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CD4+ T cells에서 백개자가 IFN-γ 와 IL-4 생성에 미치는 영향

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Effect of *Sinapis alba* L. on expression of interferon-gamma and interleukin-4 production in anti-CD3/anti-CD28-stimulated CD4(+) T cells

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ABSTRACT

Objective : *Sinapis alba* L. (SA) is a korean traditional herbal medicine that is usually used to prevent or treat inflammatory diseases, such as respiratory infection and rheumatoid arthritis. However, the effects of SA supplementation in vitro on serum antibody levels, splenocyte and peritoneal macrophage immune responses have not yet been determined. In this study, we examined the effect of SA on the production of Th1/Th2 cytokines.

Methods : Splenocytes were isolated from naive C57BL/6 mice. Cells were enriched for CD4+ cell populations by first staining the cells with anti-CD4 (BD PharMingen, Calif, USA). CD4+ T cells were selected on a (CS) column, and the flow-through was collected as CD4+ T cells. Isolated cells were activated by overnight incubation on 24-well plates coated with 1μ g/mL anti-CD3, 1μ g/mL anti-CD28 and with SA (100μ g/mL). Primary macrophages were collected from the peritoneal cavities of mice (8-week-old female C57BL/6). The peritoneal macrophages were washed and plated with RPMI-1640 overnight for the experiments. After 48-hours cultures, samples were centrifuged at 2000 rpm for 10 minutes, and the supernatants were stored at -80℃. Mouse IL-4, IFN-γ and TNF-α were quantified using ELISA kits (BioSource International, Camarillo, Calif, USA) according to the manufacturer' s protocols.

Results : SA at 100ug/ml decreased the generation of Th1 cytokine (IFN-γ) by 0.5-fold. However, SA has no effect on Th2 (IL-4) production.

Conclusions : These results suggest that SA may play an important role in the control of T-cell-mediated autoimmunity by down-regulation of Th1 cytokine (especially IFN-γ , TNF-α). These data may contribute to the design of new immunomodulating treatments for a group of autoimmune diseases.

Key words : *Sinapis alba* L. (SA), IFN-γ , TNF-α , Th1, Th2

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Introduction

T helper 1(Th1) cells were characterized by the production of interferon-gamma(IFN-γ) and were critical in the cell-mediated immunity, but Th2 cells were characterized by the production of interleukin-4(IL-4), and played an important role in humoral immunity. Based on cytokines production, fully differentiated Th lymphocytes are divided into at least two distinct subsets. Th1 lymphocytes were characterized by the production of IFN-γ concerning in cell-mediated immune response. Th2 lymphocytes, on the other hand, were characterized by the production of IL-4 concerning humoral immune response[1].

The mechanisms by which CD4+ Th cells are responsible for both humoral and cell-mediated immunity remained unclear until evidence has been provided that repeated stimulation of murine CD4+ Th cells with given antigens resulted in the outgrowth of cells with polarized patterns of cytokine secretion (Th1 or Th2)[2]. The Th1 phenotype is associated with inflammation, whereas the Th2 phenotype combats inflammation to some extent. Many studies, most of which relied on animal experiments, led to the belief that rheumatoid arthritis (RA) was a Th1-driven disease[3]. The excess of pro-inflammatory cytokines and relative deficiency in anti-inflammatory cytokines defines the Th1/Th2 imbalance, which was believed to drive several autoimmune diseases (known as Th1 diseases) such as RA, diabetes, and Crohn' disease[3]. The pivotal factors in this paradigm were the pro-inflammatory cytokine TNF-α and the Th1 subset of helper T-cells[4,5]. One of the main results of Th1/Th2 imbalance is production of TNF-α, which triggers a cascade of events leading to chronic inflammation and destruction of bone and cartilage.

Sinapis alba L., which belongs to Cruciferae, is a perennial crop that is commonly used as a condiment and a spice due to its pungent taste and peculiar flavor. The seeds of Sinapis alba L. (commonly called white or yellow mustard) have been used as a spice and as an herbal in alternative medicinal practices[6]. Mustard seed and its components have been demonstrated to possess antineoplastic activity[7].

S. alba is also known to exert a wide variety of biological activities, including antineoplastic, antimicrobial, and insecticidal functions[8,9].

However, no studies evaluating the activity of S. alba L. seeds on Th1/Th2 balance have been conducted to date. The purpose of this study is to determine whether SA may influence the direction of the Th1/Th2 balance as evaluated on the basis of splenic CD4+ T cell producing cytokine synthesis patterns in mice.

