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Baicalin Ameliorates Dysimmunoregulation in Pristane-Induced Lupus Mice: Production of IL-6 and PGE_2 and Activation of T cells

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Abstract – Systemic lupus erythematosus (SLE) is a systemic inflammatory autoimmune disease characterized by abnormalities in T cell immunoregulation and hyperreactivity of B cells, leading to autoantibody production and multiorgan injuries. We investigated the effect of baicalin on aberrant immunoregulation in pristane-induced lupus mice. Mice received *i.p.* a single injection of 0.5 ml of pristane or PBS, and approximately 3 months later, were used as a pristane-induced lupus model or healthy controls. The pristane-induced lupus mice and healthy mice were randomly divided into three groups: healthy mouse group (healthy control), pristane-primed lupus control group (lupus control), and baicalin (BAC)-treated pristane-primed lupus mouse group (BAC-treated lupus). The pristane-induced lupus mice and healthy mice were administrated orally with BAC 50 mg/kg or PBS once in a day for 10 ds. These results demonstrated that levels of serum IL-6, LPS-induced production of IL-6, PGE_2 and NO by macrophages, PGE_2-stimulated production of IL-6 by macrophages and IFN-γ by thymocytes, and an overexpression of splenic NKT cells and CD69+CD4+ T cells were downregulated in BAC-treated lupus compared to lupus control, while reduced apoptosis of splenic CD4+ T cells were upregulated. Therefore, these findings suggest that BAC may attenuate autoimmunity and disease activity in lupus via downregulation of aberrant activation of T cells and inhibition of overproduction of IL-6 and PGE_2 in pristane-induced lupus mice.
Keywords – baicalin, lupus, IL-6, PGE_2, apoptosis, T cell activation, NKT cell

Introduction

Systemic lupus erythematosus (SLE) is a T cell-dependent autoimmune disease characterized by an overproduction of systemic proinflammatory cytokines and abnormalities in the function, regulation, and interactions of immune cells, with T and B cells, leading to autoantibody production and multiple organ injuries (Hoffman, 2004; Kyttaris *et al*., 2005; Nagy *et al*., 2005; Takeuchi *et al*., 2005). The overproduction of proinflammatory cytokines, such as IL-6, IL-10, and IFN-γ, is associated with lupus T cell-mediated autoantibody production (Dean *et al*., 2000; Theofilopoulos *et al*., 2001). IL-6 is especially known as a biomarker in SLE, which is associated with disease activity, B cell differentiation, and autoantibody production in lupus (Davas *et al*., 1999; Liang *et al*., 2006; Chun *et al*., 2007). PGE_2 plays a pivotal role in the development of local inflammatory responses and tissue injuries in lupus (Herrera-Acosta *et al*., 1987; Tsai *et al*. 1994). It is also demonstrated that elevated PGE_2 receptors EP2 and EP4 contributed to the upregulation of IL-6 in pristane-treated mice (Akaogi *et al*., 2006). Recently, endogenous PGE_2 has been shown to mediate the overproduction of IL-6, IL-10, and IFN-γ in pristane-induced lupus mice (Chae *et al*., 2008).

Activated T cells in lupus have been reported to be resistant to apoptosis (Xu *et al*., 2004; Chae and Shin 2007). The decreased spontaneous apoptosis and decreased activation-induced cell death of T cells in SLE has resulted in an enhanced help to B cells and a potentially decreased regulatory function (Crispin *et al*., 1998; Ishikawa *et al*., 1998; Kyttaris *et al*., 2005). It has also been reported that the activation of natural killer T cells (NKT cells) augments helper T (Th) cell 1-type immune responses and autoantibody secretion that contribute to lupus development in lupus-prone NZB/W mice (Zeng *et al*., 2003; Takahashi and Strober, 2008). Therefore, the overproduction of IL-6 and PGE_2, and abnormalities in the function and regulation of T cells are thought to be therapeutic targets in the lupus pathogenesis.

Baicalin is a flavonoid compound purified from the medicinal plant *Scutellaria baicalensis* Georgi. Baicalin has been reported to have antioxidant, anti-inflammatory,

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and anti-cancer properties *in vivo* and *in vitro* (Gao *et al*., 1999; Li-Weber, 2009). Baicalin has been reported to have protective effects via the promotion of apoptosis of inflammatory cells in the spinal cords of rats with autoimmune encephalomyelitis (Xu *et al*., 2011). Hao *et al.* (2011) reported that baicalin suppresses the expression of toll like receptor (TLR)2/4 and NF-B in chlamydia trachomatis-infected mice. It was previously observed that Scutellariae radix methanol extract 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 model with chronically inflammatory lupus-like syndrome (Chae *et al*., 2007). However, the effects of baicalin on inflammatory autoimmunity and abnormalities in the regulation of T cells in lupus remain unknown.

