Section: Animal Cell Technology

Lipopolysaccharide-stimulated activation of murine DC2.4 cells is attenuated by *n*-butylidenephthalide through suppression of the NF-κB pathway

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Abstract

Modulation of dendritic cell (DC) fate and function may be one practicable approach for the treatment of inflammatory and autoimmune diseases. The natural compound *n*-butylidenephthalide (BP) derived from *Angelica sinensis*. The aim of this study was to evaluate the potential for BP to modulate lipopolysaccharide (LPS)-stimulated activation of cultured murine DC2.4 cells. BP (40 μg/ml) significantly decreased the secretion of interleukin-6 and tumor necrosis factor-α by LPS-stimulated DC2.4 cells ($P < 0.01$). LPS-induced major histocompatibility complex class II ($P < 0.05$), CD86 ($P < 0.01$) and CD40 ($P < 0.01$) expression on DC2.4 cells was also inhibited by BP. The endocytic capacity of LPS-stimulated DC2.4 cells was increased by BP ($P < 0.01$). The antigen-presenting capacity of LPS-stimulated DC2.4 cells was decreased by BP ($P < 0.05$). Moreover, we confirmed BP attenuates the responses of LPS-stimulated activation of DCs via suppression of NF-κB-dependent pathways.

Keywords *Angelica sinensis*; *n*-Butylidenephthalide; Dendritic cells; Immunomodulation; Nuclear factor-kappa B

Introduction

Dendritic cells (DCs) are the most important professional antigen-presenting cells. In the innate defenses, DCs can use pathogen-associated molecular patterns (PAMPs) that are regular constituents of bacteria and viruses to recognize microbial components. One PAMP, lipopolysaccharide (LPS) derived from the cell walls of Gram-negative bacteria, associates with LPS-binding protein (LBP) and CD14 and then specially binds to Toll-like receptor 4 (TLR4) on DCs. After TLR4 engagement, DCs go through maturation process, including secretion of proinflammatory mediators (e.g., IL-6 and TNF-α), up-regulation of surface molecules (e.g., MHC class II, CD40 and CD86), loss of endocytotic/phagocytic function, and acquisition of the capacity to migrate to secondary lymphoid organs, which enhances their antigen-presenting competence and initiates specific T cell immune responses (Banchereau et al. 2000; Reis e Sousa 2006).

Engagement of TLR4 activates several intracellular signaling pathways, including IκB kinase (IKK)-nuclear factor-κB (NF-κB) pathway and three mitogen-activated protein kinase (MAPK) pathways: c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) 1 and 2, and p38, which coordinate the induction of various genes related to DC maturation (Guha & Mackman 2001; Kaisho & Tanaka 2008; Lu et al. 2008).

DCs are key modulators in inflammation and immunity, controlling their maturation may be a useful approach for treating inflammatory and autoimmune diseases (Figdor et al. 2004). Therefore, an active area of research is the manipulation of DCs as pharmacological targets to screen novel biological modifiers of immune responses (Chen et al. 2009; Kim & Jobin 2005; Liu et al. 2007; Mitsui et al. 2010; Rhule et al. 2008; Yu et al. 2009). *Angelica sinensis* (AS), also called dong quai, is one of the most commonly used traditional Chinese medicine. It has been showed to

include a number of anti-inflammatory substances (Chao et al. 2009). Chao et al. (2010) reported that the components of AS ethyl acetate extract including ferulic acid and Z-ligustilide have anti-inflammatory activity through inhibition of the NF-κB pathway of macrophage on murine model.

The compound *n*-butylidenephthalide (BP), derived from the chloroform extract of AS, has been examined for its antispasmodic action (Ko 1980), anti-platelet effect (Teng et al. 1987), antianginal effect (Ko et al. 1998), anti-atherosclerotic treatment (Mimura et al. 1995), and tumor suppression effects (Lin et al. 2008; Tsai et al. 2005; Wei et al. 2009). However, no study has described immunomodulatory effects of BP. In the current study, we hypothesized that BP inhibits DC maturation. To test the hypothesis, the effects of BP on the maturation of DC and the possible molecular mechanism were studied.

