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Short communication

Echinacea purpurea extracts promote murine dendritic cell maturation by activation of JNK, p38 MAPK and NF-κB pathways

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ABSTRACT

Dendritic cells (DCs) comprise a system of highly professional antigen presenting cells (APCs) which connect innate and adaptive immunity by undergoing dramatic shift in their maturation state. Phytomedicine *Echinacea purpurea* extracts (EE) could modulate murine dendritic cell fate and function. However, the underlying mechanism of EE on DCs development and maturation remains limited. In this study, immature DCs were induced phenotypic maturation with up-regulated expression of key accessory molecules and the phagocytic activity was decreased after being treated with EE (400 μg/ml) for 48 h. We found that TLR1/2, JNK, p38-MAPK and NF-κB pathways were activated following EE exposure. Notably, JNK activation was demonstrated to be associated with increased IFN-γ response while p38-MAPK pathway exhibited immuno-regulatory effects via induction of IL-10 and TGF-β1. Furthermore, it was verified that NF-κB signaling was responsible for EE-induced synthesis of IFN-γ, IL-12 and TGF-β1, but not for IL-10 induction. These results indicate that EE have the immunomodulatory potency to promote both phenotypic and functional maturation of BMDCs via modulating the activation of JNK, p38-MAPK and NF-κB pathways. Our findings contributed to the current understanding of the immunoregulatory function of EE and the mechanism of DCs maturation.

1. Introduction

Phytomedicine *Echinacea purpurea* (*E. purpurea*) has been used traditionally throughout North America for the treatment of various infectious diseases. Currently, *E. purpurea* products have become one of the most popular herbal medicines globally. Several clinical trials against upper respiratory tract infections demonstrate positive results (Hudson, 2012; Jawad et al., 2012). Moreover, a number of studies have reported the potent antiviral and antimicrobial activities of certain standardized *E. purpurea* preparations against viruses and pathogenic bacteria involved in respiratory infections (Sharma et al., 2009; Sharma et al., 2010). Besides, enhanced phagocytic activity and increased production of tumor necrosis factor-alpha (TNF-α) in macrophages was observed following *E. purpurea* extracts (EE) treatment (Steinmüller et al., 1993). However, EE have been shown to inhibit the production of various inflammatory mediators in epithelial cells and T lymphocytes (Sasagawa et al., 2006; LaLone et al., 2007; Cech et al., 2010; Lalone et al., 2010). More recently, an interesting study reported that ethanolic EE could itself stimulate the pro-inflammatory cytokine TNF-α production in RAW 264.7 macrophage-like cells, and meanwhile suppress the generation of TNF-α in response to LPS exposure, suggesting a dual effect of EE on immune cell function (Todd et al., 2015). All these phenomenons indicate that EE may serve as a powerful immuno-modulator that can play double important roles in regulating the host immune responses.

Dendritic cells (DCs) are professional antigen presenting cells that connect innate and adaptive immunity by undergoing dramatic shift in their maturation state (Steinman and Hemmi, 2006).

Abbreviations: BMDCs, bone marrow-derived DCs; TLR, Toll-like receptor; MAPKs, mitogen-activated protein kinases; NF-κB, nuclear factor-κB; EE, *Echinacea pupurea* extracts; ERK1/2, extracellular signal-regulated kinases 1 and 2.

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After encountering inflammatory stimuli, immature DCs undergo maturation, evolving from antigen-capturing cells to T cell-priming cells (Dudek et al., 2013). Meanwhile, activated DCs can secrete a diversified panel of cytokines that initiate adaptive immune responses or induce tolerance (Tan and O'Neill, 2005). This maturation process has been reported to be highly dependent on the Toll-like receptors (TLRs)-mediated mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB) signal transduction pathways, upon exposure to TLR agonists such as bacterial LPS (Dowling et al., 2008). Accumulating evidence suggests that EE are involved in modulating cell fate, differentiation and expression of specific immune-related genes in DCs (Wang et al., 2006a; Benson et al., 2010), but the mechanism of EE on DCs maturation and activation is still obscure. In this paper, we therefore aimed to investigate the effects of EE on the maturation process of bone marrow-derived dendritic cells (BMDCs), and the mechanisms underlying these effects were also examined. LPS, a well-known stimulus for DC activation, was used as a positive control. We demonstrated that EE could promote both phenotypic and functional maturation of BMDCs and EE-mediated cell maturation involves activation of MAPKs and NF-κB pathways.