Pristane-induced lupus BALB/c mice are characterized by slightly elevated levels of plasma IL-6 and autoantibody production, which is associated with the induction of lupus-like autoimmunity (Shacter *et al*., 1992; Satoh and Reeves, 1994). Recently, pristane-induced lupus mice showed immunoregulatory abnormalities of T cells and hyperreactivity of B cells in the *ex vivo* immune responses (Chae and Shin, 2007). In the present study, we investigated the effect of baicalin (BAC) on aberrant immunoregulation in pristane-induced lupus mice.

Experimental

Animals – Adult female BALB/c mice at 3-4 weeks of age were purchased from the Dae-Han Biolink (Chungbuk, Korea), and were maintained in our animal facility on a regular 12-h light-dark cycle under a temperature of 22 ± 2 ºC and a relative humidity of 55 ± 5% with water and food available *ad libitum*. Mice 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), and approximately 3 months later, were used as a pristane-induced lupus model or healthy controls. The care and treatment of the mice were in accordance with the guidelines established by the Public Health Service Policy on the Humane Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee.

Plant material extraction – The roots of *Scutellaria baicalensis* were purchased from Bohwadang at Jeonju, Chonbuk, Korea. A voucher specimen was deposited in the herbarium of the college of pharmacy, Woosuk University (WSU-10-014). The dried plant material (300 g) was extracted three times with MeOH at room temperature and filtered. The extracts were combined and evaporated in vacuo at 40 ºC. The resultant methanolic extract (65 g) was partitioned as *n*-hexane (7 g), methylene chloride (4 g), EtOAc (7 g), *n*-BuOH (21 g), and H2O soluble fractions. Sephadex LH-20 (MeOH) column chromatography of *n*-BuOH soluble extract provided six fractions (B1-6). Fraction B2 was purified by Lobar-A column ($CHCl_3$-MeOH-H_2O, 100 : 8 : 1) to give baicalin (150 mg). The ^{1}H- and ^{13}C-NMR spectra were determined on a JEOL JMN-EX 400 spectrometer. The TLC was carried out on pre-coated silica gel F_{254} plates (Merck, Darmstadt, Germany), and the silica gel for column chromatography was Kiesel gel 60 (230 - 400 mesh, Merck). The column used for LPLC was the Lobar A (Merck Lichroprep Si 60, 240 - 10 mm). All the other chemicals and solvents were of analytical grade and they were used without further purification. Baicalin (MeOH); 220 - 222 ºC, ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 12.50 (1H, s, 5-OH), 8.008.03 (2H, m, H-2', 6'), 7.557.53 (3H, m, H-3', 4', 5'), 7.00 (1H, s, H-8), 6.95 (1H, s, H-3), 4.98 (1H, d, J = 10.9 Hz, H-1"); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 182.7 (C-4), 171.7 (C-6"), 163.7 (C-2), 151.9 (C-7), 149.4 (C-9), 146.7 (C-5), 132.1 (C-4'), 131.0 (C-6), 131.0 (C-1'), 129.3 (C-3', 5'), 126.5 (C-2', 6'), 106.4 (C-10), 104.9 (C-3), 100.9 (C-1"), 94.5 (C-8), 75.9 (C-3"), 74.5 (C-5"), 73.0 (C-2"), 72.2 (C-4").

Administration of baicalin – The pristane-induced lupus mice and healthy mice were randomly divided into three groups: healthy mouse group (healthy control), pristane-primed lupus control group (lupus control), and baicalin (BAC)-treated pristane-primed lupus mouse group (BAC-treated lupus). The pristane-induced lupus mice and healthy mice were administrated orally with BAC 50 mg/kg or PBS once a day for 10 ds.

Preparation of serum – Blood was harvested from hearts under anesthetics in BAC-treated lupus, lupus control, and healthy control. The blood was allowed to clot for 2 h at room temperature, and then centrifuged for 20 min at 2000 × g. The sera were collected and stored at ≤ −20 ºC for cytokine assays.

Preparation of lymphoid cells – Splenocyte and thymocyte suspensions were prepared from BAC-treated lupus, lupus control, and healthy control using Hanks' balanced salt solution (HBSS: Gibco Co., Grand Island, N.Y., U.S.A.). Erythrocytes in the single cell suspensions were briefly lysed with sterile red blood cell lysing buffer solution (Sigma). Subsequently, the cells were washed with HBSS and resuspended in RPMI 1640 complete medium supplemented with 10% fetal bovine serum

(FBS) and penicillin (10 U/mL)-streptomycin (10 μg/mL) at a density of 1×10^7 cells/mL.

Preparation of macrophages – Peritoneal macrophages from BAC-treated lupus, lupus control, and healthy control were harvested by peritoneal lavage with ice-cold sterile physiological PBS, 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 DMEM (Invitrogen, Carlsbad, CA), supplemented with 10% FBS (Sigma) and 1 × antibiotic/antimycotic (Invitrogen). Peritoneal macrophages were allowed to adhere for 2 h at 37 °C, 5% CO_2 incubation, the non-adherent cells were then removed by washing with PBS, and the macrophages were resuspended in fresh culture medium.