Materials and Methods

Chemicals

Synthesized BP (mol wt, 188.23, 95% purity) was purchased from Lancaster Synthesis Ltd. (Newgate Morecambe, UK) and dissolved in dimethyl sulfoxide (DMSO) to 100 mg/ml and stored at -20°C as a master stock solution. Final concentrations of DMSO in all BP-treated cultures were 0.1% (v/v). RPMI 1640-L-glutamine medium, fetal bovine serum (FBS), sodium pyruvate, penicillin-streptomycin, nonessential amino acid, and 2-mercaptoethanol (2-ME) were purchased from Invitrogen (Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), LPS (from *Escherichia coli* 055:B5), mitomycin C and other chemicals were purchased from Sigma. Fluorescein isothiocyanate (FITC)-conjugated antibody to mouse MHC class II and CD86/B7-2 were purchased from GenWay Biotech (San Diego, CA, USA). Phycoerythrin (PE)-conjugated antibody to mouse CD40 was purchased from BD Biosciences (San Jose, CA, USA). The antibodies for IKKα/β, IκBα, JNK,

ERK1/2, and p38 and their phosphorylated forms were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody for mouse β-actin was purchased from Millipore (Billerica, MA, USA).

Cell culture

DC2.4 cells, an immature DC cell line established previously from the culture of C57BL/6 mice bone marrow progenitors (Shen et al. 1997), were kindly provided by Dr. C. L. Chu (Immunology Research Center, National Health Research Institutes, Taiwan). The cells were cultured in complete RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM nonessential amino acid, 100 mg streptomycin/ml, 100 U penicillin/ml, and 50 μM 2-ME at 37°C with 5% CO_2. Cells were passaged every 3 days and used for experimentation at 60–80% confluence.

MTT assay

Cell viability was analyzed by MTT assay. In brief, DC2.4 cells were plated in 96-well plates at 1×10^4 cells/well. After 24 h, the cells were treated with serially diluted BP and incubated for another 24 h. Final concentrations of DMSO in all BP-treated cultures were 0.1% (v/v). MTT solution was added to each well (500 μg/ml) and allowed to react with the cells for 1 h at 37°C. For the solubilization of formazan, media were discarded and DMSO was added to each well. The absorbance of the dissolved solutions was detected using a SpectraMax M2 Microplate Reader (Molecular Devices, Silicon Valley, CA, USA) at 570 nm.

Cell activation and treatment

According to the results of MTT assay, DC2.4 cells (1×10^6) were further pretreated with 20 or 40 μg BP/ml in 1 ml complete media in a 35-mm plate. Final concentrations of DMSO in all BP-treated cultures were 0.1%

(v/v). After 24 h incubation, the cells washed twice in phosphate-buffered saline (PBS), followed by stimulation with LPS (1 μg/ml) for another 24 h. Media and cells were collected for subsequent evaluation of DC activation and analysis of protein expression. Three replicates were included in each experiment.

Cytokine assay

Media of cultured DCs were harvested and kept at -80°C. The secretion of IL-6 and TNF-α in media of cultured cells was measured by enzyme-linked immunosorbent assay (ELISA) kits purchased from Invitrogen (Grand Island, NY, USA). The cytokine concentration was evaluated according to the manufacturer's protocol.

Flow cytometry

The expression of surface molecules on DC2.4 cells was analyzed by BD LSR II flow cytometry (BD Biosciences, San Jose, CA, USA). In brief, a total of 1×10^5 cells were collected and suspended in cold PBS containing 2% (v/v) FBS and 0.1% (w/v) sodium azide (NaN_3), blocked with rat IgG on ice for 15 min at 4°C, and then washed, followed by incubation with FITC-conjugated anti-mouse MHC class II antibody, CD86 antibody, or PE-conjugated anti-mouse CD40 antibody, or isotype controls for 40 min at 4°C in the dark. After incubation, the cells were washed and fixed in PBS containing 2% (w/v) paraformaldehyde and analyzed with FACSDiva software (BD Biosciences, San Jose, CA, USA). The data were collected for 10000 cells/sample.

Endocytosis assay

To analyze the endocytic capacity of DC, we incubated BP-pretreated, LPS-stimulated DCs (1×10^6) with 1 mg FITC-dextran (42 kDa, Sigma)/ml in the fresh medium at 37°C for 1 h. After incubation, cells

were washed twice with cold PBS buffer and analyzed by BD LSR II flow cytometry (BD Biosciences, San Jose, CA, USA). Control experiments were performed at 4°C for 1 h. The data were collected for 10000 cells/sample.

NF-κB assay

BP-pretreated DC2.4 cells were stimulated with LPS for 24 h and nuclear protein extracted using the Nuclear Extraction Kit (Affymetrix-Panomics, Santa Clara, CA, USA). The protein level of extracts was calculated using the RC DC Protein Assay Kit (Bio-Rad Life Science, Hercules, CA, USA). NF-κB p65 binding activity was determined using the Universal EZ-TFA Transcription Factor Assay Colorimetric kit according to the manufacturer's instructions (Millipore, Billerica, MA, USA). In brief, a double-stranded biotinylated oligonucleotide, containing the consensus sequence for NF-κB p65, was mixed with nuclear extract provided directly in the streptavidin-coated plate. Any inactive, unbound material was washed away and the bound NF-κB p65 detected with a specific primary antibody. A horseradish peroxidase (HRP)-conjugated secondary antibody was then used for detection, and NF-κB p65 specific binding was quantified by absorbance (450 nm) using a SpectraMax M2 Microplate Reader (Molecular Devices, Silicon Valley, CA, USA).