Materials and methods

1) Plant material and preparation of extracts

The sample of the seeds of SA was collected from Sangji Oriental Medical Hospital(Wonju, Korea) in April, 2008. The voucher specimens (SA) are deposited in our laboratory(Department of Herbology, College of Oriental Medicine, Sanji University Wonju 220-702, Republic of Korea). Plant material (200g) was extracted three times with distilled water. Then, the extract was filtered and evaporated on a rotatory evaporator(Rotary evaporator, BUCHI B-480, Switzerland) and finally dried by a freeze drier(Freeze dryer, EYELA FDU-540, Japan) to yield the extracts SA (17g).

2) Animals

Seven to eight-week-old male C57BL/6 mice were obtained at Daehan Biolink Co. LTD. (Eumsung, Republic of Korea). All animal procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee, Korea Research Institute of Bioscience and Biotechnology (Daejeon, Republic of Korea).

3) Isolation of CD4+ T cells

As per the modified protocol previously

described[10], splenocytes were isolated from naive C57BL/6 mice. Cells were enriched for CD4+ cell populations by first staining the cells with anti-CD4 (BD PharMingen, Calif, USA). CD4+ T cells were selected on a (CS) column, and the flow-through was collected as CD4+ T cells. Isolated cells were activated by overnight incubation on 24-well plates coated with 1 μg/mL anti-CD3 (datasheet: MCA500GA; description: Rat antimouse CD3; specificity: CD3; format: purified; product type: monoclonal antibody; clone: KT3; isotype: IgG2a; quantity: 0.25mg, AbD Serotec.), 1μg/mL anti-CD28 (datasheet: MCA1363; description: Hamster antimouse CD28; specificity: CD28; format: purified; product type: monoclonal antibody; clone: 37.51.1; isotype: IgG; quantity: 0.2 mg) and with SA (100μg/mL) added to RPMI medium supplemented with 1 unit/mL penicillin, 1μg/mL streptomycin, 20mM L-glutamine, 50mM-mercaptoethanol, and 5% FBS for 48 hours after stimulation.

4) Macrophage culture

Primary macrophages were collected from the peritoneal cavities of mice (8-week-old female C57BL/6) after an intraperitoneal (i.p.) injection of 3 mL of 1% thioglycolate broth (Sigma, St. Louis, MO) 3 days before harvesting. The peritoneal macrophages were washed with 1×PBS (Ca2+ and Mg2+ free) and plated with RPMI-1640 containing 10% FBS, 100 U/mL of penicillin and 100 μg/mL of streptomycin overnight for the experiments. Mouse peritoneal macrophages (1×10^6 cells/ml) were incubated with two concentrations of SA (100, 50 ㎍/㎖) and lipopolysaccharide (LPS) (1 ㎍/ml) was treated for 24 hours.

5) Cytokine measurements

After 48-hours cultures for IL-4, IFN-γ and 24-hours for TNF-α, samples were centrifuged at 2000 rpm for 10 minutes, and the supernatants were stored at −80℃. Mouse IL-4, IFN-γ and TNF-α were quantified using ELISA kits (BioSource International, Camarillo, Calif, USA) according to the manufacturer's protocols.

6) Quantitative real-time polymerase chain reaction

To study anti-inflammatory effects of SA on cytokine gene expression from isolated CD4+ T cells, quantitative real-time polymerase chain reaction (PCR) was performed after quantitative normalization for each gene by densitometry using β-actin or Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Applied Biosystems) gene expression. Briefly, total cellular RNA was extracted from the CD4+ T cells by the phenol-chloroform method (RNAsolB Tel-Test Inc., Friendswood, TX, US) according to the manufacturer's instructions. cDNA was synthesized from 3 ㎍ of total RNA using a ReverTraAce-a-cDNA synthesis kit (Toyobo Co., Ltd, Osaka, Japan) according to the manufacturer's instructions. Real-time quantitative PCR was performed using the Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, Foster, CA, US) with the following primer sequences: mouse transforming growth factor-β (TGF-β), 5'-TGGAGCAACATGTGGAACTC-3' and 5'-CTGCCGTACAACTCCAGTGA-3' β-actin, 5'-TGGAATCCTGTGGTCCATGAAAC-3' and 5'-GTCACAGTCAGCTGTATAGGG-3'.

