Natural Product Sciences
15(4) : 222-228 (2009)

Bamboo Culm Extract Downregulated Activation of NKT- and B- cells and Production of IL-6 in Pristane-Induced Lupus Mice

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Abstract – Lupus is characterized by immunoregulatory abnormalities between T- and B-cells leading to autoantibody production and multiorgan injuries. We investigated whether bamboo culm extract (BC) ameliorates aberrant activation of T cells and B cells and attenuate production of IL-6 in pristane-induced lupus mice. Lupus was induced by *i.p.* a single injection of 0.5 ml of pristane in female BALB/c mice, which, later about 4 months, were used as a lupus model. The pristane-induced lupus mice and healthy mice were injected *i.p*. with BC 5 μl/kg or PBS once a day for 3 weeks. These results demonstrated that BC significantly decreased levels of serum and BAL IL-6 and production of IL-6 by macrophages with/without LPS, and downregulated expression of NKT cell and CD86+ CD45R/B220+, but not CD80+CD45R/B220+ and CD69+CD4+ in the splenocytes in pristane-induced lupus mice. Moreover, BC greatly increased Con A-stimulated production of IL-6, IL-10 and IFN-γ by splenocytes obtained from pristane-induced lupus mice. Therefore, our findings suggest that BC may ameliorate lupus pathogenesis in pristane-induced lupus mice via downregulation of aberrant activation of NKT cells and B cells and inhibition of production of IL-6.
Keywords – Bamboo culm, lupus, IL-6, CD86, NKT cell

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by abnormal immunoregulation between T- and B- cells. T cell tolerance loss, B cell hyperactivity, and overproduction of proinflammatory cytokines in lupus result in autoantibody production and multiorgan injuries (Kyttaris *et al*., 2005; Nagy *et al*., 2005; Chae and Shin, 2007). Overproduction of proinflammatory cytokines, such as IL-6, IL-10, and IFN-γ, was associated with autoantibody production and multiple organ injuries in lupus (Llorente *et al*., 1995; Theofilopoulos *et al*., 2001; Chun *et al*., 2007). IL-6, a biomarker in SLE, was associated with disease activity, B lymphocyte hyperactivity, and autoantibody production in lupus (Richards *et al*., 1998; Davas *et al*., 1999; Ishihara and Hirano, 2002). Anti-IL-6 monoclonal antibody showed to inhibit autoimmune responses in a murine model of SLE (Liang *et al*., 2006).

Aberrant T cell function associated with CD4+ T cells provides overproduction of proinflammatory cytokines and helping B cells produce autoantibodies in lupus (Reininger *et al*., 1996; Takeuchi *et al*., 2005). Hyperactivity of T cells in SLE leads to potentially decreased regulatory function (Crispin *et al*., 1998). It has been reported that CD4+ T cells expressing early activation of CD69 in murine lupus play a possible abnormal regulatory role in cytokine imbalance (Ishikawa *et al*., 1998). It has also been reported that activation of natural killer T cells (NKT cells) augmented Th1-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). CD80 and CD86, markers of APC cell activation, play important roles in most features of systemic lupus, including autoantibody production and renal pathology (Nakajima *et al*., 1995; Kinoshita *et al.,* 2000).

Bamboo, which has been used as folk remedies traditionally in the Orient, has antioxidant, anti-inflammatory, anti-fatigue, anti-tumor, and neuroprotective activity (Zhang *et al*., 2006; Ito *et al*., 2007; Lee *et al.,* 2008; Seki *et al.,* 2008). Oxidative stress has been known to play an important role in many autoimmune pathological conditions and in inflammatory diseases (Chapple, 1997). Lee *et al*. (2008) reported that bamboo culm extract ameliorates cell adhesion molecule

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expression and NF-κB activity through suppression of the oxidative stress. However, effect of bamboo culm on the lupus-like chronic inflammatory autoimmunity remains still unknown.