Cell culture – RAW 264.7 cells were purchased from the Korean Cell Bank (Seoul, Korea). RAW 264.7 cells were pretreated with various concentrations of BAC (5, 50, 250) in complete DMEM media for 1 h and then cultured for 24 h for IL-6 and NO production in the presence or absence of LPS 1 μg/mL (Sigma Chemical Co., St., Louse, MO) at 37 °C in a humidified atmosphere containing 5% CO_2. Splenocytes (1×10^6 cells/mL), thymocytes (1×10^6 cells/mL), and peritoneal macrophages (0.5×10^6 cells/mL) obtained from BAC-treated lupus, lupus control, and healthy control were cultured in the presence or absence of LPS 5 μg/mL or PGE_2 5 for 24 h or 36 h at 37 °C, 5% CO_2 incubation. The cell supernatants were stored at −70 °C for cytokine, PGE_2, and NO assay.

Cytokine assay – The concentrations of cytokines, including IL-6 and IFN-γ in serum and the cell supernatants, were determined by using cytokine monoclonal antibodies (BD Biosciences Pharmingen, 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.

PGE_2 immunoassay – PGE_2 concentration in the cell supernatants was determined by using a monoclonal antibody/enzyme immunoassay kit from Cayman Chemical, according to the manufacturer's instructions. Concentrations of PGE_2 were measured at 405 nm using ELISA.

NO assay – The concentrations of NO (nitric oxide) in the supernatants harvested from the culture were assayed by adding 100 μL of freshly prepared Griess reagent to 100 μL of the sample in 96-well plates, and the absorbance was then read at 540 nm after 10 minutes using ELISA.

Flow cytometry analysis – Splenocytes from BAC-treated lupus, lupus control, and healthy control 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-CD3 (Pharmingen), FITC-labelled anti-CD69, PE-labelled anti-CD4, and PE-labelled anti-NK1.1. The cells were incubated for 30 min in the dark, washed, and fixed with 1% paraformaldehyde until analysis. Cells were acquired (10,000 events per group) and analyzed for two-parameter immunofluorescence using flow cytometry (Coulter, EPICS/ML, USA).

TUNEL assay – Splenocytes from BAC-treated lupus, lupus control, and healthy control were cultured for 24 h at 37 °C, and 5% CO_2. The cells were harvested, washed, and preincubated with anti-Fc receptor MAb 2.4G2. The cells (1.0×10^6 cells/0.1 mL) were stained with PE-labelled anti-CD4, incubated for 30 min in the dark, and washed. The cells were then fixed in 4% paraformaldehyde for 10 min, washed in PBS, and then permeabilized in 1% saponin for 2 min on ice. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay was carried out for apoptotic cell detection by labeling free 3'-OH DNA ends with fluorescein-labeled dUTP, using the enzyme terminal deoxynucleotidyl transferase (TdT) (*In situ* cell death detection kit, Fluorescein, Roche, U.S.A.). The cells were then incubated at 37 °C for 1 h in the dark, and washed and resuspended in PBS for flow cytometry analysis (Coulter, EPICS/ML).

Statistical analysis – All data were expressed as means ± standard deviation (S.D.). 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 statistically significant.

Results and Discussion

Baicalin reduced levels of serum IL-6 in pristane-induced lupus mice – IL-6 has been reported to be a crucial biomarker in SLE and is a therapeutic target in the lupus pathogenesis (Liang *et al*., 2006; Chun *et al*., 2007). Elevation of serum IL-6 is correlated with the disease activity in SLE (Davas *et al*., 1999). It has been reported that elevated IL-6 was correlated with the induction of differentiation to autoantibody-forming cells in lupus and anti-IL-6 monoclonal antibody inhibited autoimmune responses in a murine model of SLE (Liang *et al*., 2006). Pristane-induced lupus BALB/c mice are characterized by slightly elevated levels of plasma IL-6 (Shacter *et al*., 1992). In this study, blood was harvested from hearts

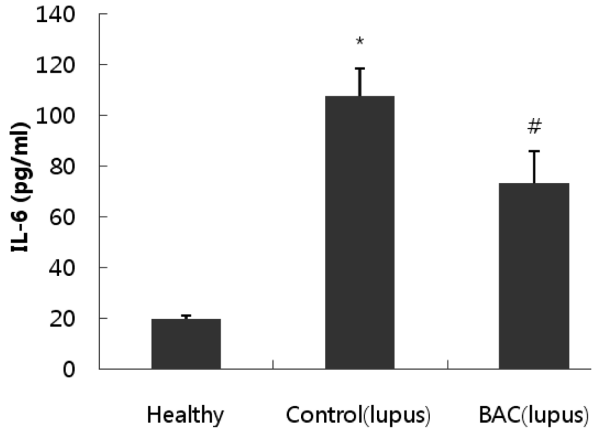

Fig. 1. Baicalin reduced levels of serum IL-6 in pristane-induced lupus mice.