2. Materials and methods

2.1. Chemicals and reagents

E. purpurea extracts (EE) used in this study were purchased from Shandong Qilu Animal Health Co., Ltd. Chemical composition of EE were: cichoric acid (3.045%), caftaric acid (1.575%), chlorogenic acid (0.065%), dodeca-2E, 4E, 8Z, 10E/Z-tetraenoic acid isobutylamide (1.635%). There was no detectable endotoxin contamination as tested by Endospecy (<0.10 endotoxin units/ml). Lipopolysaccharide (LPS, *Escherichia coli* 0111: B4, Ultrapure) and FITC-dextran (40,000 Da) were obtained from Sigma Chemical Co. (St. Louis, USA). Granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 were purchased from PeproTech Inc. (Rocky Hill, USA). Anti-mouse antibodies FITC-CD11c, -CD40, -CD80, -CD83, -CD86 and *anti*-NF-κB p65 were purchased from Biolegend (San Diego, USA). The ELISA kits for IFN-γ, IL-12p70, IL-10 and TGF-β1 were from eBioscience (San Diego, USA). Antibodies against phospho-ERK1/2, ERK1/2, phospho-JNK, JNK, phospho-p38 MAPK, p38 MAPK, LaminB1, IκBα, β-actin and HRP-conjugated IgG were obtained from Santa Cruz Biotech (USA). Inhibitors BAY 11-7082, SP600125 and SB203580 were obtained from Beyotime Biotechnology (Haimen, China).

2.2. Bone marrow-derived dendritic cells (BMDCs)

C57BL/6 mice (female, 6–8 weeks old) were obtained from Shanghai SLAC Laboratory Animal Company. BMDCs were prepared as previously described (Lee et al., 2010) with modifications. Briefly, the femur and tibia of mice were flushed with 5 ml of cold sterile PBS. After lysing red blood cells, bone marrow cells were incubated at 37 °C in complete RPMI media supplemented with 10 ng/ml GM-CSF, 10 ng/ml IL-4, 10% FBS, 100 μg/ml streptomycin, and 100 U/ml penicillin. Fresh medium was replaced every other day, and non-adherent cells were transferred to new plates. After 7 days of culture, non-adherent cells were collected and the purity of $CD11c^+$ (murine DC lineage-specific marker) cell was >90% as examined by flow cytometry (FACS).

2.3. Flow cytometry analysis

BMDCs (1×10^6 cells/ml) were cultured in 12-well plates. Expression of cell surface molecules was determined after 48 h by treatment with PBS, EE (400 μg/ml), or LPS (50 ng/ml), respectively. Cells from different treatment groups were collected and suspensions were blocked with 5% normal goat serum for 1 h at 4 °C and then stained with antibodies against CD40, CD80, CD83, and CD86 for 1 h at 4 °C. After washing, the fluorescence signals were determined immediately by a FACScan flow cytometer (Becton-Dickinson) equipped with an argon laser with emission at 488 nm. At least 10,000 events were collected from the cell gate.

2.4. Phagocytosis assay

BMDCs were seeded into 12-well plates at a density of 1×10^6 cells/ml per well. To assess the phagocytic activity of BMDCs, cells were pretreated with EE (400 μg/ml) for 48 h and then incubated with FITC-dextran (100 μg/ml) at 37 °C for 1 h. After incubation, cells were washed 3 times with PBS, and the quantitative uptake of FITC-dextran by the cells was determined using FACS.

