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Honokiol[Q1] Inhibits LPS-Induced Maturation and Inflammatory Response of Human Monocyte-Derived Dendritic Cells

CHIA-YANG LI,[1] LOUIS KUOPING CHAO,[2] SHU-CHI WANG,[1] HON-ZU CHANG,[3] MIN-LUNG TSAI,[4] SHIH-HUA FANG,[4] PEI-CHUN LIAO,[5] CHEN-LUNG HO,[6,7] SHUI-TEIN CHEN,[8,9] WEI-CHUNG CHENG,[1] CHI-SHIUN CHIANG,[1] KUO-FENG HUA,[5,10]** AND IAN C. HSU[1]*

[1]*Department of Biomedical Engineering and Environmental Sciences, National Tsing Hua University, Hsinchu, Taiwan*

[2]*Department of Cosmeceutics, China Medical University, Taichung, Taiwan*

[3]*Institute of Nanoengineering and Microsystem, National Tsing Hua University, Hsinchu, Taiwan*

[4]*Institute of Athletes, National Taiwan Sport University, Taichung, Taiwan*

[5]*Institute of Biotechnology, National Ilan University, Ilan, Taiwan*

[6]*Division of Wood Cellulose, Taiwan Forestry Research Institute, Taipei, Taiwan*

[7]*Department of Forestry, National Chung Hsing University, Taichung, Taiwan*

[8]*Institute of Biochemical Sciences, College of Life Science, National Taiwan University, Taipei, Taiwan*

[9]*Institute of Biological Chemistry and Genomics Research Center, Academia Sinica, Taipei, Taiwan*

[10]*Graduate Institute of Drug Safety, School of Pharmacy, China Medical University, Taichung, Taiwan*

Honokiol (HNK) is a phenolic compound isolated from the bark of *houpu* (*Magnolia officinalis*), a plant widely used in traditional Chinese and Japanese medicine. While substantial evidence indicates that HNK possesses anti-inflammatory activity, its effect on dendritic cells (DCs) during the inflammatory reaction remains unclear. The present study investigates how HNK affects lipopolysaccharide (LPS)-stimulated human monocyte-derived DCs. Our experimental results show that HNK inhibits the inflammatory response of LPS-induced DCs by (1) suppressing the expression of CD11c, CD40, CD80, CD83, CD86, and MHC-II on LPS-activated DCs, (2) reducing the production of TNF-α, IL-1β, IL-6, and IL-12p70 but increasing the production of IL-10 and TGF-β1 by LPS-activated DCs, (3) inhibiting the LPS-induced DC-elicited allogeneic T-cell proliferation, and (4) shifting the LPS-induced DC-driven Th1 response toward a Th2 response. Further, our results show that HNK inhibits the phosphorylation levels of ERK1/2, p38, JNK1/2, IKKα, and IκBα in LPS-activated DCs. Collectively, the findings show that the anti-inflammatory actions of HNK on LPS-induced DCs are associated with the NF-κB and mitogen-activated protein kinase (MAPK) signaling pathways.

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For thousands of years, herbal medicine has been used for the treatment of many diseases. *Houpu* (*Magnolia officinalis*) is an important medicinal herb widely used to treat fever, headache, anxiety, and nervous disturbance in Chinese and Japanese medicine (Squires et al., 1999). Honokiol (HNK, $C_{18}H_{18}O_2$, MW = 266.33) is an active compound isolated from the *houpu* (Watanabe et al., 1983; Liou et al., 2003b). HNK has been reported to have several pharmacological functions, including anti-inflammatory (Liou et al., 2003a; Lee et al., 2005; Munroe et al., 2007; Chiang et al., 2009), anti-oxidant (Lo et al., 1994; Dikalov et al., 2008), anti-bacterial (Park et al., 2004), and apoptotic functions (Yang et al., 2002; Ahn et al., 2006). It also inhibits cancer cell proliferation, both in vitro (Yang et al., 2002; Battle et al., 2005; Ishitsuka et al., 2005; Tse et al., 2005) and in vivo (Bai et al., 2003).

Dendritic cells (DCs) are considered to be the principal antigen-presenting cells, and they play a prominent role in the development of T-cell immune responses (Banchereau and Steinman, 1998; Banchereau et al., 2000). The development of DCs comprises two functional stages. Immature DCs are primarily localized in the peripheral tissues. Their main function

Chia-Yang Li and Louis Kuoping Chao contributed equally to this work.