Western blot analysis

To isolate total protein extracts, cells were washed with cold PBS and lysed them in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% (v/v) Nonidet P-40; 0.25% (w/v) sodium deoxycholate; 1 μg leupeptin/ml; 1 μg pepstatin/ml; 1 μg aprotinin/ml; 1 mM phenylmethylsulfonyl fluoride; 1 mM ethylenediaminetetraacetic acid; 1 mM NaF, and 1 mM Na_3VO_4). The protein level of extracts was calculated using the RC DC Protein Assay Kit (Bio-Rad Life Science,

Hercules, CA, USA). One hundred micrograms protein was mixed with sample buffer (100 mM Tris-HCl, pH 6.8; 2% (w/v) sodium dodecyl sulfate [SDS]; 10% (v/v) glycerol; 5% (v/v) 2-ME; and 0.01% (w/v) bromophenol blue), incubated at 100°C for 5 min, and loaded onto a 10% SDS-polyacrylamide gel. Proteins separated on the gels were transferred onto polyvinylidene difluoride membranes (PerkinElmer, Boston, MA, USA). The membranes were incubated with 1× Tris-buffered saline (TBS) (Amresco, Solon, OH, USA) containing 0.1% (v/v) Tween 20 and 5% (w/v) nonfat dry milk for 30 min at room temperature and subsequently stained with antibodies against IKKα/β, IκBα, JNK, ERK1/2, and p38 and their phosphorylated forms. After overnight incubation at 4°C, the blots were washed three times with a wash buffer (TBS-0.1% [v/v] with Tween-20), 10 min each at room temperature, and then incubated for 1 h with a secondary HRP-conjugated goat-anti-mouse or goat-anti-rabbit antibody from PerkinElmer (Boston, MA, USA). The blots were washed five times and developed using the enhanced chemiluminescence procedure, as specified by the manufacturer (PerkinElmer, Boston, MA, USA). Signals were assessed using a UVP BioSpectrum Imaging System (Upland, CA, USA).

Allogenic mixed lymphocyte reaction (MLR)

Splenocytes from the spleens of BALB/c mice (8-week-old) were isolated. BP-pretreated, LPS-stimulated DC2.4 cells were harvested, washed, and incubated with 25 μg mitomycin C/ml for 30 min at 37°C. Finally, the cells were washed and diluted with the prepared splenocytes in a ratio of 1:50 and 1:100 in U-bottomed 96-well culture plates for 4 day. Cell proliferation was determined by MTT assay.

Statistical analysis

All statistical analyses are expressed as mean ± standard deviation (SD) from three independent tests. The number of replicates for each test is three. Differences between two means were determined by student's *t*-test. Values of $p < 0.05$ were determined to be statistically significant.

Results and discussion

In this study, the immunomodulatory effects of BP were examined using the immature murine DC line, DC2.4. An MTT assay was used to assess the cytotoxicity of BP, and the viability of these cells was not considerably changed by 24-h treatment with up to 40 μg BP /ml. Therefore, in the experiments following this assay, cells were incubated with BP at concentrations of up to 40 μg/ml (Fig. 1).

Fig. 1

Maturation of DCs includes changes in their morphological, phenotypic, and functional properties. IL-6 and TNF-α are two important proinflammatory cytokines that induce expression of costimulatory/accessory molecules on DCs and enhance DC interactions with T cells (Banchereau et al. 2000; Reis e Sousa 2006). DC2.4 cells were pretreated with 20 or 40 μg BP/ml for 24 h, followed by stimulation with LPS (1 μg/ml) for another 24 h. In unstimulated DC2.4 cells, 40 μg BP/ml did not change IL-6 and TNF-α release (Fig. 2). The treatment of LPS promoted a 10-fold ($P < 0.001$) and 3-fold increase ($P < 0.001$) in the secretion of IL-6 and TNF-α, respectively. BP appreciably decreased the secretion of both IL-6 and TNF-α in a concentration-dependent manner. At 40 μg BP/ml, LPS-stimulated IL-6 secretion decreased by about 32% ($P < 0.01$) and TNF-α secretion by about 22% ($P < 0.01$).