2.5. Quantitative real-time PCR analysis

Total RNA was isolated from BMDCs and reverse transcripted using PrimeScript II 1st Strand cDNA Synthesis Kit (TAKARA). The cDNA samples were then tested for the expression of TLR1 and TLR2 by RT-PCR performed as previously described (Mao et al., 2015). The following primers were used: β-actin forward 5′-CGTTGACATCCGTAAAGACC-3′ and reverse 5′-AACAGTCCGCCTAGAAGCAC-3′; TLR1 forward 5′-CAACAGTCAGCCTCAAGCATT-3′ and reverse 5′-CCATAAGCATCTCCTAACACCAG-3′; TLR2 forward 5′-GCTGGAGGTGTTGGATGTTAG-3′ and reverse 5′-AGGATAGGAGTTCGCAGGAG-3′. Results were normalized to β-actin expression and relative quantification was calculated using the $2^{-\Delta\Delta CT}$ method.

2.6. Western blotting analysis

Cytosolic and nuclear protein extracts were isolated using a Nuclear and Cytoplasmic Protein Extraction Kit according to the manufacturer's instruction. Western blots were carried out as previously described (Mao et al., 2015) with phospho-ERK1/2, ERK1/2, phospho-JNK, JNK, phospho-p38 MAPK, p38 MAPK, NF-κB p65, LaminB1, IκBα and β-actin primary antibodies, and HRP-conjugated secondary antibodies. LaminB1 was used as the nuclear marker while β-actin was used as a cytoplasmic marker.

2.7. Cytokine assay

BMDCs (1×10^6 cells/ml) were pretreated with NF-κB inhibitor BAY11-7082 (20 μM), JNK inhibitor SP600125 (20 μM) or p38-MAPK inhibitor SB203580 (20 μM) at 37 °C for 1 h and then incubated with EE (400 μg/ml) for another 24 h. Supernatants from cultured cells were analyzed for the production of immunostimulatory cytokines, IFN-γ and IL-12p70, and of immunosuppressive cytokines, IL-10 and TGF-β1, using ELISA kits according to the manufacturer's instructions.

2.8. Statistical analysis

All data are expressed as mean ± SD of three independent experiments. Statistical significance was determined using two-tailed Student's t-test with GraphPad Prism (San Diego, CA). Values of $P < 0.05$ were determined to be significant.

3. Results

3.1. Determination of EE on cell-surface molecules expression and phagocytic activity of BMDCs

Firstly, we examined the viability of BMDCs treated with EE for 48 h ranging from 0 to 800 μg/ml and found that 100, 200 and 400 μg/ml EE all showed no significant cytotoxicity (data not shown). Next, to evaluate whether EE (400 μg/ml) promotes DCs maturation, the key accessory molecules CD40, CD80, CD83 and CD86 of BMDCs, which are associated with antigen presentation and T-cell immunity induction, were determined. Typically, immature DCs exhibit lower levels of these markers, whereas mature DCs enhance their expression following activation. As shown in Fig. 1A, the percentage of CD40, CD80, CD83 and CD86 expression was significantly upregulated ($p < 0.05$; from 22.95± 1.03%, 45.98± 0.71%, 11.74± 1.35% and 24.23± 1.33% in control to 31.85± 0.57%, 56.16± 0.98%, 19.70± 1.18% and 34.39± 0.81% in EE-treated group, respectively). The impact of EE on the phagocytic activity of BMDCs was also determined. As demonstrated in Fig. 1B, BMDCs pretreated with EE for 48 h exhibited markedly reduced endocytosis of FITC-dextran compared to control group. Similarly, BMDCs also displayed lower FITC-dextran internalization after LPS exposure. These results confirm that EE stimulation could effectively induce the maturation of BMDCs.

3.2. Functional evaluation of EE on the activation of signal pathways in BMDCs

To examine whether EE activates TLRs pathways, we detected the gene expression of TLRs (TLR1~5, TLR7, and TLR9) and MyD88 adaptor in BMDCs treated with EE for 3 h, 6 h, 12 h and 24 h, respectively. As shown in Fig. 2A, TLR1 expression increased significantly after EE or LPS stimulation for 6 h and 12 h as compared to control group. Meanwhile, after 3 h, 6 h and 12 h of EE treatment, the mRNA levels of TLR2 in BMDCs were significantly higher than those in untreated cells (Fig. 2B). However, EE exposure failed to activate the gene expression of TLR3~5, TLR7, TLR9 and MyD88 in BMDCs (data not shown).

