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Regulatory Effect of Scutellariae Radix on the Proinflammatory Cytokine Production and Abnormal T-Cell Activation *in Vitro* in Pristane-Induced Lupus Mice

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Abstract – *Scutellaria baicalensis* is known as a herbal medicine with anti-inflammatory and anti-oxidative activities. However, effect of *Scutellaria baicalensis* on lupus pathogenesis that is characterized by overproduction of proinflammatory cytokines and abnormalities in regulation, function, and interaction of immune cells remains unclear. We investigated effects of Scutellariae radix methanol extract (SBMeOH) on the production of proinflammatory cytokines and abnormal activation of T cells *in vitro* in pristane-induced lupus BALB/c mice. These results demonstrated that SBMeOH significantly decreased the LPS-stimulated production of TNF-α, IL-6, and IL-10 by splenic and peritoneal macrophages and IL-6 and IL-10 by splenocytes from pristane-induced lupus mice. SBMeOH significantly downregulated the Con A-stimulated overproduction of IL-6, IL-10, and IFN-γ by splenocytes from pristane-induced lupus mice. Also, SBMeOH significantly attenuated the Con A-induced expression of CD4+ T cells and CD69+CD4+ T cells but not CD8+ T cells in pristane-induced lupus mice. Our findings indicate that SBMeOH may ameliorate lupus pathogenic inflammation and autoimmunity via downregulation of proinflammatory cytokine production and abnormal activation of T cells.
Keywords – TNF-α, IL-6, IL-10, IFN-γ, CD4, CD8, CD69, lupus, pristane, Scutellariae radix

Introduction

Scutellaria baicalensis and its flavonoids have been widely used as a traditional medicine in Asia. *Scutellaria baicalensis* and the flavonoids derived from *Scutellaria baicalensis* have also been reported to have anticancer, antioxidative, and anti-inflammatory effects (Huang *et al*., 2006; Scheck *et al*., 2006). The flavonoids also inhibit aggregation of platelets and permeability of capillary vessels, and have antibacterial and anti-angiogenic effects (Kowalczyk *et al*., 2006).

Scutellaria baicalensis and some of its constituents, such as baicalein, baicalin or wogonin, have been studied on their anti-inflammatory effect. Flavonoids baicalein from *Scutellaria baicalensis* inhibited *in vitro* production leukotriene C4 (Butenko *et al*., 1993). Some flavonoids derived from *Scutellaria baicalensis* inhibited COX-2 gene expression or PGE_2 production in LPS-induced RAW 264.7 cells (Chen *et al*., 2001; Woo *et al*., 2006). Baicalin inhibited age-associated NF-kB activation (Kim *et al*., 2006). Wogonin attenuated skin inflammation *in vivo* (Chi *et al*., 2003), had neuroprotective effect through decreased production of inflammatory cytokines such as TNF-α and IL-6 in LPS-stimulated microglial cells (Piao *et al*., 2004), and had protective effect on endotoxin-induced lethal shock (Van Dien *et al*., 2001).

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by altered immunoregulation interplaying between overactive B cells, overproduction of proinflammatory cytokines, such as TNF-α, IL-10, IFN-γ and IL-6, and defects in regulation of activation and subsequent proliferation of T cells (Hoffman, 2004; Kyttaris *et al*., 2005), leading to production of autoantibody to contribute to multiple organ injuries and high mortality (Dean *et al*., 2000). Recent research has demonstrated that their activation thresholds for T cell signaling

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play an important role in lupus patients with active pathogenesis (Hoffman, 2004). However, whether *Scutellaria baicalensis* regulates lupus inflammatory pathogenesis characterized by proinflammatory cytokine overproduction and abnormally activated T cells remains unclear.

Pristane is a good inducer of lupus-like syndrome in female BALB/c mice (Richards *et al*., 1998). Recently, it had been reported that immunoregulatory abnormalities of T cells and hyperactivity of B cells had exhibited *in vitro* in pristane-induced lupus mice (Chae and Shin, 2007). We observed that Scutellariae radix methanol extract (SBMeOH) down-regulated the overproduction of proinflammatory cytokines and abnormal activation of T cells in pristane-induced lupus BALB/c mice that were used as a useful model with chronically inflammatory lupus-like syndrome.