Fig. 2

Communications between surface molecules on DCs and their ligands are important for the full activation of T cells. The MHC Class II on the DCs presents the peptide of antigen to helper T cell. CD86 is the

most important molecule for the amplification of T-cell responses (Fujii et al. 2004). The interaction of CD40 and DCs results in an activation signal, increasing antigen presentation and the expression of other costimulatory molecules (Fujii et al. 2004). Because of the practical immunomodulatory effects of BP on LPS-stimulated cytokine secretion, the potential for BP to regulate expression of surface molecules on activated DCs was analyzed by flow cytometry. DC2.4 cells were pretreated with 20 or 40 μg BP/ml for 24 h, followed by stimulation with LPS (1 μg/ml) for another 24 h. The expression of MHC class II, CD40 and CD86, was assessed according to fluorescence intensity. LPS-stimulated DC2.4 cells considerably increased the expression of MHC class II, CD40 and CD86 ($P < 0.01$) (Fig. 3). Unstimulated DC2.4 cells treated with BP did not affect MHC class II, CD40 and CD86 expression. BP treatment (40 μg/ml) inhibited MHC class II ($P < 0.05$), CD40 ($P < 0.01$) and CD86 ($P < 0.01$) expression on DC2.4 cells stimulated with LPS (Fig. 3).

Fig. 3

The capacity for antigen uptake in immature DCs is efficient but lost on maturation (Banchereau et al. 2000; Reis e Sousa 2006). Thus, the suppression of DC maturation could be assessed by measuring endocytosis assay. We analyzed the antigen uptake of DC2.4 cells by using flow cytometry. BP-pretreated, LPS-stimulated DC2.4 cells incubated with FITC-dextran (1 mg/ml) at 37°C for 1 h were compared with a negative control incubated at 4°C for 1 h. Increased dextran uptake was observed in BP-pretreated (40 μg/ml) DC2.4 cells ($P < 0.01$) (Fig. 4a). BP markedly enhanced the endocytosis of DC2.4 cells.

Fig. 4a

To assess the effect of BP-treated DC2.4 cells on their Antigen-presenting capacity, we used MLR and splenocytes from BALB/c mice as responder T cells. As shown in Fig. 4b, BP (40 μg/ml)-pretreated, LPS-stimulated DC2.4 cells had low stimulatory capacity as compared

Fig. 4b

with untreated DC2.4 cells ($P < 0.05$), indicating that BP treatment inhibited the antigen-presenting capacity of stimulated DCs. This result was well-matched with those obtained in the endocytosis assay, because an increase in endocytosis is corresponding with a decrease in the antigen-presenting ability. Therefore, both phenotypic and functional maturation of DC2.4 cells was attenuated by BP treatment.

Translocation of NF-κB from the cytosol to the nucleus is essential for LPS-induced activation of DCs (Guha & Mackman 2001; Kaisho & Tanaka 2008; Lu et al. 2008). Given that BP inhibited the LPS-activation of DCs in our studies, we assessed the effects of BP on NF-κB p65 nuclear levels. DC2.4 cells were pretreated with 20 or 40 μg BP/ml for 24 h, followed by stimulation with LPS (1 μg/ml) for another 24 h. Samples were collected and the nuclear extracts analyzed for NF-κB p65 binding activity. LPS-stimulated DC2.4 cells had increased NF-κB p65 levels ($P < 0.001$) (Fig. 5). BP considerably inhibited NF-κB p65 activity in the LPS-stimulated DCs in a concentration-dependent manner (40 μg BP/ml, $P< 0.01$). Fig. 5

Maturation of DCs is involved in the activation of multiple signaling pathways (Guha & Mackman 2001; Kaisho & Tanaka 2008; Lu et al. 2008). To further investigate the effect of BP in IKK-NF-κB and/or MAPK pathways, we studied the phosphorylation levels of major signaling factors involved in the activation of DC by using Western blot analysis. As shown in Fig. 6, LPS strongly induced IKKα/β and IκBα phosphorylation and promoted IκBα degradation in DC2.4 cells, which is blocked in BP-treated cells in a concentration-dependent manner ($P < 0.01$). LPS-induced JNK, ERK1/2, and p38 phosphorylation was not blocked by BP treatment. Thus, BP regulated the activation of NF-κB signal transduction pathways that were involved in DC maturation through the direct blockade of IKK Fig. 6

activity.

In conclusion, our research showed that BP attenuates the responses of LPS-stimulated activation of DCs. The inhibitory effects of BP might result from suppression of IKK-NF-κB-dependent pathways. Therefore, modulation of DCs by BP may be an attractive strategy in the treatment of inflammatory and autoimmune diseases.