Then, the phosphorylation levels of MAPKs (ERK, JNK and p38) and the nuclear translocation of NF-κB subunit p65 were examined by western blot analysis. As demonstrated in Fig. 2C, treatment with EE provoked a rapid increase in the phosphorylation of JNK and p38 in BMDCs, and the phosphorylation reached its peak at 30 min of treatment and then declined to basal level within 60 min. However, EE did not induce phosphorylation of ERK in BMDCs. Furthermore, EE also triggered a marked increase in NF-κB p65 levels in the nucleus of BMDCs (Fig. 2D). Meanwhile, IκBα protein (a potent inhibitor of NF-κB) decreased gradually in the cytoplasm within 60 min of EE stimulation. Taken together, these results demonstrated that EE treatment could activate TLR1/2, MAPKs (JNK and p38) and NF-κB pathways in BMDCs.

3.3. Effects of EE on cytokines production in BMDCs via JNK, p38 MAPK and NF-κB pathways

To explore whether cytokines are produced in the process of EE-mediated BMDCs maturation, the expression levels of pro-inflammatory (IFN-γ and IL-12) and anti-inflammatory (IL-10 and TGF-β1) cytokines, were selectively detected by ELISA. The results showed that EE could increase the secretion of IFN-γ, IL-12, IL-10, and TGF-β1 (Fig. 2E–H). We next examined the involvement of JNK and p38 MAPK transduction pathways in EE-induced cytokine response using specific inhibitors: BAY11-0782 (NF-κB inhibitor), SP600125 (JNK inhibitor), and SB203580 (p38 inhibitor). As shown in Fig. 2E, pretreatment with JNK inhibitor significantly decreased EE-induced IFN-γ production in stimulated cells, while inhibition of p38 resulted in unanticipated increase in IFN-γ level. Moreover, JNK inhibition did not alter IL-12 response while p38 suppression could enhance IL-12p70 secretion (Fig. 2F). Furthermore, suppression of p38, but not JNK, dramatically down-regulated the synthesis of IL-10 and TGF-β1 (Fig. 2G–H). In addition, BAY11-0782 (NF-κB inhibitor) pretreatment significantly down-regulated levels of IFN-γ, IL-12 and TGF-β1 with EE exposure, indicating an involvement of NF-κB in the modulation of those cytokines. Noticeably, NF-κB inhibition did not alter IL-10 generation, suggesting that NF-κB may not be involved in IL-10 regulation in BMDCs treated with EE.

4. Discussion

As a top-selling herbal medicine and food supplement, EE have drawn increasing attention for its multiple immuno-modulatory effects, resulting either from a synergistic effect of different components or activities of individual constituents. Here, we demonstrated that EE treatment promoted maturation of DCs, as evidenced by increased accessory molecules expression and reduced phagocytic activity, consistent with previous observations (Wang et al., 2006a; Benson et al., 2010).

TLRs-mediated MAPKs and NF-κB activation has been shown to be involved in the process of DC activation and maturation (Ade et al., 2007). TLR2 agonist, such as peptidoglycan, has been reported to potently activate members of the MAPK family (ERK, JNK and p38-MAPK) in human DCs (Re and Strominger, 2001). In the present study, EE could significantly up-regulate TLR1 and TLR2 gene expressions. These findings suggested that the promoting effect of EE on DCs maturation could be mediated via a TLR1/2-dependent pathway. Furthermore, EE could markedly induce the phosphorylation of JNK and p38-MAPK, but not ERK in BMDCs. These data were in agreement with a previous study, showing that *Echinacea* alkylamides could activate JNK and p38 signaling cascade and finally up-regulate the expression of pro-inflammatory cytokine (Gertsch et al., 2004).

