads (Miltenyi Biotec, Germany) as directed by the manufacturer. In brief, CD4 T cells were separated by passing the cell suspension over a magnetic-activated cell sorter MS column held in MACS magnetic separator (Miltenyi Biotec, Germany). The CD4 T cells adhering to the column were then used for further assays.

**Viability and Proliferation Assay** For the dose response of AR, splenocytes (1 \( \times 10^5 \) cells/ml) were plated in 96-well plates in the presence of various AR concentrations in 0.2 ml of RPMI-1640 containing 10% FBS for 24 h. For the proliferation assay, purified CD4 T cells (1 \( \times 10^5 \) cells/ml) were grown in 96-well plates coated with anti-CD3 Ab. Cells were added with anti-CD28 (2 \( \mu \text{g/ml} \)) and cultured with AR in 0.2 ml of RPMI-1640 containing 10% FBS for 48 h. To determine the viability or proliferation capacity of CD4 T cells, the CellTiter 96\(^{TM}\) non-radioactive cell proliferation assay (Promega, U.S.A.) was used as described by the manufacturer. The assay is based upon the cellular conversion of the tetrazolium salt MTS \( [3-(4,5\text{-dimethylthiazol-2-yl})-5\text{-} (3\text{-carboxymethoxyphenyl})-2H\text{-tetrazolium}] \) into a formazan product in the tissue culture medium that can be measured at 490 nm.

**In Vitro Th1 and Th2 Cell Polarization** CD4 T cells (1\( \times 10^5 \) cells/ml) were resuspended in complete medium (RPMI 1640 with 10% FBS, 1% penicillin–streptomycin) and activated with plate-bound 10 \( \mu \text{g/ml} \) anti-CD3 and 2 \( \mu \text{g/ml} \) anti-CD28. For Th1 cell differentiation, naïve CD4 T cells were incubated with 5 ng/ml rIL-12, 10 \( \mu \text{g/ml} \) anti-IL-4 and 10 ng/ml rIL-2. For Th2 cell differentiation, 10 ng/ml rIL-4 and 10 \( \mu \text{g/ml} \) anti-IL-12 were added to the culture medium in the presence of 10 ng/ml of rIL-2. AR was added at the start of culture. Cells were cultured for 3 d and supernatants were collected and stored at \(-20^{\circ}\text{C} \) for cytokine analysis.

**Cytokine Assays** Flat-bottom 96-well plates were coated overnight at 4\(^{\circ}\text{C}\) with coating IL-4 mAb or IFN-\( \gamma \) mAbs. The primary mAbs were discarded and the plates were blocked with Assay Diluent (Pharmingen, San Diego, CA, U.S.A.) for 1 h at room temperature. The plates were washed 3 times with wash buffer (0.05% Tween 20 in PBS) and blotted on a paper towel. Diluted samples or IL-4 and IFN-\( \gamma \) standards were added in triplicate. The plates were incubated for 2 h at room temperature. The supernatant was discarded and the wells were washed 5 times with wash buffer. IL-4 or IFN-\( \gamma \) detecting mAb plus Avidin-HRP was added and incubated for 1 h at room temperature. After washing, tetramethylbenzidine substrate solution (Pharmingen, San Diego, CA, U.S.A.) was added. The color was allowed to develop for 30 min in the dark before the reaction was quenched with a stop solution (0.2 M \( \text{H}_2\text{SO}_4 \)). The plates were read at 450—570 nm and the sample concentrations were determined with the help of a standard curve.

**In Vivo T Cell Activation** AR extract was suspended in PBS and given orally to mice at a dose of 1.25 g per day per kg. The herbal medicine was administered for 7 consecutive days. Intact and control mice were treated with the same amount of PBS. On day 8, mice were injected intravenously with a single dose of anti-CD3 (4 \( \mu \text{g} \) in 0.1 ml of PBS). Mice were sacrificed 90 min after this injection. Blood was collected immediately by cardiac puncture and sera were stored at \(-20^{\circ}\text{C} \). Spleens were then removed and prepared for cell culture. Splenocytes were adjusted to 5 \( \times 10^6 \) cells/ml and cultured for 4 h in 24 well plates. After culture, supernatants were collected and stored at \(-20^{\circ}\text{C} \) for cytokine analysis.

