# The effect of Astragali Radix Ethanol extract on Murine CD4 T cells' Cytokine Profiles in vitro

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Astragali Radix(AR), one of the strong tonic herbs, is known to improve immunological responses in mice and human. In this study, AR's Qi-reinforcing effect was examined in the context of $CD4^+$ T cells' TCR/CD3 induced activation responses. In order to evaluate the direct effect of AR on helper T cells, $CD4^+$ T cells are isolated using magnetic bead and their proliferation and CD69 expression in AR treated medium were assessed with anti-CD3/anti-CD28 activation for 48h. CD4 T cells' proliferation was slightly increased but there was little effect on CD69 expression. RT PCR and ELISA equally demonstrated that IL-2 and IL-4 production was increased but IFN-γ was down-regulated. This shows AR ethanol extract favors Th2 cytokine profile under neutral conditions.

Key words : Astragali Radix(AR), CD4 T cell, IL-2, IL-4, IFN-γ

## Introdution

The destiny of T cells is determined by contact with their cognate antigen bound to self major histocompatibility complex (MHC) on antigen presenting cells (APC)[1]. Antigenic stimulation causes T cell activation initiated by the T cell receptor(TCR)/CD3 complex and costimulatory molecules[2]. This involves proliferation of the corresponding T cells and their cytokine production, leading to the recruitment of other effector cells, and eliminating the harmful substance[3]. In the course of activation, cytokines produced by CD4 T helper (Th) cells divide their immune responses[4]: Interferone (IFN -γ) is the major cytokine induced in Th1 cells, mediating delayed type hypersensitivity and targeting intracellular pathogens, while Interleukin-4 (IL-4) and IL-5 are secreted by Th2 cells, providing help to B cells. In terms of pathology, Th 1 predominance is observed in organ-specific autoimmune disease, whereas Th 2 response is implicated in allergies.

The dry roots of Astragali membranaceus Bunge, known as Hwangi in Korea, have been widely used for Qi-tonifying effects in clinical practice[5]. Such concept can be interpreted as increased activity of immune cells and their cytokine production. There are numerous studies on Astragali root's (AR) immunological activities [6],[7],[8],[9],[10]; Immunoglobulin(Ig) production, cytotoxic T cells' activity, and IL-1 production by monocytes were enhanced in vitro; in vivo assay, AR increased the percentage of lymph node CD4 T cells and type I cell-mediated response.

However, most studies relating to herbal products, compared the proliferative capacity of herbal products with that of general mitogens such as Conconavalin A or Lipopolysaccharide[7],[9],[10]. Since they are utilized to experimentally stimulate T cells polyclonally, such comparison would be irrelevant if one wishes to emphasize the effect of any herbs on boosting effector functions by immune cells. Here our study tests whether AR ethanol extract increases anti-CD3/CD28 antibody induced CD4 T cell's activity and if so, how it directs the Th1/Th2 cytokine profiles.

## Materials and Methods

### 1. Mice

Male BALB/cAnTacSamfBR mice at 8 weeks of age were purchased from Samtaco.

### 2. Preparation of AR ethanol extract

The dry root of 6- year-old Astragali membranaceus Bunge was purchased from the Korean Association of Crude Medicinal Herbs. Our sample is a local variety cultivated in Jungsun, Korea and classified into high-grade on the Korean market. The root was powdered to 785.35 g and extracted with

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70, 80, 90, and then 100% ethanol using sonication for 10 min. The total supernatant collected on extraction was concentrated at 60℃ and evaporated to dryness in vacuo. 22.201g (2.22%) of extract powder was obtained. The sample was dissolved in PBS and sterilized by passing through 0.22-㎛syringe filter. The endotoxin level (in EU/mg) of the sample was tested through use of the Limlus ameabocyte lysate assay (Charles River Endosafe, U.S.A.). Its level was below 0.3 EU/mg.

3. Cell Purification and culture

Splenocytes were prepared from BALB/c mice and treated with red blood cell lysing buffer (BD Pharmingen, U.S.) CD4 T cells were purified by magnetic cell sorting using the MACS system (Miltenyi, Germany), as described 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. The CD4 T cells adhering to the column were cultured in RPMI medium containing 10% FBS. Cells were stimulated with immobilized anti-CD3/CD28 Ab (Pharmingen) and incubated for 48 hr at 37℃ in 5% $CO_2$ with AR at various concentrations.

4. Proliferation assay

To measure the proliferation capacity of CD4 T cells in the presence of AR, cells were placed in a 96 well for 48 hr and then treated with the tetrazolium salt MTS {3-(4,5- dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl)-2H-tetrazolium} (Promega, U.S.)