Pro-inflammatory cytokine gene expression was analysed with SYBR Green PCR Mastermix (Applied Biosystems) and a final concentration of 200 nM of primers, using β-actin as the internal standard. The TaqMan probes used in the real-time polymerase chain reaction (PCR) for Foxp3 (pn.4331182, FAM dye-labeled, ABi, USA) and GAPDH (pn.4352339E, VIC dye-labeled, ABi, USA) were selected using Assays-on-Demand Gene Expression Products (Applied Biosystems, Foster City, CA). GAPDH was used as an endogenous control.

The following PCR parameters were used: 2 min at 50℃, 10 min at 94℃, then 40 cycles of 1 min at 94℃, and 1 min at 60℃. The amount of SYBR Green was measured at the

end of each cycle. The cycle number at which the emission intensity of the sample rose above baseline was referred to as the RQ (relative quantitative) and was proportional to the target concentration. Real-time PCR was performed in duplicate and analysed by an Applied Biosystems 7500 Fast Real-Time PCR system manual (threshold: 0.05, baseline: 6-5 cycles). To generate standard curves for pro-inflammatory cytokine and β-actin, serially diluted cDNA (1/1~1/16) was prepared and real-time PCR was performed as above. RQ evaluation by real-time PCR was determined and expressed for various samples.

7) Statistical Analysis.

For statistical analysis of data, P-values were analyzed using a one-way analysis of variance (ANOVA) or unpaired Student' t-test followed by Dunnett' multiple comparison test (SPSS version 14.0 statistic software). Results were considered statistically significant when P values were 〈0.05, 〈0.01, 〈0.001 (*p〈0.05, **p〈0.01, ***p〈0.001).

Results

1. Effect of IFN-γ cytokine production in isolated CD4+ T cells.

Firstly, we examined how SA makes an effect toward CD4+ T cells in vitro. As per the modified protocol previously described in materials and methods, CD4+ T cells were selected on a CS column, and the flow-through was collected as CD4+ T cells. Isolated cells were activated by overnight incubation on 24-well plates coated with 1μ g/mL anti-CD3, 1μ g/mL anti-CD28, and with SA (100μ g/mL) added to RPMI medium supplemented with 1unit/mL penicillin, 1μ g/mL streptomycin, 20mM L-glutamine, 50mM-mercaptoethanol, and 5% FBS for 48 hours after stimulation. After 48-hour culture, samples were centrifuged at 2000 rpm for 10 minutes. Mouse IFN-γ and IL-4 cytokines production were quantified using ELISA kits according to the manufacturer' s protocols. As you see Fig. 1, SA decreased IFN-γ cytokines, it has been suggested that SA may be has suppressing activities of CD4+ T cells, inducing rheumatoid arthritic inflammation(Fig. 1).

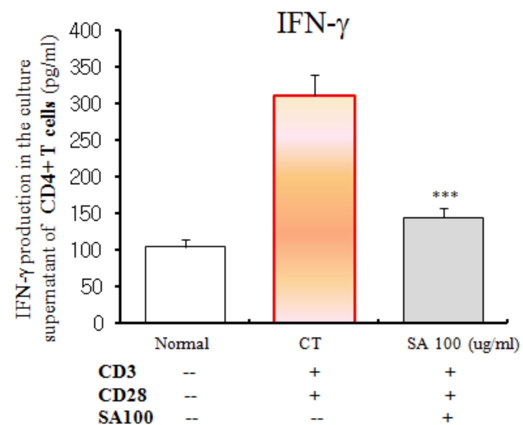

Fig.1. Effects of SA on IFN-γ cytokines in CD4+ T cells isolated from Naive C57BL/6 Mice (n=5, Normal: Normal C57BL/6 mice; CT: a-CD3/CD28+ CD4+ T cells; SA 100: a-CD3/CD28+ CD4+ T cells + SA (100 μ g/mL).

2. Effect of IL-4 cytokine production in isolated CD4+ T cells.

Mouse IL-4 cytokines production were quantified using ELISA kits according to the manufacturer' protocols. As you see Fig. 2, SA has no effect on Th2 (IL-4) production.