Experimental

Animals – Adult female ICR and BALB/c mice at 3-4 weeks of age were purchased from the Dae-Han Experimental Animal Center (Taejeon, Korea), and had been maintained in our animal facility on a regular 12-h light-dark cycle under a temperature of 22 ± 2 °C and relative humidity of 55 ± 5% with water and food available *ad libitum*. BALB/c mice were received *i.p.* a single injection of 0.5 mL of pristane (Sigma Chemical Co., St., Louse, MO, U.S.A.) or PBS (phosphate-buffered saline). Adult female ICR mice, 6 to 10-mo-old BALB/c mice as a pristane-induced lupus model and PBS-treated mice as a normal were used for experiment.

Plant material extraction – The crude extract was obtained from ground mature roots of Scutellariae radix using 99% methanol. The Scutellariae radix methanol extract (SBMeOH) was dried and quantified for the total amount of crude extract. A stock solution was prepared at 100 mg of solid per ml in dimethyl sulphoxide (DMSO; Sigma) and was further diluted with RPMI 1640 immediately before treatment of the cells to achieve concentrations of 0.01, 0.10, and 1.00 mg/mL.

Preparation of lymphoid cells – Splenocyte suspensions were prepared from normal and pristane-induced lupus mice using Hanks' balanced salt solution (HBSS: Gibco Co., Grand Island, N.Y., U.S.A.). Erythrocytes in the single cell suspensions were lysed by brief treatment with sterile red blood cell lysing buffer solution (Sigma). Subsequently, the cells were washed with HBSS and resuspended into a suspension of 1×10^7 cells/mL with RPMI 1640 complete medium supplemented with 10% fetal bovine serum (FBS) and penicillin (10 U/mL)-streptomycin (10 μg/mL).

Preparation of macrophages – Peritoneal macrophages from normal and pristane-induced lupus mice were harvested by peritoneal lavage with ice-cold sterile physiological saline 3 days after the *i.p.* injection of the mice with 2 mL of sterile 3% thioglycollate broth. Cells were washed, and resuspended in complete RPMI 1640 medium. Splenic and peritoneal macrophages were allowed to adhere for 2 h at 37 °C, 5% CO_2 incubation, and then the nonadherent cells were removed by washing with PBS, and the macrophages were resuspended in fresh culture medium.

Cell culture – Splenocytes (1×10^6 cells/mL) and peritoneal macrophages (1×10^6 cells/mL) from normal and pristane-induced lupus mice were cultured in complete RPMI 1640 medium for 6 h, 24 h, or 48 h in the presence or absence of LPS 10 μg/mL (Sigma) or Con A 2 μg/mL at 37 °C, 5% CO_2 incubation. The cell supernatants were then harvested and stored at –70 °C for cytokine assay.

Cytokine assay – The concentrations of TNF-α, IL-6, IL-10, and IFN-γ in supernatants of splenocytes and peritoneal macrophages were determined using ELISA with cytokine monoclonal antibodies (BD Biosciences Pharmingen, San Diego, CA, U.S.A.). All measurements were carried out in duplicate. The results were measured in picograms per milliliter at 450 nm using an ELISA microplate reader (Molecular Devices Co., Ltd., U.S.A.). The lower limit of sensitivity for each of the ELISA was equal to or smaller than 5 pg/mL.

Flow cytometry analysis – Splenocytes (4×10^6 cells/mL) from normal and pristane-induced lupus mice were cultured in complete RPMI 1640 medium for 48 h in the presence or absence of Con A 2 μg/mL at 37 °C, 5% CO_2 incubation. The cells were harvested, washed, and preincubated with anti-Fc receptor monoclonal antibody (MAb) 2.4G2. The cells (1×10^6 cells/0.1 mL) were directly stained with fluorescein isothiocyanate (FITC)-labelled anti-CD4 or CD69 (Pharmingen) and phycoerythrin (PE)-labelled anti-CD8 or CD4. The cells were incubated for 30 min in the dark, washed, and fixed with 1% paraformaldehyde until analysis. Cells were acquired (5,000 events per group in the lymphocyte gate) and analyzed for two-parameter immunofluorescence using flow cytometry (Coulter, EPICS/ML).

Statistical analysis – All data were expressed as means ± standard error (S.E.). Experiments were always run in duplicate and repeated at least twice. Analysis of variation and Student's *t*-test were used to determine statistical significance, and $p < 0.05$ was considered to be statistically significant.