**Statistical Analysis** Statistical evaluation of the results was performed by an independent \( t \)-test. The results were considered significant at a value of \( p<0.05 \).

**RESULTS**

**Standard Material Analysis** The standard curve was calibrated by using the linear regression derived from the peak area. The regression equation (correlation coefficient, \( R^2 \)) of formononetin was \( y = 4.80e + 007x + 2.31e + 005 \) (0.9979), which exhibited good linearity. The content of formononetin in 1 g of AR ethanol extract was \( 0.27 \pm 0.033 \) mg, which corresponds to 0.03% of the whole extract (Fig. 1).

**Dose Response of AR Extract and Proliferation of CD4 T Cells Stimulated with Anti-CD3/CD28** Spleen lymphocytes die in 2 d in vitro unless they are provided with cytokines or antigenic stimulus. Figure 2A illustrates the
Dose dependent effect of AR on spleen lymphocyte viability at 24 h using the MTS assay. The peak concentration was 50 mg/ml, which was twice more viable than the controlled cell. However, CD4 T cell proliferation in response to AR treatment, while stimulated with anti-CD3/CD28, was not significantly altered (Fig. 2B), although at 50 μg/ml, a slight increase was observed.

Effect of AR Extract on Cytokine Secretion in Activated CD4 T Cells

Cytokine production is another feature of activated T helper cells. CD4 T cells were stimulated with anti-CD3/CD28 in the presence of AR but without rIL-2 or any other exogenous cytokines. After 2 d, supernatants were collected and levels of IL-4 and IFN-γ were measured via ELISA. As shown in Fig. 3, AR significantly decreased the levels of IFN-γ in a dose-dependent manner but it increased the IL-4 secretion. Thus, AR appears to favor Th2 cell differentiation in vitro when no exogenous supplement is available.

Effect of AR Extract on Cytokine Secretion in Th1/Th2 Polarized Cells in Vitro

In addition to testing the effects of AR on CD4 T cells activated only with anti-CD3/CD28, we further examined its effect on in vitro Th1/Th2 polarization. CD4 T cells were cultured for 3 d in Th1 or Th2 polarizing conditions with rIL-2 and other necessary exogenous cytokines. AR (50, 100 μg/ml) was added at the start of the polarization process. Our ELISA data (Fig. 4) showed that AR decreased the amount of IFN-γ in a dose-dependent manner but it increased the IL-4 secretion. Thus, AR appears to favor Th2 cell differentiation in vitro when no exogenous supplement is available.

Effect of AR Extract on Cytokine Secretion in Anti-CD3 Antibody Injected Mouse

When anti-CD3 antibody is administered in vivo, T cell dependent cytokine production is rapidly induced without a predominance of either Th1 or Th2 type cells. Another set of experiments investigated the effect of orally administered AR on Th1/Th2 cytokine profiles. AR was orally given to mice for 7 d, and on day...
The present study demonstrated the effects of Astragali Radix extract on mouse CD4 T cells’ Th1/2 cytokine profiles. Our experiments showed that AR treatment elicited a decreased IFN-γ production but enhanced IL-4 secretion, favoring the Th2 development pathway with both in vitro and in vivo conditions. During the Th1/Th2 differentiation process, AR consistently inhibited IFN-γ secretion in the Th1 subset, while it augmented IL-4 levels in the Th2 subset.

Investigations of the Th1/Th2 paradigm enable a better understanding of different immune responses and the pathogenesis of certain immunological disorders. The best example of a Th1 pattern in cytokine production is a microbial infection in which the Th1 signature cytokine IFN-γ activates inflammatory leukocytes to destroy the microbes. Pathogenic Th1 responses are associated with organ-specific disorders, autoimmune disease and some forms of chronic inflammation. In contrast, the Th2 cytokine IL-4 plays a major role in promoting IgE production and is thus strongly associated with allergic reactions and airway hypersensitivity. Ultimately the balance of Th1/Th2 cytokine production is clinically useful in treating immunologically dysregulated states.