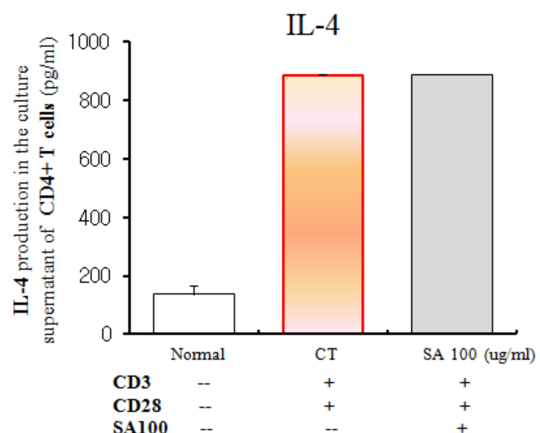

Fig.2. Effects of SA on IL-4 cytokines in CD4+ T cells isolated from Naive C57BL/6 Mice (Normal: Normal C57BL/6 mice; CT: a-CD3/CD28+ CD4+ T cells; SA 100: a-CD3/CD28+ CD4+ T cells + SA (100 μ g/ml).

3. Effect of TNF-α cytokine production in isolated peritoneal macrophages.

To investigate the anti-inflammatory effect of SA, we examined the effect of SA on pro-inflammatory cytokines production. Effects of SA on peritoneal macrophages, there were marked reduction TNF-α cytokine production in high concentration (SA 100 μg/ml) compared to control group (Fig. 3). However, there was no remarkable change in low concentration(SA 50 μg/ml).

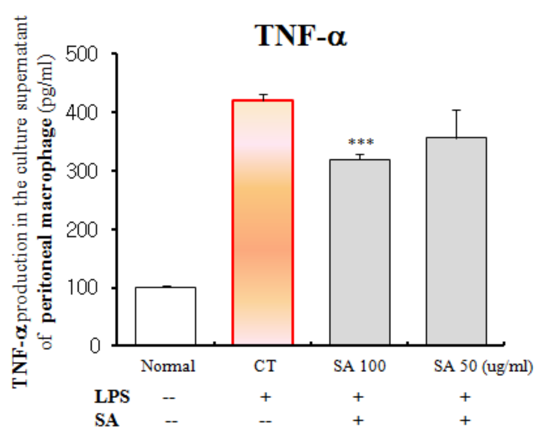

Fig.3. Effects of SA on TNF-α cytokines in peritoneal macrophages isolated from Naive C57BL/6 Mice. (Normal: Normal C57BL/6 mice; CT: LPS + peritoneal macrophages; SA 100: LPS + peritoneal macrophages + SA (100 μg/ml); SA 50: LPS + peritoneal macrophages + SA (50 μg/ml).

4. Detection of mRNA from CD4+ T cells by SYBR Green real-time polymerase chain reaction assay.

To study anti-inflammatory effects of SA on cytokine gene expression from isolated CD4+ T cells, quantitative real-time polymerase chain reaction (PCR) was performed after quantitative normalization for each gene by densitometry using β-actin or Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Applied Biosystems) gene expression. As shown in Fig. 4, the mRNA for Foxp3, TGF-β were significantly increased in the CD4+ T cells treated with SA (100 μg/mℓ).

PCR products for Foxp3, TGF-β amplified from CD4+ T cell RNA preparations, were increased in the SA treated groups compared with control groups (anti-CD3, 1 μg/mL anti-CD28 treated group)(Fig. 4).

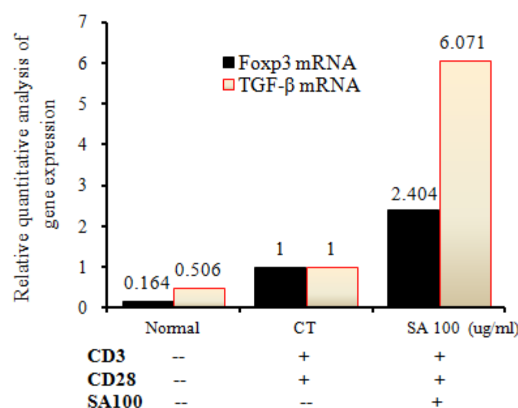

Fig.4. Effects of SA on Foxp3, TGF-β mRNA expression in CD4+ T cells.
As previously described in materials and methods, isolated CD4+ T cells were treated with SA (100 μg/mℓ). PCR products for Foxp3, TGF-β amplified and analyzed by real-time PCR. (Normal: Normal C57BL/6 mice; CT: a-CD3/CD28+ CD4+ T cells; SA 100: a-CD3/CD28+ CD4+ T cells + SA (100 μg/ml). The results are expressed as the RQ(relative quantitive).