Results and Discussion

Effect of SBMeOH on the *in vitro* production of splenic cytokines in ICR mice – *Scutellaria baicalensis* and some of its components, such as baicalein, baicalin, and wogonin, have been reported their anti-inflammatory effects both *in vivo* and *in vitro* (Van Dien *et al*., 2001; Piao *et al*., 2004; Kim *et al*., 2006). We investigated effect of SBMeOH on the production of cytokines associated with inflammatory responses and cell-mediated immune responses in normal mice. In this study, splenic macrophages and splenocytes (each 1×10^6 cells/mL) from normal ICR mice at age 6-8 weeks were cultured for 24 h in the presence of LPS 10 μg/mL 30 min after SBMeOH treatment for IL-6 and IL-10, and for 48 h in the presence of Con A 1 μg/ml for IL-2 and IFN-γ, respectively. IL-6 is generally well-known as a proinflammatory cytokine but IL-10 as an anti-inflammatory cytokine. Our results were observed that SBMeOH significantly attenuated LPS-stimulated production of IL-6 by splenic macrophages in a dose-dependent manner in normal ICR mice but increased IL-10 (Fig. 1A), which supports previous reports about anti-inflammatory effects of *Scutellaria baicalensis* and some of its components. IL-2 and IFN-γ induce T cell proliferation and macrophage activation associated with cell-mediated immune responses. SBMeOH remarkably enhanced Con A-stimulated production of IL-2 and IFN-γ by splenocytes (Fig. 1B). Therefore, these data indicate that SBMeOH has anti-inflammatory effect with induction of increased cell-mediated immune responses in normal mice.

Effect of SBMeOH on the LPS-induced production of proinflammatory cytokines by immune cells from pristane-induced lupus mice – Abnormalities in the function, regulation, and interactions of immune cells, with T and B lymphocytes, result in immune-complex-mediated deposition and inflammatory organ damage in SLE (Takeuchi *et al*., 2005). Macrophages are thought to trigger lupus pathogenic inflammation through overproduction of proinflammatory cytokines. Higher levels of the TNF-α and IL-6 were maintained in lupus patients with active disease than patients with inactive disease (Davas *et al*., 1999). Anti-DNA autoantibody production in pristane-induced lupus has been reported to be dependent on IL-6 (Richards *et al*., 1998). IL-10, which is produced at a high level by B lymphocytes and monocytes of patients with SLE, contributes to B lymphocyte hyperactivity and autoantibody production (Llorente *et al*., 1995). Blocking IL-6 and IL-10 may decrease autoantibody production and induce normalization of T cell dysfunction in lupus. LPS strongly induces production of IL-6 and IL-10 *in vivo* and LPS-induced in pristane-induced lupus mice compared to normal mice (Chae *et al*., 2006). In present study, we investigated effect of SBMeOH on the *in vitro* production of proinflammatory cytokines by immune cells from pristane-induced 6 to 10-mo-old lupus BALB/c mice. Splenic and peritoneal macrophages and splenocytes (each 1×10^6 cells/mL) from pristane-induced lupus BALB/c mice were cultured for 6 h for TNF-α and for 24 h for IL-6 and IL-10 in the presence of LPS 10 μg/mL 30 min after SBMeOH treatment. In Fig. 2, our observation showed that SBMeOH significantly attenuated the LPS-enhanced production of IL-6 by splenic and peritoneal macrophages at 0.01 and 0.10 mg/mL, and TNF-α and IL-10 at 0.10 mg/mL in pristane-induced lupus mice. In Fig. 3, these results also demonstrated that SBMeOH at 0.10 mg/mL remarkably downregulated LPS-stimulated

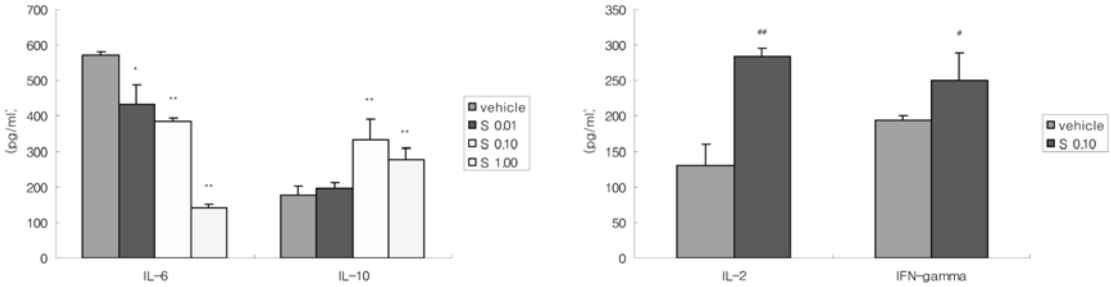

Fig. 1. Effect of SBMeOH on the *in vitro* production of splenic cytokines in ICR mice.