Acknowledgments

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Legends

Fig. 1

Effect of *n*-butylidenephthalide (BP) on the viability of mouse DC2.4 cells. DC2.4 cells were treated with serially diluted BP for 24 h. Final concentrations of DMSO in all BP-treated cultures were 0.1% (v/v). Cells viability were determined by MTT assay. The data represent the mean ± SD (n = 3). An asterisk (*) indicates significant differences between BP untreated control samples and BP treated samples ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$)

Fig. 2

Inhibitory effect of *n*-butylidenephthalide (BP) on interleukin (IL)-6 and tumor necrosis factor (TNF)-α secretion in lipopolysaccharide (LPS)-stimulated DC2.4 cells. DC2.4 cells were pretreated with 20 or 40 μg BP/ml. Final concentrations of DMSO in BP-treated cultures were 0.1% (v/v). After 24 h incubation, the cells were washed and followed by stimulation with LPS (1 μg/ml) for another 24 h. Media were collected and assayed for IL-6 and TNF-α concentrations using an enzyme-linked immunosorbent assay (ELISA) kit. The data represent the mean ± SD (n = 3). A hash (#) indicates significant differences between LPS-stimulated and unstimulated cells ($P < 0.001$); an asterisk (*) indicates significant differences between the LPS-stimulated control samples and BP-treated, LPS-stimulated samples ($^{*}P < 0.05$, $^{**}P < 0.01$)

Fig. 3

Inhibitory effect of *n*-butylidenephthalide (BP) on MHC class II, CD86 and CD40 expression in LPS-stimulated DC2.4 cells. DC2.4 cells were pretreated with 20 or 40 μg BP/ml. Final concentrations of DMSO in BP-treated cultures were 0.1% (v/v). After 24 h incubation, the cells were washed and followed by stimulation with LPS (1 μg/ml) for another 24 h.

The expression of MHC class II, CD86 and CD40 on DCs was determined by flow cytometry. The data represent the mean ± SD (n = 3). A hash (#) indicates significant differences between LPS-stimulated and unstimulated cells ($P < 0.01$); an asterisk (*) indicates significant differences between the LPS-stimulated control samples and BP-treated, LPS-stimulated samples ($^{*}P < 0.05$, $^{**}P < 0.01$)

Fig. 4

a Promoting effect of *n*-butylidenephthalide (BP) on antigen uptake in LPS-stimulated DC2.4 cells. DC2.4 cells were pretreated with 20 or 40 μg BP/ml. Final concentrations of DMSO in BP-treated cultures were 0.1% (v/v). After 24 h incubation, the cells were washed and followed by stimulation with LPS (1 μg/ml) for another 24 h. Endocytic activity of DC2.4 was assessed by flow cytometry after treatment with fluorescein isothiocyanate (FITC)-dextran (1 mg/ml) at 37°C for 1 h. The control was assessed after FITC-dextran treatment at 4°C. The data represent the mean ± SD (n = 3). A hash (#) indicates significant differences between LPS-stimulated and unstimulated cells ($P < 0.01$); an asterisk (*) indicates significant differences between the LPS-stimulated control samples and BP-treated, LPS-stimulated samples ($^{*}P < 0.05$, $^{**}P < 0.01$). **b** *n*-butylidenephtalide (BP) inhibits activation of naïve allogeneic T lymphocytes by DC2.4 cells. BP-pretreated, LPS-stimulated DC2.4 cells incubated with 25 μg mitomycin C/ml for 30 min at 37°C. Finally, the cells were washed and diluted with the prepared splenocytes in a ratio of 1:50 and 1:100 in culture plates for 4 day. The proliferation of T cells was assessed by MTT assay. DC2.4 cells were sufficient fixation by mitomycin C according to DC only control (data not shown). The values of untreated DCs served as control values in the calculation of % proliferation. The data represent the mean ± SD (n = 3). A hash (#) indicates significant differences between LPS-stimulated and unstimulated cells ($P < 0.01$); an

asterisk (*) indicates significant differences between the LPS-stimulated control samples and BP-treated, LPS-stimulated samples ($^{*}P < 0.05$).