Blood was harvested from hearts under anesthetics in BAC-treated lupus [BAC(lupus)], lupus control [Control(lupus)], and healthy control (Healthy). The concentrations of serum IL-6 were measured at 450 nm using ELISA. All measurements were carried out in duplicate. Each value represents the mean ± S.D. $^{*}(p < 0.05)$: Significantly different from the value in healthy control. $^{\#}(p < 0.05)$: Significantly different from the value in pristane-induced lupus controls.

under anesthetics in BAC-treated lupus, lupus control, and healthy control. As shown in Fig. 1, these results demonstrated that levels of serum IL-6 decreased remarkably in BAC-treated lupus compared to lupus control. Therefore, these findings indicate that BAC may attenuate disease activity and autoimmune response in lupus via the inhibition of systemic IL-6 production.

Baicalin inhibited LPS-induced production of IL-6 *ex vivo* in pristane-induced lupus mice – It has been reported that BAC has anti-inflammatory and antioxidant properties (Gao *et al*., 1999; Li-Weber, 2009). In this study, we investigated whether BAC inhibits the production of IL-6 by immune cells. RAW 264.7 cells were pretreated with various concentrations of BAC in complete media for 1 h and then cultured for 24 h for cytokine production in the presence or absence of LPS 1 μg/mL at 37 ºC, 5% CO_2. These results demonstrated that BAC remarkably inhibited the LPS-induced production of IL-6 in RAW 264.7 cells in a concentration dependent manner (Fig. 2 A). Anti-DNA antibody production has been reported to be dependent on the IL-6 production by murine peritoneal macrophages in pristane-induced lupus mice (Shacter *et al*., 1992; Richards *et al*., 1998). Here, peritoneal macrophages and splenocytes from BAC-treated lupus, lupus control, and healthy control were cultured for 24 h and 36 h in the presence or absence of LPS 5 μg/mL, respectively. These results demonstrated that the LPS-induced production of IL-6 by macrophages (Fig. 2 B) and splenocytes (Fig.

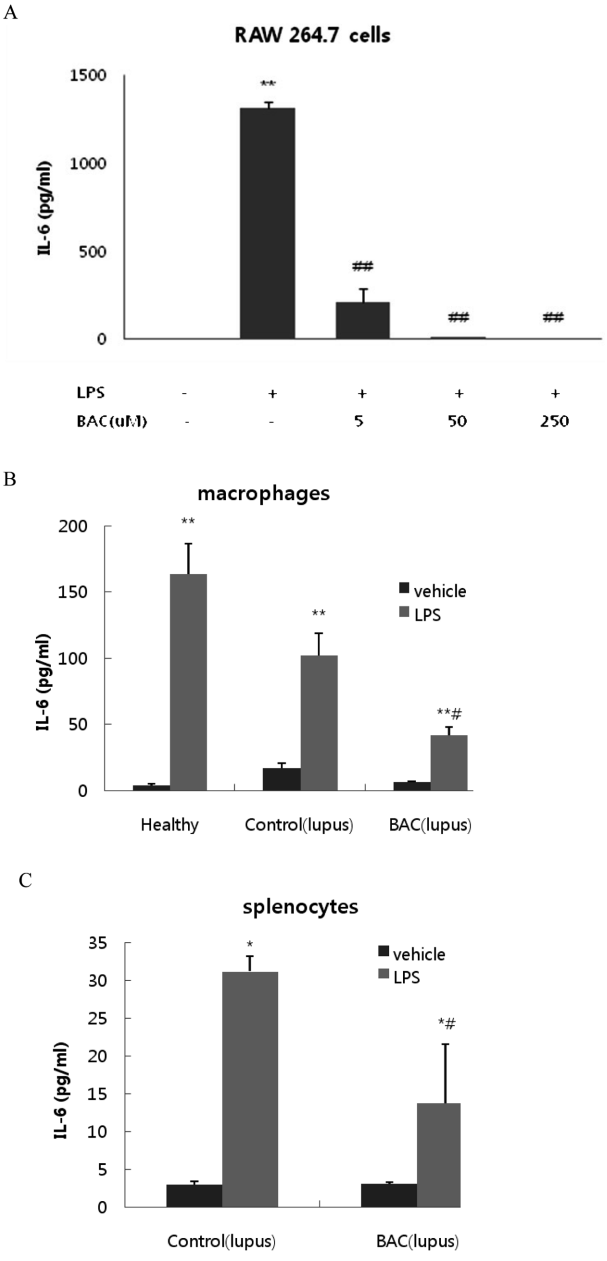

Fig. 2. Baicalin attenuated LPS-induced production of IL-6 *ex vivo* in pristane-induced lupus mice.

A: Inhibitory effect of BAC on the LPS-induced production of IL-6 by RAW 264.7 cells. B: The LPS-induced production of IL-6 by peritoneal macrophages from BAC-treated lupus, lupus control, and healthy control. C: The LPS-induced production of IL-6 by splenocytes from BAC-treated lupus and lupus control. The cell supernatants were determined by using cytokine monoclonal antibodies. Each value represents the mean ± S.D. Other legends and methods are the same as in Fig. 1. $^{*}(p < 0.05)$ and $^{**}(p < 0.01)$: Significantly different from the value in vehicle-treated negative control. $^{\#}(p < 0.05)$ and $^{\#\#}(p < 0.01)$: Significantly different from the value in LPS-treated positive (lupus) control.