Scutellariae radix methanol extract: SBMeOH. S 0.01: SBMeOH 0.01 mg/mL; S 0.10: SBMeOH 0.10 mg/mL; S 1.00: SBMeOH 1.00 mg/mL. Splenic macrophages (1×10^6 cells/mL) from normal ICR mice were cultured for 24 h for IL-6 and IL-10 in the presence of LPS 10 μg/mL 30 min after SBMeOH treatment (Fig. 1A), and splenocytes (1×10^6 cells/mL) for 48 h for IL-2 and IFN-γ in the presence of Con A 1 μg/mL (Fig. 1B). Concentrations of cytokines in supernatants of splenic macrophages or splenocytes were measured at 450 nm using ELISA. All measurements were carried out in duplicate. Each value represents the mean ± S.E. * ($p < 0.05$) and ** ($p < 0.01$): Significantly different from the value in each vehicle-treated control.

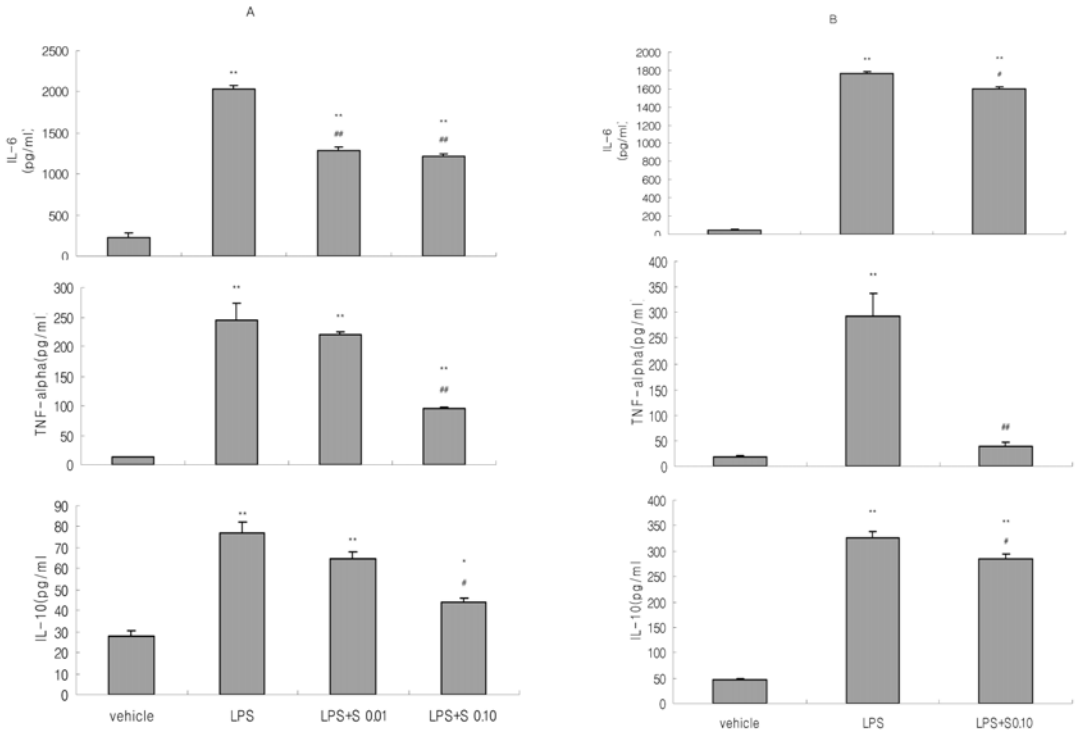

Fig. 2. Effect of SBMeOH on the LPS-induced production of proinflammatory cytokines by macrophages from pristane-induced lupus mice.

Peritoneal macrophages (A) and splenic macrophages (B) from pristane-induced lupus mice were cultured for 6 h for TNF-á and for 24 h for IL-6 and IL-10 in the presence or absence of LPS 10 μg/mL 30 min after SBMeOH treatment. Each value represents the mean ± S.E. Other legends and methods are the same as in Fig. 1. * ($p < 0.05$) and ** ($p < 0.01$): Significantly different from the value in each vehicle-treated control. $^{\#}$ ($p < 0.05$) and $^{\#\#}$ ($p < 0.01$): Significantly different from the value in LPS-treated immune cells.