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*Correspondence to: Kuo-Feng Hua, Institute of Biotechnology, National Ilan University, 1, Sec. 1, Shen-Lung Road, I-Lan 260, Taiwan. E-mail: kuofenghua@niu.edu.tw

**Correspondence to: Ian C. Hsu, Department of Biomedical Engineering and Environmental Sciences, National Tsing Hua University, 101, Section 2, Kuang-Fu Road, Hsinchu 30013, Taiwan. E-mail: ichsu@mx.nthu.edu.tw

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is antigen phagocytosis and processing. Following antigen uptake, DCs migrate to the lymphoid organs, where they become potent antigen presenters and thereby activate T cells (Cella et al., 1997b). The maturation of DCs is critical to initiate the adaptive immune response since fully mature DCs can induce antigen-specific T-lymphocyte responses and control the differentiation of T cells toward Th1 or Th2 immunity (Seder and Paul, 1994; Abbas et al., 1996). Fully mature DCs exhibit high surface expression of MHC-II and costimulatory molecules such as CD40, CD80, and CD86 (Chambers and Allison, 1999; Quah and O'Neill, 2005) but decreased capacity to internalize antigens (Cella et al., 1997a; Lin et al., 2005). Additionally, CD83, a specific marker of DC maturation, is upregulated (Reddy et al., 1997). The expression of MHC-II, CD40, CD80, and CD86 on DCs is essential for T-lymphocytes activation (Banchereau and Steinman, 1998; Lechmann et al., 2001). The integrin CD11c is known as a marker of DCs and has been proposed to function in phagocytosis, cell migration, cytokine production, and inflammation (Sadhu et al., 2007; Georgakopoulos et al., 2008). Many stimuli are able to induce DC maturation, including proinflammatory cytokines, the CD40 ligand, and pathogen–associated materials such as lipopolysaccharide (LPS), bacteria DNA, and the unmethylated DNA CpG motif (Banchereau et al., 2000).

Toll like receptors (TLRs) recognize conserved structural motifs on microorganisms known as pathogen-associated molecular patterns (PAMPs). LPS, an integral component of the outer membrane of gram-negative bacteria, can trigger an acute inflammatory response, leading to a series of pathological events such as septic shock (Martich et al., 1993). DCs play an important role in the elimination of pathogens and provide a link between innate and adaptive immunity (Banchereau and Steinman, 1998; Banchereau et al., 2000). They are extremely sensitive to even low concentration of LPS and respond to this cue by releasing inflammatory mediators (Verhasselt et al., 1997). DC activation by LPS eventually triggers an antigen-specific T-cell response (Rieser et al., 1998). Briefly, LPS is a potent activator of DCs and triggers the production of proinflammatory cytokines such as IL-1, IL-6, TNF-α, and IL-12 and promotes DC maturation via the TLR4 signaling pathway (Roake et al., 1995; de Jong et al., 1999; Kelleher and Beverley, 2001), which in turn triggers the signaling cascades that result in the activation of NF-κB and mitogen-activated protein kinase (MAPK; Akira and Takeda, 2004).

Previous reports have demonstrated that HNK has anti-inflammatory effects, whereby it inhibits reactive oxygen species-related inflammatory responses in neutrophils by inhibiting the activity of NADPH oxidase, myeloperoxidase, and cyclooxygenase, and subsequently enhancing the GSH activity (Liou et al., 2003b). Moreover, Lee et al. (2005) showed that HNK has an antioxidant effect, whereby it reduces the production of IL-8 and TNF-α, and inhibits the activation of NF-κB in *Propionibacterium acnes*-activated THP-1 cells. Munroe et al. demonstrated that HNK prevents the flare up of symptomatic collagen-induced arthritis in both CD40-LMP1 transgenic mice and their congenic C57BL/6 counterparts and inhibits CD40- and latent membrane protein-1 (LMP1)-mediated B-cell activation by abrogating the activation of NF-κB and AP-1, and inhibiting the production of TNF-α and IL-6 (Munroe et al., 2007). However, to date, no studies have reported the effects of HNK on the functions of DCs. In the present study, LPS-activated DCs were used as a model of the inflammatory stage to investigate whether HNK affects the LPS-induced inflammatory response.