Pristane induces large amount of IL-6 production by murine peritoneal macrophages, on which anti-DNA antibody production is dependent in pristane-induced lupus mice (Shacter *et al*., 1992; Satoh and Reeves, 1994; Richards *et al*., 1998). In the present study, our results demonstrated that BC downregulated aberrant activation of NKT cells and B cells and inhibited production of IL-6 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 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*. 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), and then, later about 4 months, were used as a pristane-induced lupus model or healthy controls (Shacter *et al*., 1992).

Preparation of plant extracts – Bamboo culm (*Phyllostachys bambusoides*; Gramineae, over 3 years old) was obtained from Damyang Jeonnam (Korea). The bamboo culm was washed and broken into about 5 cm fragments. The mineral material mixture that was composed of elvan, germanium, sericite, jade, and amethyst was heated at 250 - 300 °C to induce far-infrared radiation. The bamboo culm fragments were exposed to the far-infrared radiation for 6 h and then extracted. The bamboo culm extract (BC) was used as a stock solution, which was yielded 4.8 ml from fresh bamboo culm 1.2 kg.

Administration of BC – The pristane-induced lupus mice and healthy mice were injected *i.p.* with BC 5 μl/kg or PBS once a day for 3 weeks: PBS-treated healthy mice, PBS-treated pristane-induced lupus mice (lupus control), and BC-treated pristane-induced lupus mice.

Preparation of serum – Bloods were harvested from hearts under anesthetics in BC-treated lupus, lupus control, and healthy mice. 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 °C for cytokine assay.

Preparation of bronchoalveolar lavage fluids – The lungs were removed under anesthetics from BC-treated lupus, lupus control, and healthy mice. Bronchoalveolar lavage (BAL) fluids were performed twice in a total volume of 1 ml of PBS through an intratracheal polyethylene tube attached to a 1 ml-syringe and centrifuged. The BAL fluids were collected and stored at ≤ −20 °C for cytokine assay.

Preparation of lymphoid cells – Splenocyte suspensions were prepared from BC-treated lupus, lupus control, and healthy 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 in RPMI 1640 complete medium supplemented with 10% fetal bovine serum (FBS) and penicillin (10 U/ml)-streptomycin (10 μg/ml) at density of 1×10^7 cells/ml.

Cell culture – Splenocytes (each 1×10^6 cells/ml) and peritoneal macrophages (0.5×10^6 cells/ml) from healthy and pristane-induced lupus mice were cultured for 20 h in the presence or absence of LPS 10 μg/ml or Con A 2 μg/ml for 24 h 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 IL-6 in plasma, BAL and peritoneal macrophage supernatants and IL-6, IL-10, and IFN-γ in splenocyte 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.

Flow cytometry analysis – Splenocytes from BC-treated lupus, lupus control, and healthy mice 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-CD80, FITC-labelled anti-CD86, PE-labelled anti-CD69, PE-labelled anti-NK1.1, and PE-labelled anti-CD45R/B220. 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).

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

BC attenuated *in vivo* and *ex vivo* production of IL-6 in pristane-induced lupus mice – Pristane-primed female BALB/c mice develop a lupus-like syndrome characterized by lupus-specific autoantibodies starting 1-2 mo and severe immune complex-mediated glomerulonephritis starting 4 - 6 mo after pristane treatment (Satoh and Reeves 1994; Satoh *et al*., 1995). In the present study, lupus was induced by *i.p.* a single injection of 0.5 ml of pristane in female BALB/c mice, which, 4 mo after pristane treatment, were used as a lupus model. BC 5 μl/kg with *i.p.* injection once a day for 3 weeks was determined as an appropriate dose of BC associated with anti-inflammatory effect according to results of our previous experiment that vehicle, 10 μl/kg, 40 μl/kg or 160 μl/kg of BC were administrated orally once a day for 10 days in ICR mice and then 50 μl/kg of LPS was injected *i.p.* to induce acute inflammation (data not shown).