In the current study, EE treatment could stimulate the production of immuno-stimulatory cytokines (IFN-γ and IL-12) and immuno-suppressive cytokines (IL-10 and TGF-β1), indicating the functional maturation of DCs. Moreover, JNK pathway inhibition potently decreased the EE-induced IFN-γ production in stimulated cells, while suppression of p38-MAPK down-regulated IL-10 and TGF-β1 levels. Additionally, p38-MAPK seemed to be a negative regulator of IFN-γ and IL-12 since suppression of p38 resulted in elevated levels of these two cytokines in BMDCs treated with EE. These results were consistent with the work of Wang et al. (2006b), who demonstrated that p38 inhibitor successfully reduced the production of IL-10 and TGF-β and enhanced IL-12 secretion in BMDCs treated with tumor culture conditioning medium. p38 MAPK has been shown to promote the activation of cAMP response element-binding protein (CREB), a transcription factor required for anti-inflammatory immune responses and regulatory T cell (Treg) generation (Wen et al., 2010). Thus, it could be deduced that the EE-induced IL-10 and TGF-β1 secretion could be mediated through the p38 MAPK-CREB signaling axis in BMDCs. These findings indicated that JNK activation might be required for the pro-inflammatory properties of EE while p38 pathway exhibited an immuno-regulatory effect via induction of anti-inflammatory factors.

NF-κB complexes (p65/p50) regulate the expression of many cytokines (Wan and Lenardo, 2010). Matthias et al. (2008) reported that *Echinacea* and its phytochemical components exerted no effect on basal NF-κB expression while cichoric acid, an *Echinacea* root extract, increased NF-κB levels in immunostimulated human T-