Materials and Methods

Reagents

HNK (purity >98%) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The HNK contained less than 0.01 EU/ml LPS as measured using the Limulus Amoebocyte Lysate (LAL) Chromogenic Endpoint assay (Hycult Biotech, Uden, the Netherlands; data not shown). The culture medium used in this study was RPMI 1640 (Gibco-BRL, Life Technologies, Paisley, UK) supplemented with 2 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin, 0.1 mg/ml streptomycin (Gibco-BRL, Life Technologies, Paisley, UK), and 10% heat-inactivated FCS (Hyclone, Logan, UT). Recombinant human GM-CSF, recombinant human IL-4, and human soluble CD40 ligand (sCD40L) were purchased from PeproTech (Rocky Hill, NJ). LPS (*Escherichia coli* serotype O55:B5), MTT, BSA, DMSO, proteinase K, FITC–dextran (40,000 Da), FITC–LPS, and lucifer yellow were purchased from Sigma (St. Louis, MO). FITC–*E. coli* was purchased from Orpegen Pharma (Heidelberg, Germany). Mouse anti-human mAbs conjugated with FITC or PE were used to detect CD4, CD11c CD14, CD25, CD40, CD80, CD83, CD86, HLA-DR, and FoxP3. All mAbs and isotype control Abs were purchased from eBioscience (San Diego, CA). ELISA kits for detection of human TNF-α, IL-1β, IL-4, IL-6, IL-10, IL-12p70, IL-13, IL-17, TGF-β1, and IFN-γ were also purchased from eBioscience. For intracellular staining, Brefeldin A, fixation buffer, permeabilization buffer, FITC-labeled anti-human IFN-γ mAb, and PE-labeled anti-human IL-4 mAb were purchased from eBioscience. Cell proliferation was measured by using the BrdU ELISA kit that was purchased from Roche Molecular Biochemicals (Mannheim, Germany). For Western blot analysis, we used anti-ERK1 rabbit polyclonal IgG (Santa Cruz, CA; catalog No. sc-94), anti-JNK1 rabbit polyclonal IgG (Santa Cruz; catalog No. sc-474), anti-p38 rabbit polyclonal IgG (Santa Cruz; catalog No. sc-535), monoclonal anti-MAP kinase, activated (diphosphorylated ERK-1&2) IgG1 (Sigma–Aldrich, St. Louis, MO; catalog no. M9692), monoclonal anti-JNK, activated (diphosphorylated JNK) IgG1 (Sigma–Aldrich; catalog no. J4750), monoclonal anti-p38 MAP kinase, activated (diphosphorylated p38) IgG1 (Sigma–Aldrich; catalog no. M8177), anti-phospho-IκBα (Ser32/Ser36) rabbit polyclonal IgG (Millipore, Billerica, MA, USA; catalog No. 07-836), anti-IκBα mouse monoclonal IgG1 (Santa Cruz; catalog No. sc-1643) anti-phospho-IKKα (Ser176/Sser180) rabbit polyclonal IgG (Millipore; catalog No. 07-837), and anti-IKKα mouse monoclonal IgG1 (Millipore; catalog No. 05-536). The human specific TLR9 ligand (CpG oligodeoxynucleotides (CPG ODNs)-Type A) was purchased from Invivogen (San Diego, CA).

Generation of human monocyte-derived DCs

The detailed experimental procedure for generating monocyte-derived DCs was as described previously (Li et al., 2009). Briefly, whole blood samples from healthy volunteers were obtained from the Taipei Blood Center by an Institutional Review Board (IRB) approved procedure issued by Academia Sinica, Taipei, Taiwan. PBMCs were separated from the whole blood by using Ficoll-Hypaque density gradient centrifugation and then monocytes were isolated by the plastic adherence method (Thurner et al., 1999). The purity of the monocyte population was >90% as assessed by CD14 staining (data not shown). Immature DCs were generated from monocytes that were cultured for 6 days at 37°C in an incubator with 5% humidified CO_2 in RPMI 1640 culture medium supplemented with 500 U/ml recombinant human GM-CSF and 1,000 U/ml recombinant human IL-4. On days 2 and 4, half the medium was replaced with fresh medium containing recombinant human GM-CSF and recombinant human IL-4.