IL-6, a B cell differentiation factor, plays an important role in induction of differentiation to autoantibody-forming cells and anti-DNA antibody production in lupus (Richards *et al*., 1998; Ishihara and Hirano, 2002). Anti-IL-6 monoclonal antibody showed to inhibit autoimmune responses in a murine model of SLE (Liang *et al*., 2006). We evaluated whether BC 5 μl/kg inhibits IL-6 production in pristane-induced lupus mice, because bamboo culm extract inhibited NF-κB activity through suppression of the oxidative stress (Lee *et al.,* 2008). Here, BC or PBS was injected *i.p.* once a day for 3 weeks in the lupus and healthy mice. In the present study, our results demonstrated that levels of serum and BAL IL-6 were remarkably attenuated in BC-treated lupus mice compared to lupus control (Fig. 1). It has been reported that elevation of serum IL-6 is correlated with disease activity in lupus and elevation of BAL IL-6 contributes to lung injury in lupus (Davas *et al*., 1999; Chowdhary *et al*., 2007). Also, pristane induces large amount of IL-6 production by murine peritoneal macrophages, which anti-DNA antibody production is dependent on in pristane-induced lupus mice (Shacter *et al*., 1992; Richards *et al*., 1998). To investigate effect of BC on the production of IL-6, peritoneal macrophages from BC-treated lupus mice, lupus control and healthy mice were cultured for 20 h with/without LPS. We observed that BC

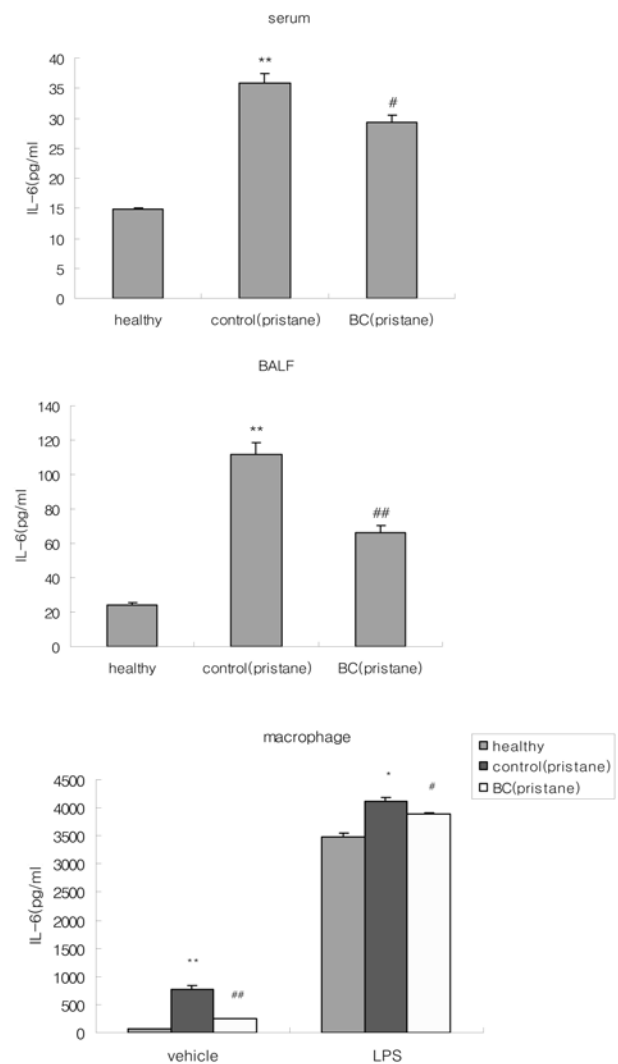

Fig. 1. Regulatory effect of BC on the production of IL-6 in pristane-induced lupus mice.
Bloods were harvested from hearts under anesthetics in BC-treated lupus, lupus control, and healthy mice. The BAL fluids (BALF) were harvested from lungs removed under anesthetics from BC-treated lupus mice, lupus control, and healthy mice. Peritoneal macrophages (1×10^6 cells/ml) from BC-treated lupus mice, lupus control, and healthy mice were cultured for 20 h in the presence or absence of LPS. Each value represents the mean ± S.E. from 5-7 mice per each group. * ($p < 0.05$) and ** ($p < 0.01$): Significantly different from the value in healthy mice. # ($p < 0.05$) and ## ($p < 0.01$): Significantly different from the value in pristane-induced lupus controls.