Discussion

Th1 and Th2 cells are characterized by the patterns of cytokines they produce. Moreover, the Th1/Th2 concept suggests that modulation of the relative contribution of Th1- or Th2-type cytokines would make it possible to regulate the balance between protection and immunopathology as well as the development or the severity of some immunopathologic disorders.

Rheumatoid arthritis (RA) is an autoimmune disease that causes chronic inflammation in synovial tissue and joints, which leads to impaired joint function, severe pain and reduced life expectancy[11]. The pathogenesis of T cell-mediated diseases like RA has typically been explained in the context of the Th1-Th2 balance: the initiation/propagation by pro-inflammatory cytokines, and downregulation by

Th2 cytokines[12].

Although macrophages and neutrophils are the major sources of mediators of inflammation, CD4 T cells play a key role in the initiation and perpetuation of CIA by producing IFN-γ [13]. Once activated, CD4+ T cells differentiate into specialized effector cells and become the central regulators of specific immune responses. In RA a number of observations are consistent with the hypothesis that CD4+ T cells play a dominant role in the immunopathogenesis of the disease[14]. Activated CD4+ T cells can be found in the inflammatory infiltrates of the rheumatoid synovium[15]. CD4+ T cells play an important role in a variety of animal models of inflammatory arthritis[16].

In this study, SA down-regulated the production of the inflammatory cytokines IFN-γ but didn't increased the levels of Th2 cytokines IL-4(Fig. 1, 2). Our results demonstrate that the treatment of SA to splenic CD4+ T cells results in a decreased CII-specific Th1-mediated response.

Several inflammatory cytokines, particularly TNF-α, IL-1 and IL-6, are known to play key roles in the induction and perpetuation of inflammation in macrophages. These cytokines play pivotal roles in the induction of the innate immune response as well as in the determination of the magnitude and nature (Th1 *vs* Th2) of the acquired immune response[17]. One of the main consequences of Th1/Th2 imbalance is production of TNF-α, which triggers a cascade of events leading to chronic inflammation and destruction of bone and cartilage. As shown in Fig. 3, SA exhibits anti-inflammatory function by inhibiting the production of TNF-α in macrophages, and these results suggest that SA acts as anti-inflammatory agent influencing these pro-inflammatory cytokine.

Foxp3 is predominantly expressed in the CD4+CD25+Treg(regulatory T) cells and is recognized to be a specific marker for Treg cells. In humans, as in mice, the function of FoxP3 has been linked to suppressor T cell development and function[18]. Foxp3+ T regulatory (Treg) cells control all aspects of the immune response and rheumatoid arthritis. Davidson et al. have reported that TGF-β induced Foxp3+ cells have full immunoregulatory capacity[19]. TGF-β is another immunosuppressive cytokine, and studies have shown that it may be responsible for both the expansion of Treg cells[20]. The role of CD4+CD25+ regulatory T cells in the pathogenesis and regulation of arthritis has been best studied in a mouse model of RA, collagen-induced arthritis (CIA)[20]. TNF-α is abundantly present in the sera and joints of patients with RA. It has been shown that TNF-α inhibits the suppressive function of nTreg cells and TGF-β induced Treg cells. The inhibitory effects of TGF-β on T cell proliferation, therefore, promote Treg rather than T effector cell differentiation. It is well known that TGF-β inhibits Th1 and Th2 differentiation[21].

In our present study, PCR products for Foxp3, TGF-β amplified from CD4+ T cell RNA preparations, were increased in the SA treated groups compared with control groups (anti-CD3, 1 μg/mL anti-CD28 treated group)(Fig. 4).

The concept of modulating the Th1/Th2 balance as a treatment for chronic autoimmunity has been successfully applied in a number of animal models of autoimmune diseases. If Treg cells could balance the immune system completely, there would be no occurrence of immune system-related disorders. Aside from the use of anti-rheumatic drugs to alter the Th1/Th2 balance, several other strategies are under current investigation.

It has become apparent that, in this experiment, CD4+ T cell subsets with regulatory capacity, such as Th1 cells, are functionally impaired by SA. Our study will be an invention of novel anti-inflammatory agents to modulate the Th1/Th2 balance.

In conclusion, we demonstrated that SA inhibited Th1(IFN-γ) cytokine and pro-inflammatory cytokine(TNF-α) production in

LPS-stimulated mouse peritoneal macrophages, and that these effects were mediated by TGF-β and Foxp3 gene dependently. To the best of our knowledge, SA is the active Th1/Th2 balance modulator that may be beneficial for the therapy of autoimmune disorders such as rheumatoid arthritis.

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