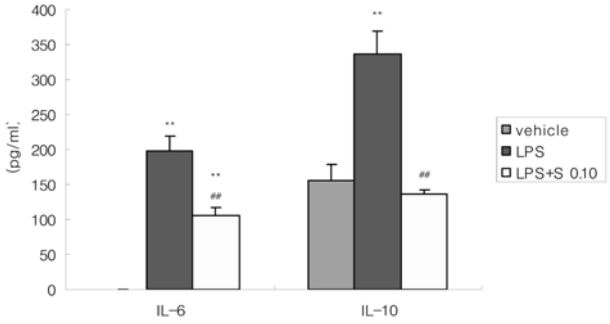

Fig. 3. Effect of SBMeOH on the LPS-induced production of splenic cytokines in pristane-induced lupus mice.

Splenocytes from pristane-induced lupus mice were cultured for 24 h in the presence or absence of LPS 10 μg/mL 30 min after SBMeOH treatment. Each value represents the mean ± S.E. Other legends and methods are the same as in Fig. 1. ** ($p < 0.01$): Significantly different from the value in each vehicle-treated control. $^{\#\#}$ ($p < 0.01$): Significantly different from the value in LPS-treated immune cells.

production of IL-6 and IL-10 by splenocytes from pristane-induced lupus mice. Therefore, these observations indicate that SBMeOH may attenuate lupus pathogenic inflammation and overactivation of B cells for autoantibody production via downregulation of TNF-α, IL-6, and IL-10.

Effect of SBMeOH on the Con A-induced production of splenic cytokines in pristane-induced lupus mice – Abnormalities in T cell function play an important role in the immune dysregulation in human disease and murine models of lupus. Production of lupus pathogenic autoantibodies is determined by the type of Th responses (Reininger *et al*., 1996). IFN-γ, a cytokine associated with Th1 response, is required for lupus-like syndrome and lymphoaccumulation in MRL-*lpr* mice, and plays prominent roles in the lupus pathogenic tissue injuries (Gerez *et al*., 1997; Balomenos *et al*., 1998). Therefore, IFN-γ may be considered as a target for modulating autoimmunity. Th2 cells, which produce IL-6 and IL-10, stimulate antibody production by B cells and upregulate humoral or allergic responses (Murphy *et al*., 2000). The elevated levels of IL-6 and IL-10 in SLE are necessary to activate B cells for autoreactive T cells and further stimulate to proliferate and produce autoantibodies (Llorente *et al*., 1995; Samoilova *et al*., 1998).