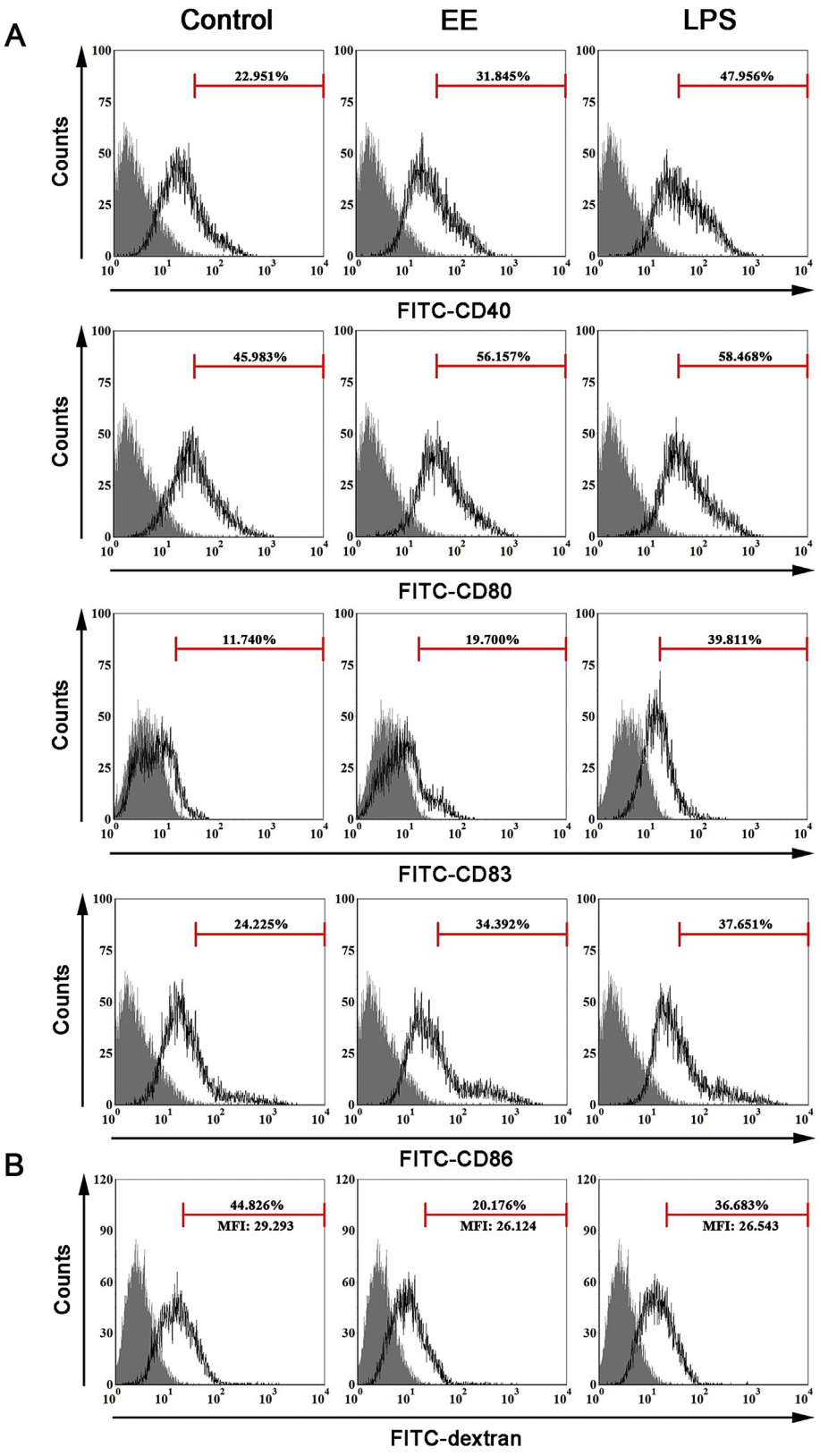

Fig. 1. Determination of surface phenotypic molecules and functional evaluation of phagocytic activity of BMDCs. (A) BMDCs were treated with PBS (blank control), EE (400 μg/ml), or LPS (50 ng/ml) (positive control) for 48 h, respectively. Cells were stained with antibodies against CD40, CD80, CD83 and CD86, and the fluorescence signals were determined immediately by a FACScan flow cytometer. At least 10,000 events were collected from the cell gate. (B) BMDCs were pretreated with PBS, EE (400 μg/ml) or LPS (50 ng/ml) for 48 h. Cells were then incubated with FITC-dextran (100 μg/ml) at 37 °C for 1 h. The quantitative uptake of FITC-dextran was determined using FACS. Different treatments are presented at the top of each block diagram. The numbers above the bracketed lines indicate the percentage of FITC-tagged cells in each panel. Data represent three independent experiments. Mean ± SD of results from three independent experiments are shown.