2 C) was significantly inhibited in BAC-treated lupus compared to lupus control. Therefore, these findings

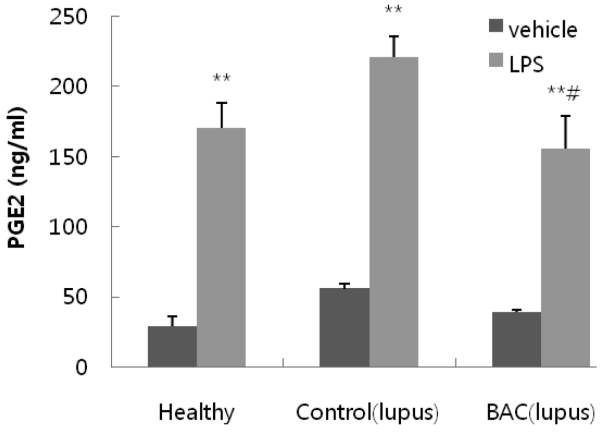

Fig. 3. Baicalin inhibited LPS-induced production of PGE_2 *ex vivo* in pristane-induced lupus mice.

The harvested peritoneal macrophages in BAC-treated lupus, lupus control, and healthy control were incubated with LPS for 24 h. Each value represents the mean ± S.D. Other legends and methods are the same as in Fig. 1. $^{**}(p < 0.01)$: Significantly different from the value in vehicle-treated negative control. $^{\#}(p < 0.05)$: Significantly different from the value in LPS-treated lupus positive controls.

indicate that BAC may inhibit the IL-6 production by immune cells, including macrophages and splenocytes in lupus.

Baicalin inhibited LPS-induced production of PGE_2 *ex vivo* in pristane-induced lupus mice – PGE_2 plays a pivotal role in the development of local inflammatory responses and tissue injuries in lupus (Herrera-Acosta *et al*., 1987; Tsai *et al*., 1994). Recently, it has been reported that levels of serum PGE_2 were remarkably enhanced in pristane-induced lupus mice compared to healthy controls (Chae *et al*., 2008). In this study, we examined whether BAC inhibits the production of PGE_2 in pristane-induced lupus mice. Peritoneal macrophages obtained from BAC-treated lupus, lupus control, and healthy control was cultured for 24 h in the presence or absence of LPS 5 μg/mL. As shown in Fig. 3, these results demonstrated that the LPS-induced production of PGE_2 by peritoneal macrophages was remarkably inhibited in BAC-treated lupus compared to lupus control, indicating that BAC may protect against local inflammatory responses and tissue injuries in lupus through the inhibition of PGE_2 production.

Baicalin inhibited PGE_2-induced production of proinflammatory cytokines *ex vivo* in pristane-induced lupus mice – Elevated IL-6 has been reported to be induced by PGE_2 in a model of inflammation (Hinson *et al*., 1996). It was also demonstrated that the elevated PGE_2 receptors EP2 and EP4 contributed to the

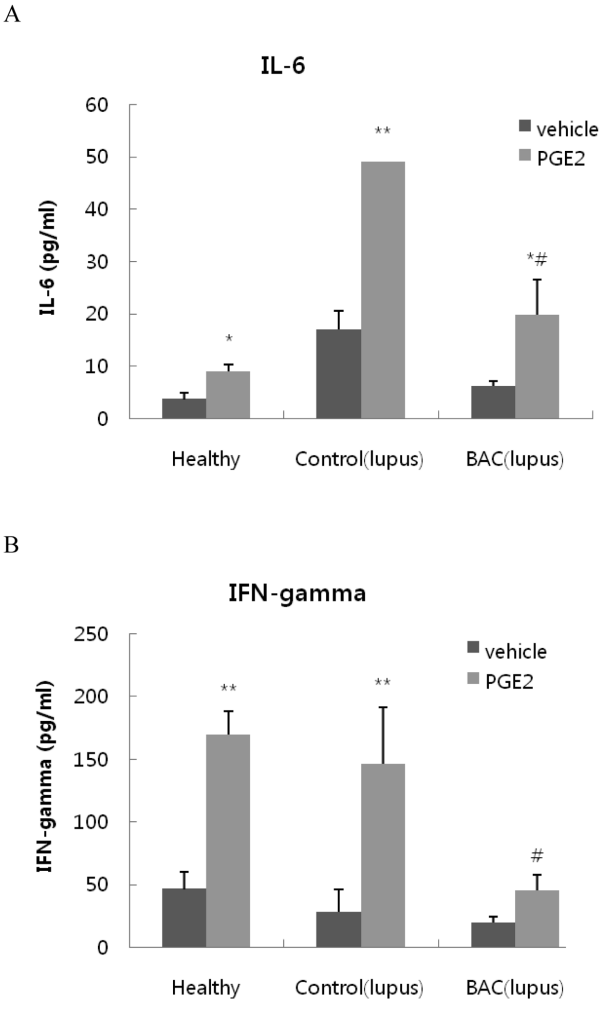

Fig. 4. Baicalin inhibited PGE_2-induced production of proinflammatory cytokines *ex vivo* in pristane-induced lupus mice.