Fig. 5

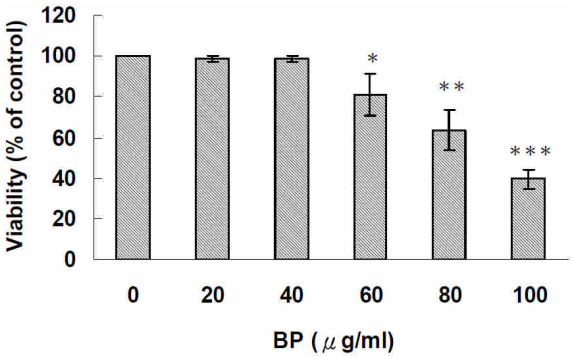
Inhibitory effect of *n*-butylidenephthalide (BP) on nuclear factor (NF)-κB p65 activity in LPS-stimulated DC2.4 cells. DC2.4 cells were pretreated with 20 or 40 μg BP/ml. Final concentrations of DMSO in BP-treated cultures were 0.1% (v/v). After 24 h incubation, the cells were washed and followed by stimulation with LPS (1 μg/ml) for another 24 h. Cells were lysed and the nuclear fraction determined for relative binding activity of NF-κB p65 using the Universal EZ-TFA Transcription Factor Assay Colorimetric kit. The data represent the mean ± SD (n = 3). A hash (#) indicates significant differences between LPS-stimulated and unstimulated cells ($P < 0.001$); an asterisk (*) indicates significant differences between the LPS-stimulated control samples and BP-treated, LPS-stimulated samples ($^{*}P < 0.05$, $^{**}P < 0.01$)

Fig. 6

Inhibitory effect of *n*-butylidenephthalide (BP) on IκB kinase α/β (IKKα/β) and IκBα phosphorylation in LPS-stimulated DC2.4 cells. DC2.4 cells were pretreated with 20 or 40 μg BP/ml. Final concentrations of DMSO in BP-treated cultures were 0.1% (v/v). After 24 h incubation, the cells were washed and followed by stimulation with LPS (1 μg/ml) for another 24 h. Phosphorylation levels of IKKα/β, IκBα, c-Jun N-terminal kinase, extracellular signal-related kinase, and p38 were analyzed by Western blot analysis. Expression of β-actin was used as an internal control. One of representative result from three independent experiments is shown. Right panel: Fold of control is relative to LPS-unstimulated cells. The data represent the mean ± SD (n = 3). A hash (#) indicates significant differences between LPS-stimulated and unstimulated cells ($P < 0.001$); an

asterisk (*) indicates significant differences between the LPS-stimulated control samples and BP-treated, LPS-stimulated samples ($^{*}P < 0.05$, $^{**}P < 0.01$)

Fig. 1


Viability (% of control)
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100
80
60
40
20
0
*
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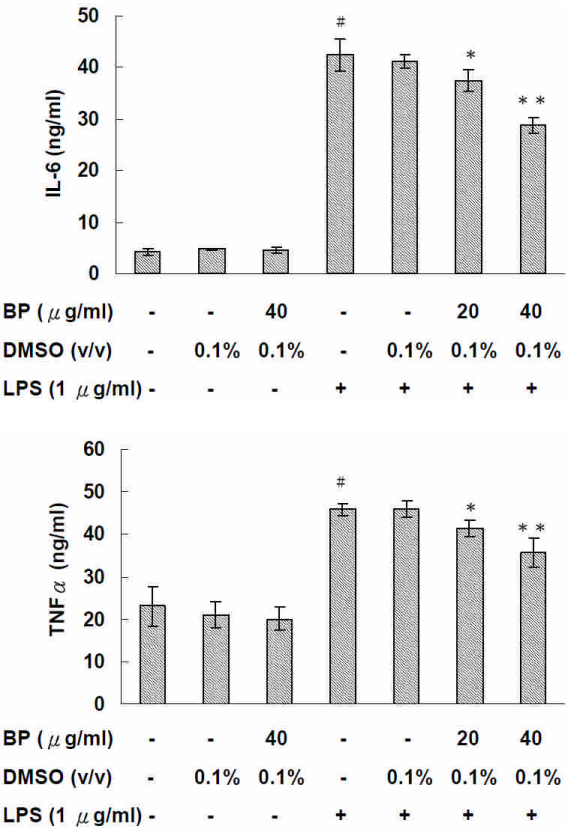
***
0
20
40
60
80
100
BP (μ g/ml)

Fig. 2


IL-6 (ng/ml)
50
40
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BP (μ g/ml) - - 40 - - 20 40
DMSO (v/v) - 0.1% 0.1% - 0.1% 0.1% 0.1%
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* *
BP (μ g/ml) - - 40 - - 20 40
DMSO (v/v) - 0.1% 0.1% - 0.1% 0.1% 0.1%
LPS (1 μ g/ml) - - - + + + +