5. Analysis of cell surface expression

For cell surface staining, cells were cultured in 24-well in the presence of AR for 48 hr. Staining for cell surface markers was performed as described by the manufacturer's instruction. In brief, cells were harvested and centrifuged 1000rpm for 5 min. After removing supernatant, the pellet was resuspended in cold wash buffer (PBS/ 0.1%NaN3/ 1%FBS), centrifuged and resuspended in 100㎕ of wash buffer and stained with PE-conjugated CD69 (Pharmingen) and incubated at 4℃ in the dark for 40 min. After washing twice, the cells were analyzed with a Becton Dickinson FACScan.

6. RT PCR

Total RNA was extracted using Trizol reagent (Gibro-BRL, Life Technologies) as described by the manufacturer. Total RNA was quantified using spectrophotometry at 260 nm. Purified RNA was electrophoresed on a 1.5% agarose gel to verify that the RNA was not degraded. RNA samples were reverse-transcribed with Superscript Ⅱreverse transcriptase (Gibro-BRL, Life Technologies), and cytokine-specific primers[19),(20)] were used to amplify selected cytokines. For each cDNA product, the optimum numberof cycles for PCR amplification was determined experimentally. Relative concentrations of IL-2, IL-2Rα chain, IL-4 and IFN-γ mRNA were determined. Primers for the "housekeeping gene", GAPDH(glyceraldehydes-3-phosphate dehyrogenase), were used in each experiment to verify that equal amounts of RNA were added in each PCR. All cytokine values were normalized individually to the corresponding GAPDH values. Amplified PCR products were resolved on a 3% agarose gel and stained with ethidium bromide. Products were quantified using the Bio-Rad Quantity One software.

7. Measurement of cytokine production by CD4 T cells.

The levels of IL-4 and IFN-γ in the culture supernatant were measured with BD OptEIA Mouse IL-2, IL-4 and IFN-γ set ( Pharmingen). The assay sensitivities were 200- 3.1 pg/ml (IL-2), 31.25-2000 pg/ml (IFN-γ) and 7.81-500 pg/ml (IL-4). The plates were read at 450-570 nm and the sample concentrations were determined with the help of the standard curve.

8. Statistical analysis

All data are expressed as the mean±S.D. Statistical differences between the groups were determined by the application of the independent T test.

## Results

1. AR effect on the proliferation and CD69 expression of CD4 T cells.

CD4 T cells from Balb/c mouse were activated with anti-CD3/CD28 at various concentrations of AR for 48 h using the MTS assay. The peak concentration was 50 ㎍/㎖ with a 10% increase and the other concentrations at 20 and 100 ㎍/㎖ showed marginal increases.

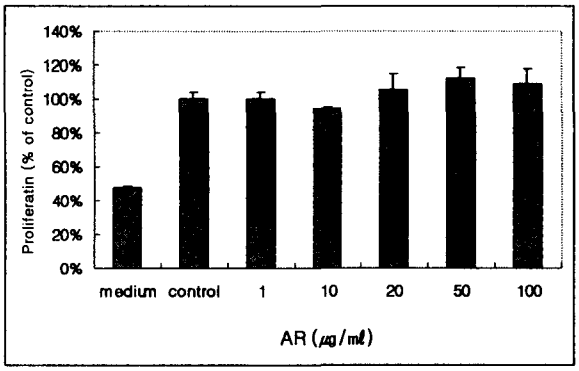


**Fig. 1. The effect of AR ethanol extract on activated CD4 T cells.** CD4 T cells from Balb/c mice were sorted and cultured in medium containing AR (1-100 ㎍/㎖) for 48 h. Cells were stimulated with anti-CD3/CD28 except those in medium alone. Proliferation was quantified by the ability to reduce the MTS. Results were expressed as the percentage of control.

As evidence for rapid T cell activation, CD69 expression by flow cytometry was evaluated. CD69 is considered a very early activation marker of T cell activation. At 20, 50 and 100 ㎍/㎖ of AR, there was no difference in CD69 expression. Taken together, AR showed little effects on the qualitative and quantitative response of CD4 T cells.