treatment attenuated production of IL-6 by peritoneal macrophages with/without LPS in pristane-induced lupus mice. Therefore, these results indicate that BC inhibited *in vivo* and *in vitro* production of IL-6 in pristane-induced lupus mice.

BC increased Con A-stimulated production of cytokines by splenocytes in pristane-induced lupus mice – T- and B- cell dysfunction in SLE includes

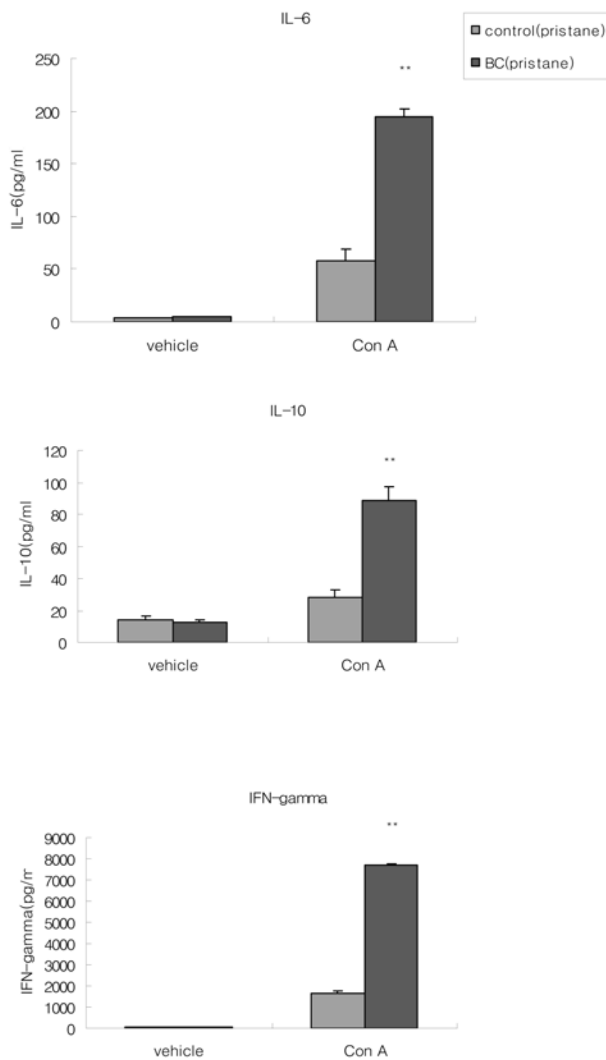

Fig. 2. Upregulatory effect of BC on the Con A-induced production of splenic cytokines in pristane-induced lupus mice. Splenocytes (1×10^6 cells/ml) from BC-treated lupus mice and lupus control were cultured for 24 h in the presence or absence of Con A. Each value represents the mean ± S.E. ** ($p < 0.01$): Significantly different from the value in each Con A-treated immune cells in lupus control.

defective *in vitro* proliferative responses to several stimuli. Con A is known to mitogenically activate T lymphocytes via the antigen receptor. Several reports demonstrated that the *in vitro* responses of lupus immune cells to mitogen as well as the *in vivo* cellular responses of lupus patients to antigen were deficient (Takeuchi *et al*., 2005). The induction of CD69 expression, an activation marker on lymphoid cells, by phytohemagglutinin has also been reported to be significantly lower in T cells from SLE patients (Portales-Perez *et al*., 1997). In this study, we examined Con A-activated functions of T cells on the production of cytokines by splenocytes from BC-treated lupus mice and lupus control. Splenocytes from BC-treated lupus mice and lupus control were cultured for 24 h in the presence or absence of Con A. As shown in Fig. 2, it was observed that Con A-stimulated production of splenic IL-6, IL-10, and IFN-γ was much greater in BC treated lupus mice compared to lupus control, indicating that BC may up-regulate deficient immune responses of lupus T lymphocytes to mitogen.