The harvested peritoneal macrophages (A) and thymocytes (B) from BAC-treated lupus, lupus control, and healthy control were incubated with PGE_2 for 36 h. Each value represents the mean ± S.D. Other legends and methods are the same as in Fig. 1. $^{*}(p < 0.05)$ and $^{**}(p < 0.01)$: Significantly different from the value in vehicle-treated negative control. $^{\#}(p < 0.05)$: Significantly different from the value in PGE_2-treated lupus positive control.

upregulation of IL-6 in pristane-treated mice (Akaogi *et al*., 2006). Recently, the endogenous PGE_2 was shown to mediate the overproduction of IFN-γ, IL-6, and IL-10, in pristane-induced lupus mice (Chae *et al*., 2008). In the present study, the peritoneal macrophages and thymocytes from BAC-treated lupus, lupus control, and healthy control were cultured for 24 h for macrophages and 36 h for thymocytes in the presence or absence of PGE_2 5 μM, respectively. The results demonstrated that the PGE_2-induced production of IL-6 by macrophages and IFN-γ by thymocytes was remarkably downregulated in BAC-treated lupus compared to lupus control (Fig. 4). IL-6, a B cell

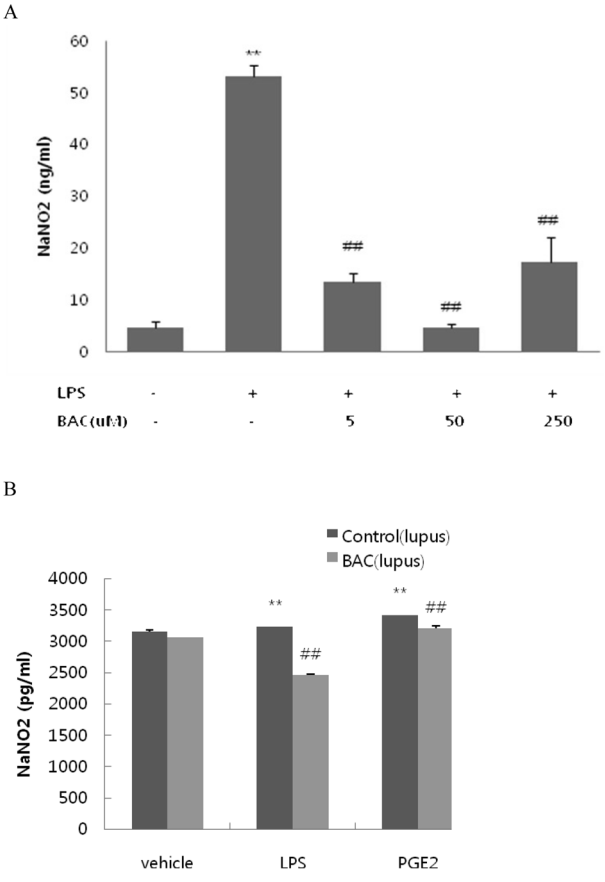

Fig. 5. Baicalin attenuates LPS-induced production of NO *ex vivo* in pristane-induced lupus mice.

A: RAW 264.7 cells were pretreated with various concentrations of BAC in complete media for 1 h and then cultured for 24 h in the presence or absence of LPS. B: The harvested peritoneal macrophages from BAC-treated lupus and lupus control were incubated for 24 h with LPS or PGE_2. The concentrations of NO in the supernatants were assayed at 540 nm using ELISA. Each value represents the mean ± S.D. Other legends and methods are the same as in Fig. 1.

differentiation factor, is correlated with the induction of differentiation to autoantibody-forming cells in lupus (Liang *et al*., 2006). It has been reported that IFN-γ is required for lupus-like disease and lymphoaccumulation in MRL-lpr mice (Balomenos *et al*., 1998) and that IFN-γ is associated with autoantibody production and immune complex-mediated organ injuries in lupus (Haas *et al*., 1998). Therefore, our findings suggest that BAC may ameliorate autoimmunity in lupus via inhibition of the PGE_2-mediated production of proinflammatory cytokines, such as IL-6 and IFN-γ.

Baicalin inhibited mitogen-induced production of NO by macrophages *ex vivo* in pristane-induced lupus mice – Lupus activity and inflammation have been associated with an increase in formed reactive nitrogen and the activity of inducible nitric oxide synthase (iNOS) (Oates and Gilkeson, 2006). Chen *et al.* (2001) have reported that baicalin inhibited iNOS gene expression. In this study, we examined whether BAC inhibits production of NO in pristane-induced lupus mice. Initially, RAW 264.7 cells were pretreated with various concentrations of BAC in complete media for 1 h and then cultured for 24 h in the presence or absence of LPS 1 μg/mL. These results demonstrate that BAC remarkably inhibited production of NO by RAW 264.7 cells in a concentration dependent manner (Fig. 5 A). In addition, the peritoneal macrophages from BAC-treated lupus, lupus control, and healthy control were cultured for 24 h in the presence or absence of LPS 5 μg/mL or PGE_2 5 μM. These data showed that the production of NO by macrophages was strongly attenuated in BAC-treated lupus compared to lupus control (Fig. 5 B). Therefore, these results suggest that BAC may inhibit production of NO in lupus macrophages.