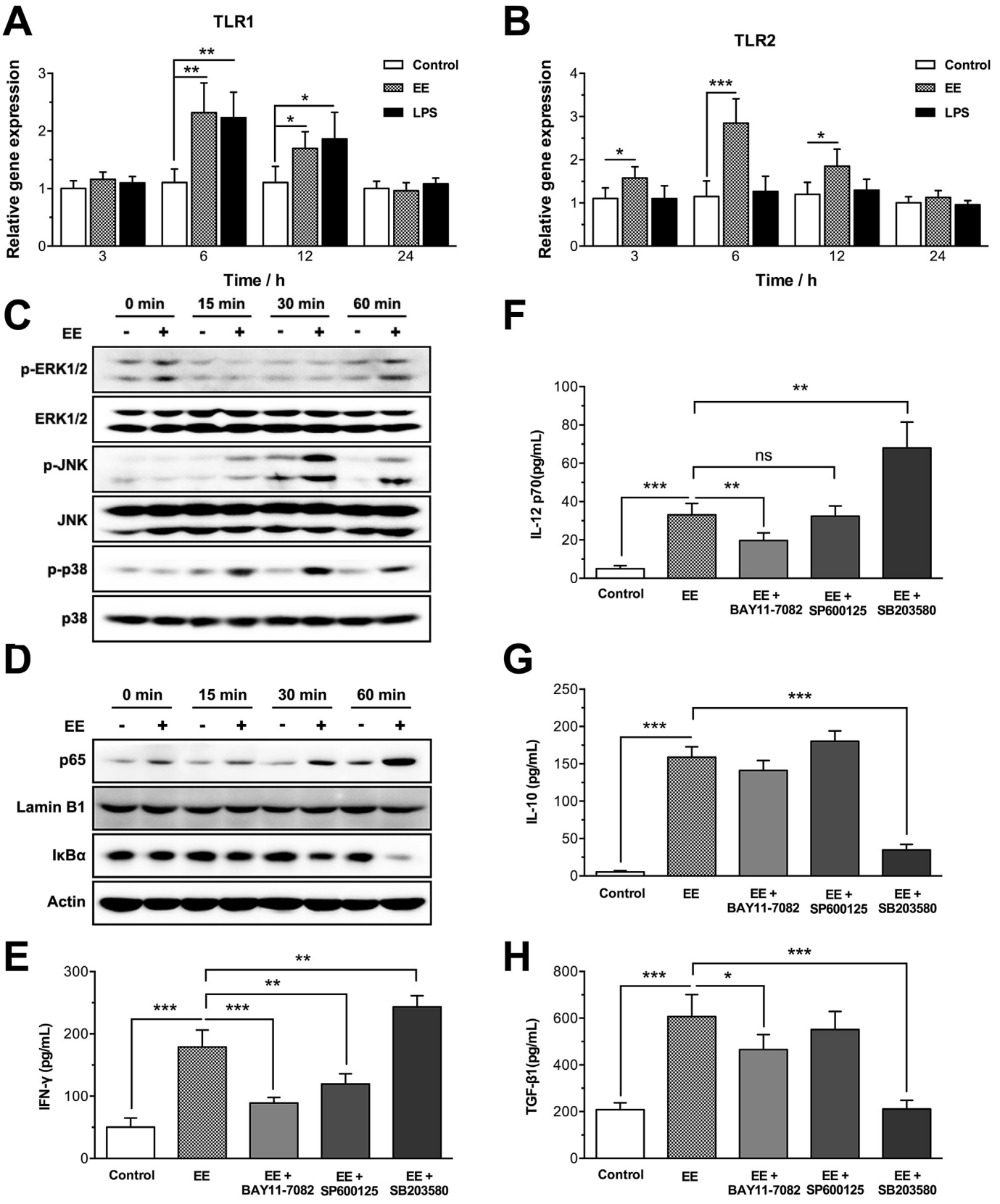

Fig. 2. Activation of TLRs, MAPKs, NF-κB and cytokine responses in BMDCs. BMDCs were treated with PBS, EE (400 μg/ml) or LPS (50 ng/ml) for the indicated time points. The gene expression of TLR1 (A) and TLR2 (B) was assessed via quantitative real-time PCR. (C and D) Cytosolic and nuclear protein extracts were collected for detection of phosphorylated ERK1/2 (p-ERK1/2), JNK (p-JNK), p38 MAPK (p-p38), NF-κB p65 and IκBα by Western blotting. LaminB1 was used as the nuclear marker while β-actin was used as a cytoplasmic marker. (E-H) BMDCs were pretreated with NF-κB inhibitor BAY11-7082 (20 μM), JNK inhibitor SP600125 (20 μM) or p38-MAPK inhibitor SB203580 (20 μM) at 37 °C for 1 h. Supernatants were collected after 24 h treatment with EE, and analyzed for the production of IFN-γ (E), IL-12p70 (F), IL-10 (G) and TGF-β1 (H) using ELISA kits. Data represent the mean ± SD (n = 5/group; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

cells. In our study, EE triggered a marked increase in NF-κB p65 levels in the nucleus of BMDCs while IκBα protein decreased gradually in the cytoplasm after EE stimulation. The discrepant effects of EE on NF-κB might be due to differences in their phytochemical profile. Further, NF-κB suppression was shown to down-regulate IFN-γ, IL-12 and TGF-β1 levels in BMDCs. Unexpectedly, NF-κB inhibition did not affect IL-10 expression, implying that NF-κB may not be the main regulator of IL-10 in BMDCs following EE exposure. Thus, our results demonstrate that NF-κB signaling is responsible for EE-induced expression of IL-12, IFN-γ and TGF-β1 in BMDCs, but not for IL-10 induction.

In conclusion, *E. purpurea* extracts utilized in our study could promote both phenotypic and functional maturation of BMDCs via modulating the activation of JNK, p38 MAPK and NF-κB pathways, indicating its potential immunomodulatory activities. These findings will provide important information for the development of *Echinacea*-based therapies.

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