Effect of BC on the *in vivo* expression of splenic CD69+CD4+ in pristane-induced lupus mice – Hyperactivity of T cells in SLE leads to potentially decreased regulatory function (Kyttaris *et al*., 2005). SLE exhibits overactivation of T cells and the increased percentage of *in vitro* expression of CD69 on lymphoid cells (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 activation of CD69 in murine lupus play a possible abnormal regulatory role for cytokine imbalance (Ishikawa *et al*., 1998). In the present study, we examined whether BC downregulates *in vivo* overexpression of CD69 on splenic CD4+ T cells in pristane-induced lupus mice. As shown in Fig. 3, these results demonstrated that BC didn't affect overexpression of CD69+CD4+ cells on splenocytes in pristane-induced lupus mice, suggesting that BC may not affect hyperactivation of Th responses with expression of CD69+.

BC attenuated *in vivo* expression of splenic NKT cell in pristane-induced lupus mice – Autoreactive T cell clones from patients with SLE support polyclonal autoantibody production (Takeno *et al*., 1997). It has been reported that abnormal activation of natural killer T cells (NKT cells) augments autoantibody secretion that contribute to lupus development in adult NZB/W mice (Zeng *et al*., 2003) and that NKT cells and innate immune B cells from lupus-prone NZB/W mice interact to generate IgM and IgG autoantibodies, leading to multiorgan injuries (Takahashi and Strober, 2008). In this study, our results showed that NKT cell expression on splenocytes were remarkably enhanced in pristane-induced lupus mice compared to healthy mice (Fig. 4), indicating that pristane-induced 4-mo lupus mice may have developed lupus pathogenesis and B cell hyperactivity leading to autoantibody production. It was also observed that NKT cell expression was greatly downregulated in BC-treated lupus mice compared to lupus control. Therefore, our findings indicate that BC may decrease autoantibody production in pristane-induced lupus mice via downregulation of NKT cell expression.