Baicalin attenuated *in vivo* expression of splenic NKT cells in pristane-induced lupus mice – NKT cells have been reported to augment Th1-type immune responses and autoantibody secretion that contribute to lupus development in adult NZB/W mice (Zeng *et al*., 2003). NKT cells and innate immune B cells from lupus-prone NZB/W mice were shown to interact to generate IgM and IgG autoantibodies, leading to multiorgan injuries (Takahashi and Strober, 2008). In the present study, we measured the expression of CD3+NK1.1+ cells (NKT cells) in splenocytes from BAC-treated lupus, lupus control, and healthy control. As shown in Fig. 6, these data demonstrated that the splenic NKT cell expression was remarkably enhanced in lupus control compared to healthy mice, while reduced in BAC-treated lupus compared to lupus control. NKT cells have also been observed to be an important source of serum IFN-γ after the polyclonal activation of T cells *in vivo* (Mendiratta *et al*., 1997). Therefore, these observations suggest that BAC may attenuate the expression of NKT cells, leading to an inhibitory production of autoantibody and an overproduction of IFN-γ in lupus.

Baicalin downregulated *in vivo* activation of splenic Th cells in pristane-induced lupus mice – Activation of T cells in SLE contributes to the potentially dysregulatory function of T cells and autoantibody production (Portales-Perez *et al*., 1997; Takeno *et al*., 1997). Development and progression of lupus is determined by the type of Th responses inducing the generation of more or less pathogenic autoantibodies (Reininger *et al*., 1996). The percentage of *in vitro* expression of CD69, an activation marker on lymphoid cells, was increased in SLE cells

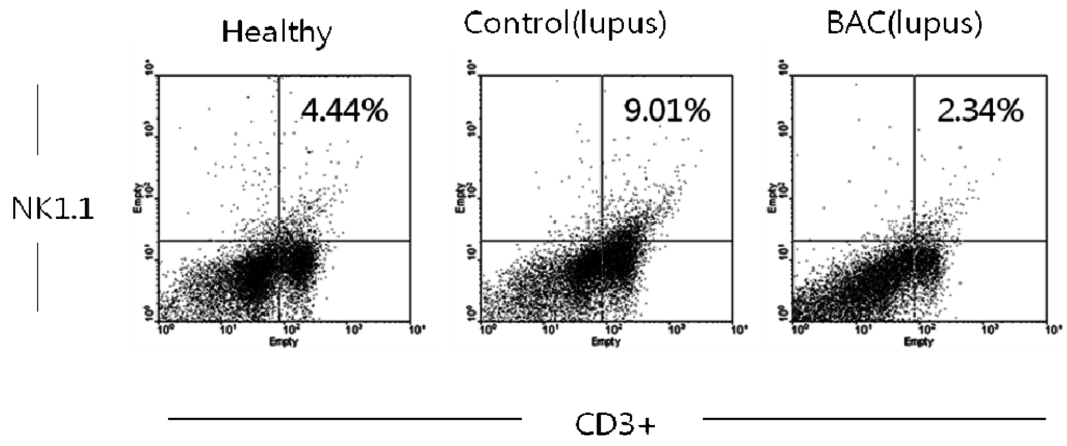

Fig. 6. Baicalin attenuates the splenic NKT cell expression *in vivo* in pristane-induced lupus mice.
The harvested splenocytes from BAC-treated lupus, lupus control, and healthy control were preincubated with anti-Fc receptor MAb 2.4G2. The cells were stained with FITC-labelled anti-CD3 and PE-labelled anti-NK1.1. The cells were incubated for 30 min in the dark, fixed with 1% paraformaldehyde, and analyzed using flow cytometry.