Fig. 3

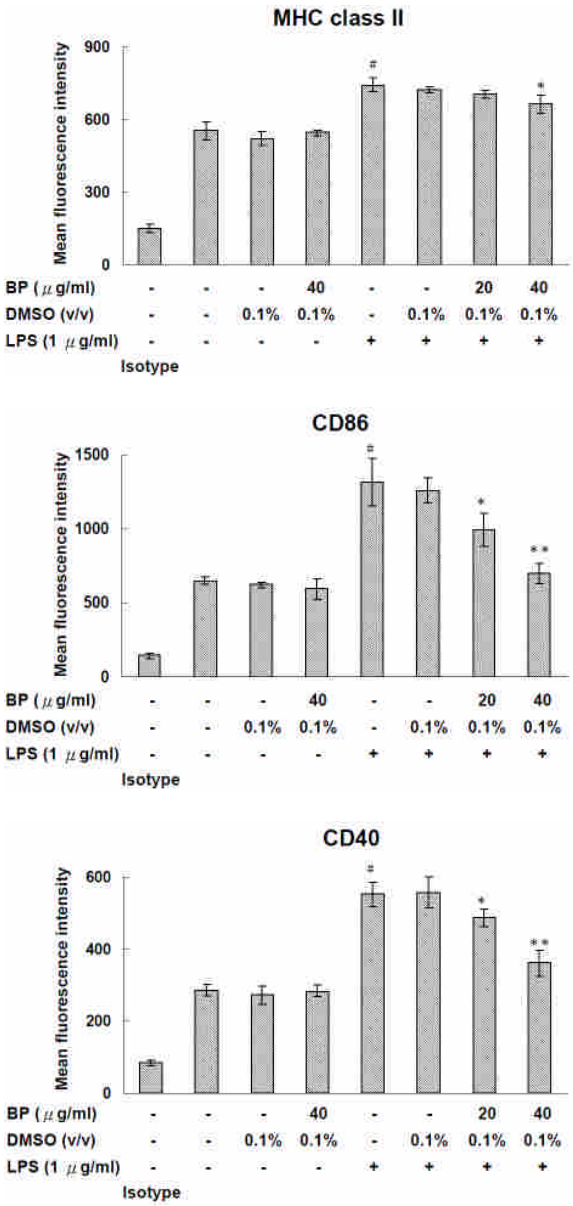
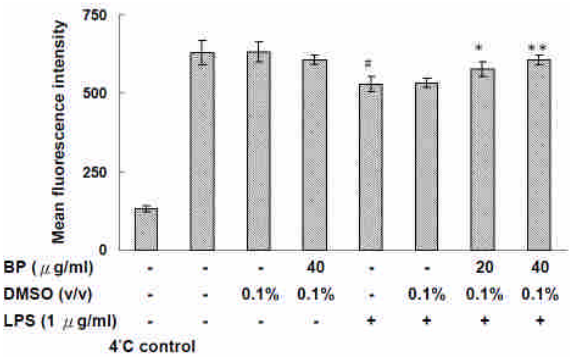
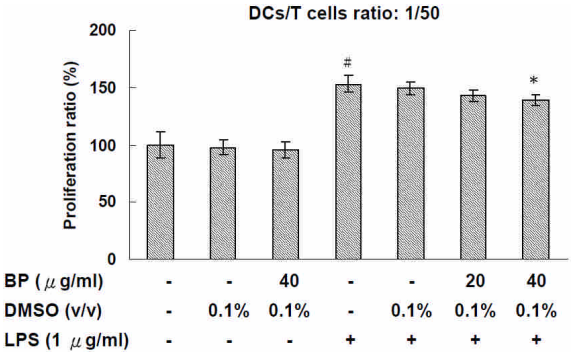

MHC class II
Mean fluorescence intensity
900
600
300
0
#
*
BP (μg/ml) - - - 40 - - 20 40
DMSO (v/v) - - 0.1% 0.1% - 0.1% 0.1% 0.1%
LPS (1 μg/ml) - - - - + + + +
Isotype
CD86
Mean fluorescence intensity
1500
1000
500
0
#
*
**
BP (μg/ml) - - - 40 - - 20 40
DMSO (v/v) - - 0.1% 0.1% - 0.1% 0.1% 0.1%
LPS (1 μg/ml) - - - - + + + +
Isotype
CD40
Mean fluorescence intensity
600
400
200
0
#
*
**
BP (μg/ml) - - - 40 - - 20 40
DMSO (v/v) - - 0.1% 0.1% - 0.1% 0.1% 0.1%
LPS (1 μg/ml) - - - - + + + +
Isotype

Fig. 4

(A)


Mean fluorescence intensity
750
500
250
0
#
*
**
BP (μg/ml) - - - 40 - - 20 40
DMSO (v/v) - - 0.1% 0.1% - 0.1% 0.1% 0.1%
LPS (1 μg/ml) - - - - + + + +
4°C control

(B)


DCs/T cells ratio: 1/50
Proliferation ratio (%)
200
150
100
50
0
#
*
BP (μg/ml) - - 40 - - 20 40
DMSO (v/v) - 0.1% 0.1% - 0.1% 0.1% 0.1%
LPS (1 μg/ml) - - - + + + +