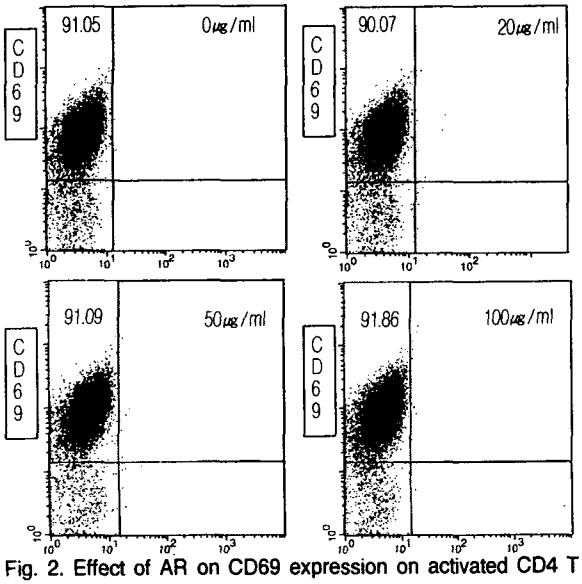


**Fig. 2. Effect of AR on CD69 expression on activated CD4 T cells.** Murine CD4 T cells were stimulated with anti-CD3/CD28 and cultured in medium containing AR ethanol extract. 48 hours later, the cells were harvested and processed for cell surface staining. Numbers on the upper left gives the percentage of positive cells and numbers on the upper right gives the concentration of AR.

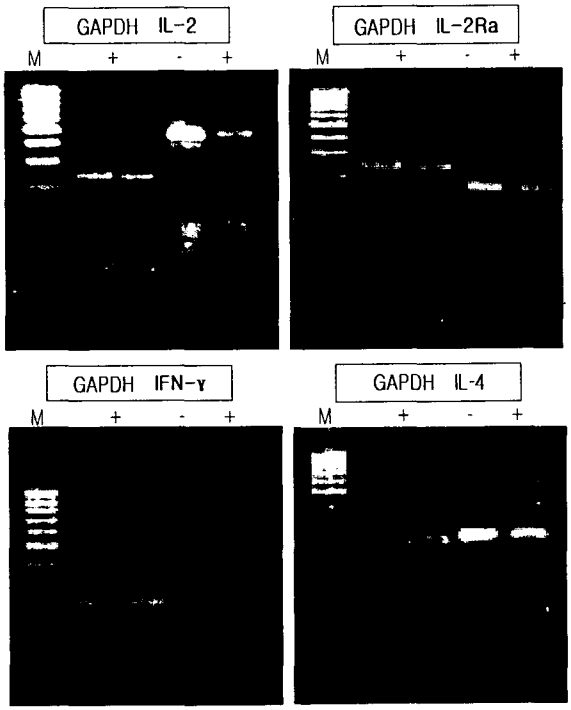


**Fig. 3. RT PCR analysis of the expression of mRNA for IL-2, IL-2R α, IFN-γ and IL-4 in AR( 50 ㎍/㎖) treated CD4 T cells.** Cells were stimulated with anti-CD3/CD28 ab. Total RNA was isolated after 48 hr of culture and processed for determination of each gene expression. The housekeeping gene GAPDH was used to assess the amount of the RNA. M:25-100 bp DNA Ladder (Bioneer, Korea). (+): AR treated cells. (-): control.

2. IL-2, IL-2R α chain, IL-4 and IFN γ expression by RT PCR.

Upon activation, CD4 T cells release IL-2 and express IL-2 receptor on their surface. In addition, they differentiate into Th1 and Th2 cells, depending on the cytokines which they produce. CD4 T cells were stimulated with anti-CD3/CD28 for 48 h in presence of AR (50 ㎍/㎖) containing medium. RT PCR analysis revealed that AR treated cells showed higher levels of IL-2 and IL-2Rα mRNA expression and that AR favors Th2 cell development by increasing IL-4 mRNA while decreasing IFN γ expression(Fig. 3.)