Recently, it showed that Con A-stimulated overpro-

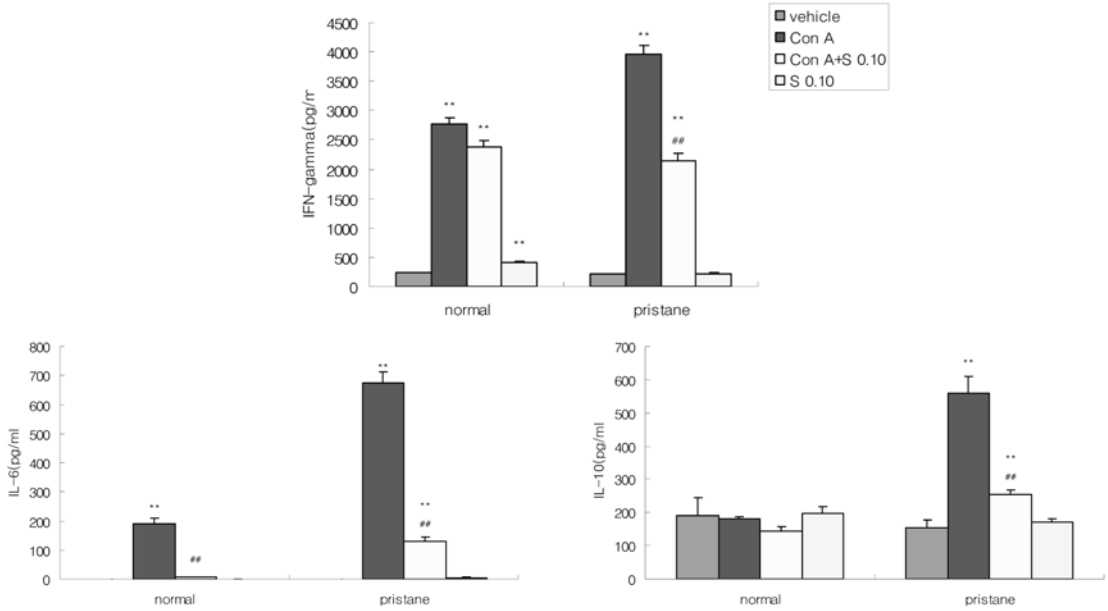

Fig. 4. Effect of SBMeOH on the Con A-induced production of splenic cytokines in pristane-induced lupus mice.
Splenocytes from pristane-primed or PBS-treated 6 to 10-mo-old BALB/c mice were cultured for 48 h in the presence or absence of Con A 2 μg/mL 30 min after SBMeOH treatment. Each value represents the mean ± S.E. Other legends and methods are the same as in Fig. 1. ** ($p < 0.01$): Significantly different from the value in each vehicle-treated control. $^{\#\#}$ ($p < 0.01$): Significantly different from the value in Con A-treated immune cells.

duction of IL-6, IL-10, and IFN-γ by splenocytes had exhibited in pristane-induced lupus mice (Chae and Shin, 2007). We investigated effect of SBMeOH on the Con A-stimulated production of proinflammatory cytokines by splenocytes by pristane-primed or PBS-treated 6 to 10-mo-old BALB/c mice. Con A has been used to activate T lymphocytes via the antigen receptor. Splenocytes from normal and pristane-induced lupus mice were cultured for 48 h in the presence or absence of Con A 2 μg/mL 30 min after SBMeOH treatment. As shown in Fig. 4, we observed that Con A-stimulated production of splenic IL-6, IL-10, and IFN-γ were remarkably upregulated in pristane-induced lupus mice compared to normal mice, indicating that *in vitro* overproduction of proinflammatory cytokines remarkably exhibits in pristane-induced lupus mice compared to PBS-treated mice. SBMeOH at 0.10 mg/mL significantly attenuated Con A-stimulated production of IL-6 but not IFN-γ and IL-10 by splenocytes from PBS-treated mice, while downregulated the Con A-stimulated overproduction of IFN-γ, IL-6, and IL-10 in pristane-induced lupus mice (Fig. 4). These findings indicate that SBMeOH may suppress Th2 type immune responses with a shift toward Th1 responses in normal state, while downregulate hyperreactivity of Th1 and Th2 responses in pristane-induced lupus mice through downregulation of IFN-γ, IL-6, and IL-10.

Effect of SBMeOH on the Con A-induced expression of CD4+ T cells and CD8+ T cells in splenocytes from pristane-induced lupus mice – It is known that CD4+ T cells function as helper cells for B cell differentiation and involve in delayed-type hypersensitivity reactions, and CD8+ T cells participate in the host response against intracellular microorganisms and mediate cytotoxic and suppressor activities. Overexpression of CD4+ cells or CD8+ cells is thought to be required for B cell hyperactivity in SLE. CD8+ lymphocytes from patients with SLE sustained, rather than suppressed, spontaneous *in vitro* production of polyclonal IgG and synergized with CD4+ cells to support autoantibody synthesis by SLE peripheral blood mononuclear cells (Linker-Israeli *et al*., 1990). We measured expression of CD4+ T cells and CD8+ T cells in splenocytes incubated with Con A 2 μg/mL for 24 h 30 min after treatment of SBMeOH 0.10 mg/mL in pristane-induced lupus mice. These results demonstrated that SBMeOH significantly decreased Con A-stimulated expression of CD4+CD8- but not CD4-CD8+ in splenic T cells from pristane-induced lupus mice (Fig. 5). CD4+ T cells play an important role in the pathogenesis of lupus (Reininger *et al*., 1996). Therefore, these data suggest that SBMeOH may downregulate expression of CD4+ T cells, leading to ameliorate abnormal hyperactivity in function, regulation