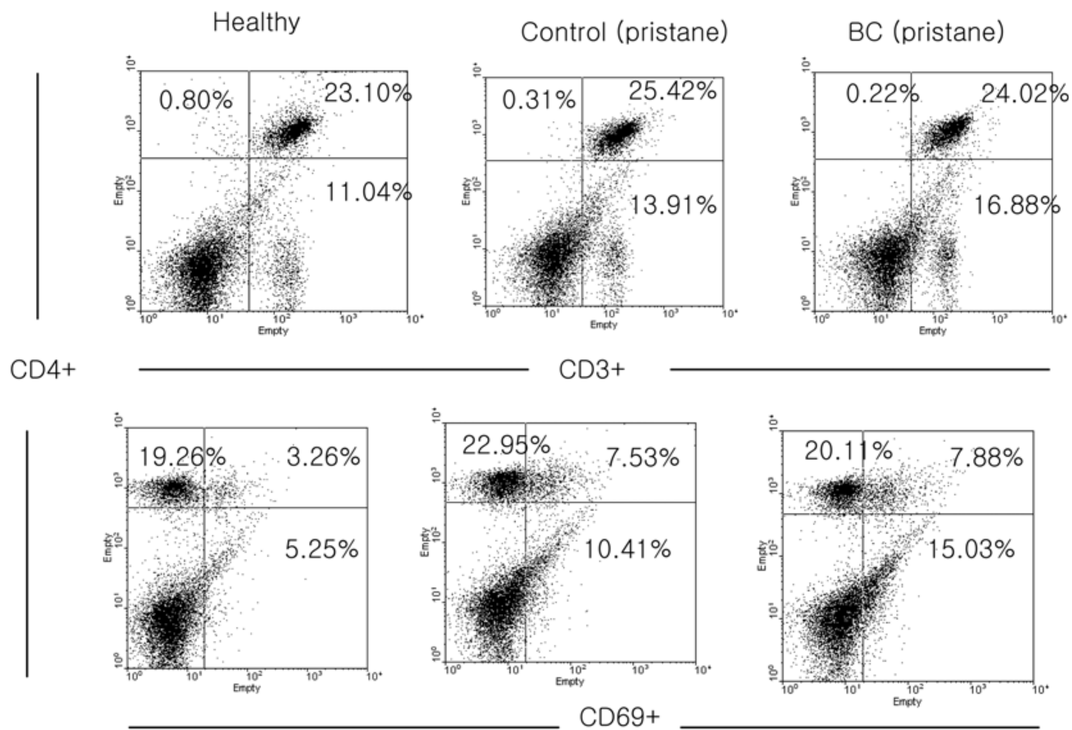

Fig. 3. Downregulatory effect of BC on the expression of CD69+CD4+ T cells on splenocytes in pristane-induced lupus mice.
The harvested splenocytes from BC-treated lupus and lupus control were preincubated with anti-Fc receptor MAb 2.4G2. The cells were incubated for 30 min in the dark, fixed with 1% paraformaldehyde, and analyzed using flow cytometry. Other legends and methods are the same as in Fig. 1.

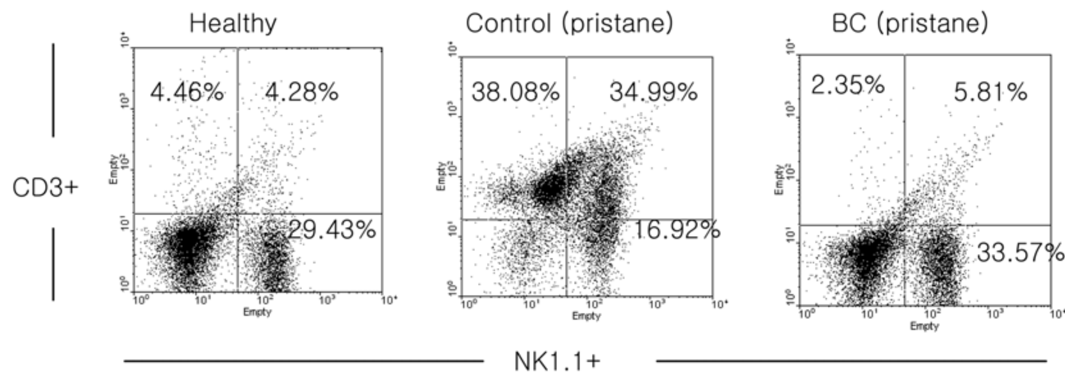

Fig. 4. Downregulatory effect of BC on the expression of NKT cells on the splenocytes in pristane-induced lupus mice.
The harvested splenocytes from BC-treated lupus, lupus control, and healthy mice were preincubated with anti-Fc receptor MAb 2.4G2. The cells were stained with FITC-labelled anti-CD3 and PE-labelled anti-NK1.1. Other legends and methods are the same as in Fig. 3.

Effect of BC on the *in vivo* overexpression of CD80 and CD86 on the splenic B cells in pristane-induced lupus mice – Lupus is characterized by humoral immune hyperactivity leading to autoantibody production through dependence on T cells. The CD80 and CD86 antigens are markers of APC cell activation including B cells. Kinoshita *et al.* (2000) demonstrated that costimulation by CD80 and CD86 is required for autoimmune disease in MRL-Faslpr mice. Nakajima *et al*. (1995) reported that autoantibody production was dependent on CD86 costimulatory molecule and treatment with CD86 monoclonal antibodies after the onset of lupus resulted in a significantly prolonged survival with reduction of autoantibody production. In this study, these results showed that expression of splenic CD86+CD45R/B220+ and CD80+CD45R/B220+ BC was remarkably enhanced in pristane-induced lupus mice (Fig. 5). In addition, BC reduced expression of CD86 in splenic B cells but not