Traditionally AR is one of the most frequently used tonic herbs in Korea and other Asian countries. Most Qi tonifying herbs are considered to promote host defense mechanisms. Their clinical applications are to boost the body’s general vitality or to treat a debilitating condition. Indeed, a number of studies have shown AR’s multiple actions on immune system responses. It exhibited a protective effect on mice infected with Japanese encephalitis virus by increasing survival rates. The polysaccharide fraction of AR enhanced system responses. It exhibited a protective effect on mice infected with Japanese encephalitis virus by increasing survival rates. The polysaccharide fraction of AR enhanced antibody production in aged mice. It was also demonstrated that AR specifically increased the production of IL-2 and IFN-γ as well as the overall cytotoxic activity of lymphocytes from mice treated with a carcinogen called BBN.

Yoshida et al. reported that an aqueous extract of AR alone was shown to stimulate only B cells instead of T cells in mice, resulting in greater B cell proliferation and Ig production. This suggests that the mitogen-like capacity of AR extract was confined to B cells. In the present study, we used ethanol extraction, which we believe is presumed to extract both polar and nonpolar substances from AR, compared with water extraction, and found that AR in up to 100 μg/ml did not influence the proliferation of anti-CD3/CD28 stimulated CD4 T cells, confirming that AR does not bear any additional mitogenic stimuli toward CD4 T cells.

Despite the lack of its proliferative capacity, AR significantly affected the Th1/Th2 cytokine secretion patterns when the anti-CD3 plus anti-CD28 stimuli were given to CD4 T cells. An in vitro treatment of AR for 48 h increased IL-4 production but decreased IFN-γ, thus favoring the Th2 development pathway. Furthermore, under Th1/Th2 polarizing conditions AR decreased IFN-γ secretion in Th1 cells while increased IL-4 levels in Th2 cells. Supporting these data, IFN-γ and IL-4 levels of serum and splenocyte culture from anti-CD3 injected mice, which were pretreated with AR for 7 d, exhibited the same secretion patterns. From our results, AR clearly directed cytokine secretion patterns toward Th2 differentiation under neutral conditions. However, it should
be noted that the mouse strain used in this study, BALB/c, tends to develop overwhelming Th2 responses, so we cannot guarantee the observations made here will occur in the general genetic background. Future studies should be performed to support the implications of this report, by using other mouse strains such as C57BL/6 that have a tendency to develop strong Th1 responses.

Currently, several transcription factors have been identified that control the differentiation of Th1/Th2 cells. One of these, T-bet, is involved with the Th1 transition. Another, GATA-3, is upregulated in Th2 cells and is selectively induced early during Th2 development. GATA-3 is induced by IL-4 through Stat6, and inhibited by IFN-γ and IL-12 through Stat1 and Stat4 respectively. GATA-3 overexpression increases Th2 cytokine expression and inhibits IL-12R expression with decreased Th1 development even with Th1-inducing conditions. GATA-3 is also reported to induce Th2 development even in Stat6-deficient mice. Overexpressed GATA-3 likely binds Th2 cytokine clusters like the conserved noncoding region (CNS-1) and intronic enhancer (IE), increasing promoter accessibility to activated transcription factors. In addition, proximal IL-4 promoter analysis demonstrated that both c-maf and nuclear factor of activated T cells (NFAT) family members transactivate the IL-4 promoter, and that this process is regulated by T cell receptor-mediated signals. An investigation of these transcription factors in the presence of AR could elucidate the detailed mechanisms that AR performs on the Th1/Th2 lineage commitment process.

In conclusion, we demonstrated that AR alters selectively Th1/Th2 cytokine secretion patterns and provided the pharmacological basis for AR’s clinical applications.

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REFERENCES