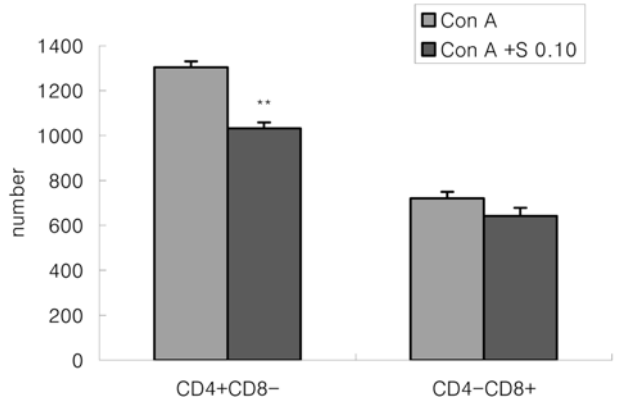

Fig. 5. Effect of SBMeOH on the Con A-induced expression of CD4+ T cells and CD8+ T cells in splenocytes from pristane-induced lupus mice.

Splenocytes from pristane-induced lupus mice were incubated with Con A 2 μg/mL for 24 h 30 min after treatment of SBMeOH 0.10 mg/mL. The harvested cells were preincubated with anti-Fc receptor MAb 2.4G2. The cells (1.0×10^6 cells/0.1 mL) were stained with FITC-labelled anti-CD4 and PE-labelled anti-CD8. The cells were incubated for 30 min in the dark, fixed with 1% paraformaldehyde, and analyzed using flow cytometry. Each value represents the mean ± S.E. ** ($p < 0.01$): Significantly different from the value in each vehicle-treated control.

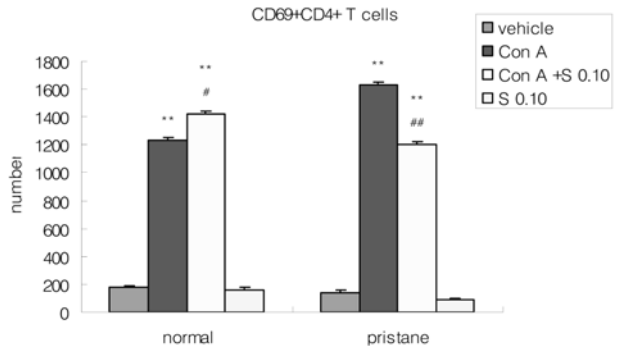

Fig. 6. Effect of SBMeOH on the expression of CD69+CD4+ T cells in Con A-stimulated splenocytes from pristane-induced lupus mice.

Splenocytes from pristane-induced lupus mice were incubated in the presence or absence of Con A 2 μg/mL for 24 h 30 min after treatment of SBMeOH 0.10 mg/mL. The harvested cells were stained with FITC-labelled anti-CD69 and PE-labelled anti-CD4. Each value represents the mean ± S.E. Other legends and methods are the same as in Fig. 5. ** ($p < 0.01$): Significantly different from the value in each vehicle-treated control. $^{\#\#}$ ($p < 0.01$): Significantly different from the value in Con A-treated immune cells.

and interaction of macrophages, T and B cells in pristane-induced lupus mice.

Effect of SBMeOH on the expression of CD69+CD4+ T cells in Con A-stimulated splenocytes from pristane-induced lupus mice – Activation of T cells is associated with lupus immune-mediated pathogenesis (Portales-Perez *et al*., 1997). The CD69 antigen is an early leukocyte activation marker rapidly induced on the surface of activated lymphocytes. It has been reported that the percentage of *in vitro* expression of CD69, an activation marker on lymphoid cells, is increased in SLE cells (Crispin *et al*., 1998), and that CD4+ T cells expressing early activation antigen CD69 in murine lupus play a possible abnormal regulatory role for cytokine imbalance (Ishikawa *et al*., 1998). We measured the number of CD69 expression in CD4+ T cells in splenocytes from pristane-induced lupus mice after 24 h incubation in the presence or absence of Con A 2 μg/mL 30 min after treatment of SBMeOH 0.10 mg/mL. As shown in Fig. 6, our results demonstrated that SBMeOH significantly attenuated Con A-induced expression of CD69+CD4+ T cells in splenocytes in pristane-induced lupus mice, suggesting that SBMeOH may attenuate the abnormal activation of Th responses leading to lupus pathogenic autoantibody production.

In conclusion, our findings indicate that SBMeOH may attenuate lupus pathogenic inflammation and ameliorate abnormalities in T cell activation for autoantibody production in pristane-induced lupus mice via downregulation of proinflammatory cytokine overproduction and abnormal activation of T cells.

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