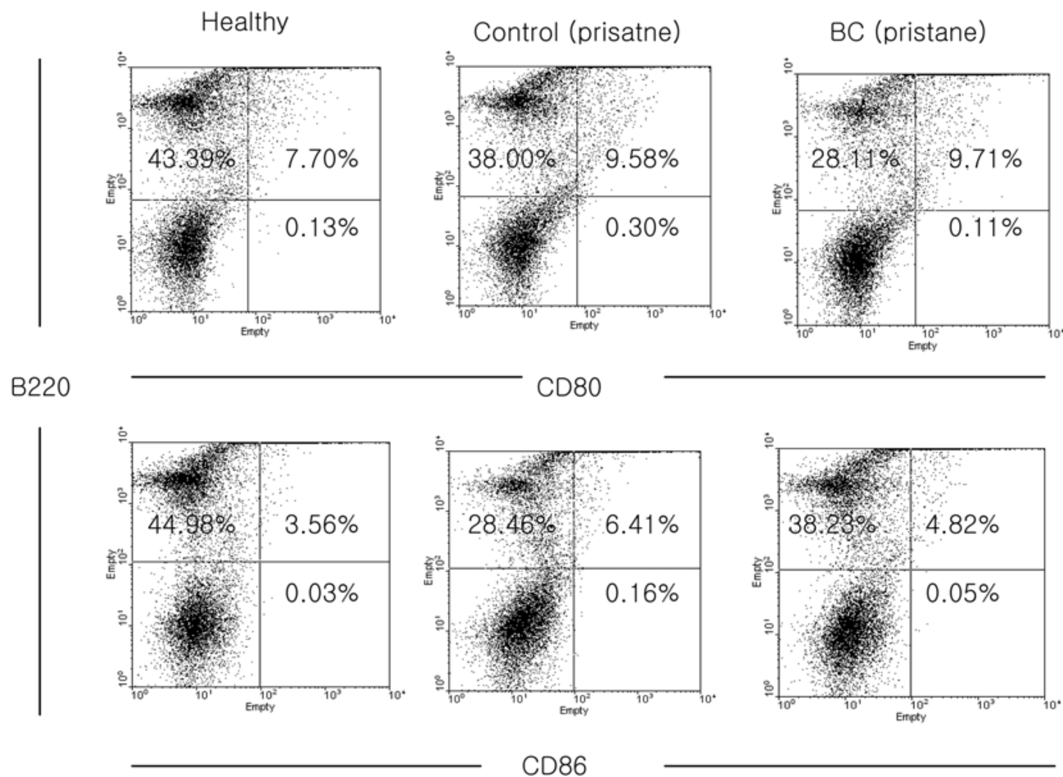

Fig. 5. Effect of BC on the expression of costimulatory molecules on the splenic B cells in pristane-induced lupus mice. The harvested splenocytes from BC-treated lupus, lupus control, and healthy mice were preincubated with anti-Fc receptor MAb 2.4G2. The cells were stained with FITC-labelled anti-CD80 or FITC-labelled anti-CD86 and PE-labelled anti-CD45R/B220. Other legends and methods are the same as in Fig. 3.

CD80 in pristane-induced lupus mice, suggesting that BC may attenuate B cell hyperactivity and autoantibody production through downregulation of expression of CD86 on B cells in lupus.

In conclusion, our findings indicate that BC may ameliorate pathogenic autoimmunity in pristane-induced lupus mice via inhibition of IL-6 production and downregulation of activation of NKT cells and B cells.

Acknowledgements

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (the Center for Healthcare Technology Development, Chonbuk National University, Jeonju 561-756, Republic of Korea) and Woosuk University (2009).

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Received November 12, 2009
Revised December 14, 2009
Accepted December 14, 2009