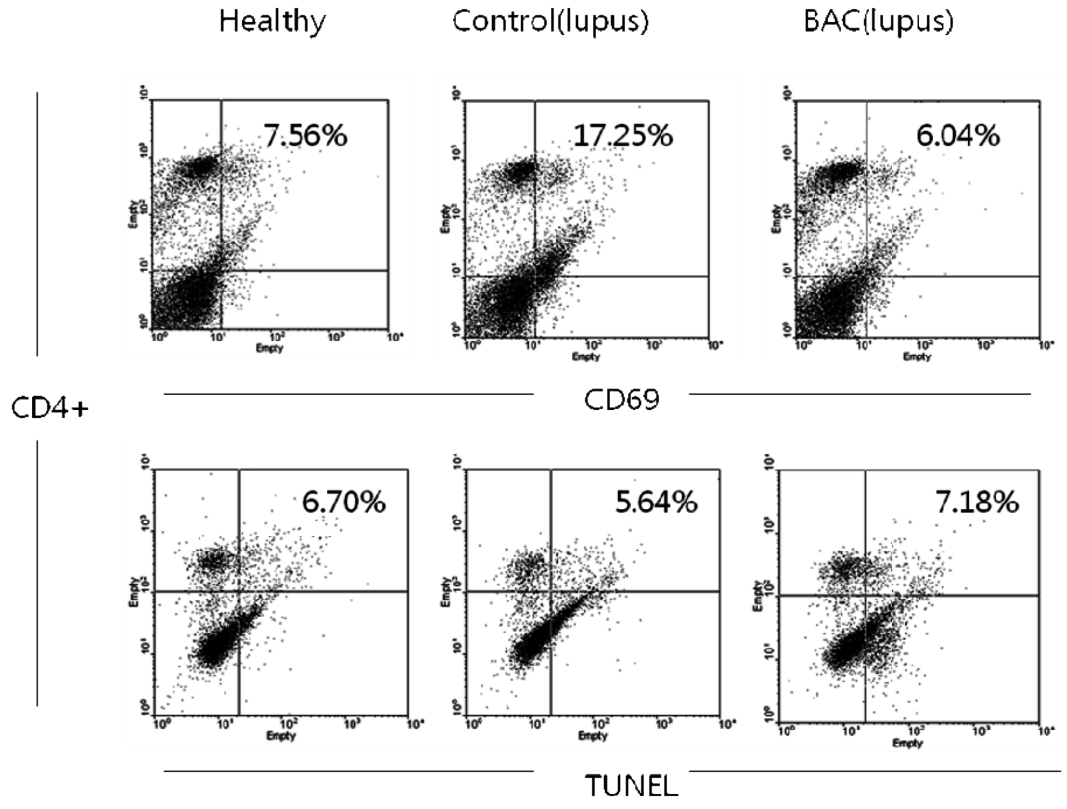

Fig. 7. Baicalin downregulated *in vivo* activation of splenic Th cells in pristane-induced lupus mice.
Splenocytes from BAC-treated lupus, lupus control, and healthy control were preincubated with anti-Fc receptor MAb 2.4G2. A: The harvested splenocytes were stained with FITC-labelled anti-CD69 and PE-labelled anti-CD4. B: The harvested splenocytes were stained with PE-labelled anti-CD4 and apoptosis of CD4+ T cells was measured using TUNEL assay. Other legends and methods are the same as in Fig. 6.

(Crispin *et al*., 1998). Pristane-induced autoimmune diseases have been reported to be CD4+ T-cell dependent (Stasiuk *et al*., 1997). CD4+ T cells expressing antigen CD69 in murine lupus played a possible abnormal regulatory role for cytokine imbalance (Ishikawa *et al*., 1998). The present study was examined whether BAC

downregulates *in vivo* overexpression of CD69 on splenic CD4+ T cells in pristane-induced lupus mice. As shown in Fig. 7, these results demonstrated that the expression of CD69+CD4+ cells in splenocytes was downregulated in BAC-treated lupus compared to lupus control.

Activated T cells in lupus have been reported to be resistant to apoptosis (Xu *et al*., 2004; Chae and Shin, 2007). Down-regulation of spontaneous apoptosis and activation-induced cell death of T cells in SLE have resulted in the enhanced help on B cells and loss of potentially regulatory functions (Kyttaris *et al*., 2005). In the present study, the apoptosis of splenic CD4+ T cells was measured in BAC-treated lupus, lupus control, and healthy control using TUNEL assay. This observation demonstrated that the apoptosis of CD4+ T cells in splenocytes decreased in lupus control compared to healthy control, while upregulated in BAC-treated lupus compared to lupus control (Fig. 7). Therefore, these results suggest that BAC may induce a decrease in activation of Th cells and an upregulation of Th cell apoptosis in lupus, leading to the amelioration of dysregulation of overactive T cells. In addition, human lupus T cells have been reported to resist inactivation and escape death by upregulating COX-2 (Xu *et al*., 2004). The immune cells of lupus mice exhibited hyperexpressed COX-2 and COX-2 inhibitors, resulting in increased cell apoptosis, decreased autoantibody production, and inhibition of the T cell response to the major lupus autoantigen (Zhang *et al*., 2007). As shown in Fig. 3, BAC inhibited LPS-induced production of PGE_2 by peritoneal macrophages in pristane-induced lupus mice, indicating that the inhibitory effect of BAC on the PGE_2 production may contribute to an increase in apoptosis and a decrease in activation of Th cells in BAC-treated lupus. Further studies are needed to determine the exact mechanism by which BAC induces a decrease in the activation of Th cells and an upregulation of Th cell apoptosis in lupus.

In conclusion, these findings indicate that BAC may attenuate the development of lupus autoimmunity and disease activity via the inhibition of production of major inflammatory mediators, such as IL-6 and PGE_2, and amelioration in dysregulation of T cells.

Acknowledgements

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