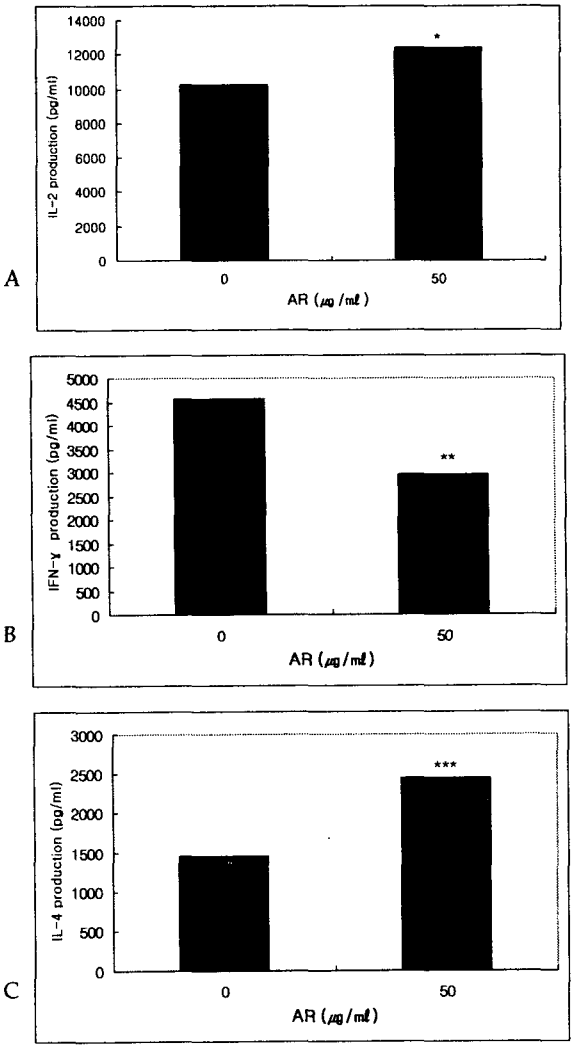


**Fig. 4. CD4 T cells from Balb/c mice were purified by magnetic cell sorting and stimulated with anti-CD3/CD28 for 48 h with or without AR ethanolic extract.** The supernatants were tested for cytokines by ELISA. There is a significant difference between groups at * $p < 0.005$, ** $p < 0.00001$, *** $p < 0.01$.

3. IL-2, IL-4 and IFN-γ production by ELISA

Cytokine production from activated CD4 T cells were measured by ELISA. In parallel with the RT PCR data, increased IL-2, IL-4 levels and decreased IFN-γ production

were observed. The IL-2 production in AR treated cells was significantly higher than that in the control cells( control 10179.69 ±472.28 pg/ml; AR 12355.54 ±481.84 pg/ml [p <0.005] ). The IL-4 production was also increased in AR treated cells ( control 1442.09±50.28 pg/ml; AR 2437.64±50.28 pg/ml [p <0.00001] ). However, the IFN-γ production was decreased in AR treated cells ( control 4553.30±283.14 pg/ml; AR 2954.47 ± 517.45 pg/ml [p <0.01 ] ).

## Discusssion

The goal of the present study was to demonstrate the sensitivity of AR to stimulated CD4 T cells by assessing cell proliferation and cytokine production during activation. AR's Qi tonifying effect is believed to strengthen the individual's weakened defense system in Oriental medicine[5]. Though the entity of Qi is still controversial, there is consensus that the Qi aspect, in many regards, is analogous to the individual's immunological status for one of the functions of Qi is to regulate the host's response to foreign, as well as, self-generated harmful substances. In this paper, the characterization of AR was attempted by evaluating cell activation capacity.

Stimulation of TCR/CD3 plus co-stimulatory signals leads to a series of intracellular signaling events, resulting in cytokine production, cell proliferation and the achievement of effector functions[2],[12]. In our previous study[13], AR increased the viability of unstimulated spleen lymphocytes and, from flow cytometric analysis, the percentage of CD4 T cells in AR treated lymphocytes was increased. With respect to activation induced proliferation, AR slightly increased the proliferation of anti-CD3/anti-CD28 stimulated CD4 T cells but showed little effect on CD69 expression. However, AR played a striking role in cytokine secretion patterns. In our RT PCR data, the expressions of IL-2 and IL-2 Rα chain mRNA were enhanced in AR treated cells. In particular, increased IL-2 production was also demonstrated in ELISA. IL-2 is one of the major cytokines produced by activated CD4 T cells. Its major function is to promote DNA synthesis and thus cell division [14] ; the interaction of IL-2 and IL-2 receptor triggers different pathways, culminating in cell proliferation[15]. Our data suggest that AR's increased IL-2 production is mediated by an IL-2 pathway level, not by the increased cell number.

AR responded differently to cytokine transcription and production. AR was in favor of Th2 cell under neutral conditions: a higher level of IL-4 expression while a lower level of IFN-γ. This provides indirect evidence that AR possibly affects Th2 cell commitment. Cytokines produced by CD4 helper T cells determine the type of immune response and effector mechanism[4],[16]. IFN -γ is one of the major cytokines secreted by Th1 cells, regulating phagocyte-mediated responses, whereas IL-4 is produced by Th2 cells, mediating humoral responses[17]. The paradigm of Th1/Th2 cytokines allows explanation for individual susceptibility to parasitic infection, virology, allergies, antitumor responses, and autoimmunity[10].

Taken together, the present study presents that AR did not show a powerful action on the proliferation and CD 69 induction on anti-CD3/anti-CD28 stimulated CD4 T cells. However, AR affects cytokine production by increasing IL-2 and IL-4 secretion and decreasing IFN-γ, possibly favoring Th2 cell development. Our results provide molecular basis for AR's therapeutic applications: by enhancing IL-4 production, AR can be administered in combination to vaccines for increased antibody production, or exert inhibitory effects on IFN-γ -mediated autoimmune disease.

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