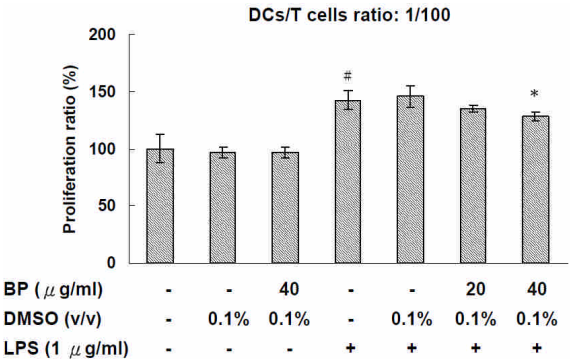

DCs/T cells ratio: 1/100
Proliferation ratio (%)
200
150
100
50
0
#
*
BP (μg/ml) - - 40 - - 20 40
DMSO (v/v) - 0.1% 0.1% - 0.1% 0.1% 0.1%
LPS (1 μg/ml) - - - + + + +

Fig. 5

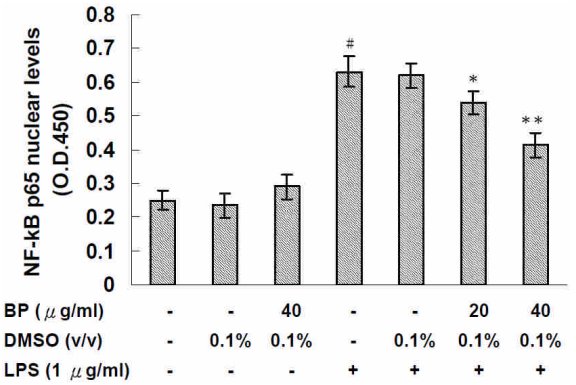
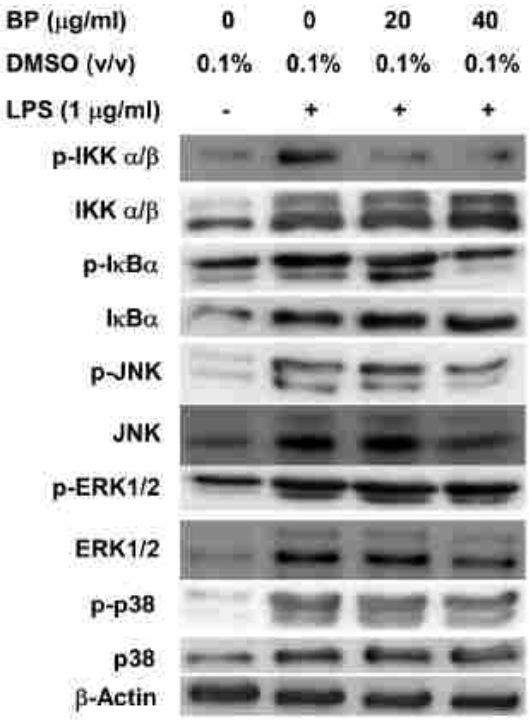

NF-kB p65 nuclear levels (O.D.450)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
#
*
**
BP (μg/ml) - - 40 - - 20 40
DMSO (v/v) - 0.1% 0.1% - 0.1% 0.1% 0.1%
LPS (1 μg/ml) - - - + + + +

Fig. 6



	Fold of control		
1.0	$33.6 \pm 9.1^{\#}$	$3.1 \pm 1.3^{**}$	$2.0 \pm 0.5^{**}$
1.0	$4.4 \pm 1.0^{\#}$	5.2 ± 0.4	5.3 ± 0.2
1.0	$4.3 \pm 1.0^{\#}$	$3.0 \pm 0.4^{*}$	$2.0 \pm 0.2^{**}$
1.0	$11.8 \pm 2.8^{\#}$	11.7 ± 3.0	12.5 ± 2.5
1.0	$12.6 \pm 3.0^{\#}$	12.8 ± 4.5	12.2 ± 2.0
1.0	$7.3 \pm 1.9^{\#}$	6.9 ± 2.0	6.3 ± 1.0
1.0	$1.5 \pm 0.2^{\#}$	1.3 ± 0.3	1.4 ± 0.3
1.0	$32.0 \pm 8.0^{\#}$	30.7 ± 7.7	29.0 ± 7.8
1.0	$72.3 \pm 19.7^{\#}$	68.7 ± 25.7	66.3 ± 25.0
1.0	$5.1 \pm 1.0^{\#}$	4.6 ± 0.4	5.0 ± 0.4