Cell culture and cell viability assay

HNK was dissolved in DMSO at a stock concentration of 100 mg/ml. It was further diluted in the culture medium at a final DMSO concentration of <0.02%. In order to minimize the effects of proteins and peptidoglycan contamination in our studies, the contaminating proteins and peptidoglycan in the LPS sample were removed by proteinase K treatment (300 μg/ml) before the experiments. Then, the proteinase K was also removed by dialysis,

and the purity of the LPS sample was determined by NMR spectroscopy (data not shown). On day 6 of DC culture, a total of 10^6 immature DCs were reseeded into a 6-well culture plate and treated with HNK at various concentrations (0, 5, 10, 15, and 20 μg/ml) in a culture for 2 days. LPS-, CpG ODNs-, and sCD40L-activated DCs were generated from immature DCs by culturing for 2 days in the presence of LPS (1 μg/ml), CpG ODNs (10 μg/ml), and sCD40L (1 μg/ml), respectively, and HNK at various concentrations (0, 5, 10, 15, and 20 μg/ml). The untreated controls (HNK, 0 μg/ml, LPS alone, CpG ODNs alone, or sCD40L alone) were treated with 0.02% DMSO as the vehicle control. The viability of the cells following these treatments was determined by the MTT assay that was performed following the manufacturer's instructions (Sigma).

Phenotypic characterization of DCs

For phenotypic analysis of DCs, the cells were incubated with FITC- or PE-labeled mAbs against human CD11c, CD14, CD40, CD80, CD83, CD86, or HLA-DR or isotype control Abs for 40 min at 4°C. The cells were then washed twice with cold PBS containing 2% FCS and analyzed by flow cytometry (Partec, Munster, Germany). For each analysis, 20,000 cells were acquired by flow cytometry and analyzed using WinMDI software (Scripps, La Jolla, CA). CD14 is a monocyte lineage specific marker, and was not presented on the DC surface (data not shown). All assays were performed in triplicate.

Analysis of endocytic activity

The phagocytic activity of DCs was measured as described previously with slight modification (Duperrier et al., 2000). Briefly, a total of 5×10^5 DCs were resuspended in 100 μl PBS containing 2% FCS and then incubated with FITC–dextran (0.1 mg/ml), FITC–*E. coli* (40:1, *E. coli*:DC ratio), or lucifer yellow (1 mg/ml) at 37°C for 30 min. Following incubation, the cells were washed four times in cold PBS that contained 2% FCS and analyzed by flow cytometry. As a control, cells cultured under each condition were left untreated in the same solution for 30 min at 4°C. All assays were performed in triplicate.

Allogeneic MLR assay

For the MLR assay, responder cells (T cells) were obtained from allogeneic PBMCs by nylon wool column purification (Polysciences, Warrington, PA). The purity of CD3-positive T cells was shown to be at least 85%, as determined by flow cytometry (data not shown). Immature DCs treated for 2 days with HNK, LPS, or a combination of LPS and HNK were used as the stimulator cells. Before the MLR experiment, the DCs were washed twice with HBSS. In the flat-bottomed 96-well plates containing 0.2 ml medium per well, 2×10^3 stimulator cells were cocultured for 3 days with a graded ratio of responder cells (1:25, 1:50, 1:75, and 1:100). Allostimulatory activity was measured following the addition of 10 μM BrdU during the last 14 h of the 3-day culture period. Proliferation of T cells was determined by measuring BrdU incorporation with the cell proliferation ELISA kit according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany). All assays were performed in triplicate.

Measurement of cytokine secretion

DC supernatants were harvested after 2 days of incubation with medium alone, LPS, or a combination of LPS and HNK. TNF-α, IL-1β, IL-6, IL-10, IL-12p70, and TGF-β1 levels in DC supernatants were quantified using ELISA kits (eBioscience). T cells were cocultured with LPS-treated, or a combination of LPS and HNK-treated DCs for 3 days. To assess the T cell differentiation, the levels of IFN-γ, IL-4, IL-10, IL-13, and IL-17 in the supernatant were measured by ELISA and normalized to total cell numbers at the end of the treatment. Cell viability was measured by the MTT assay. For intracellular staining, cells were fixed with a fixation buffer for 30 min and permeabilized with permeabilization buffer for 30 min as per the manufacturer's instructions (eBioscience). Cells were stained with FITC-labeled anti-IFN-γ and PE-labeled anti-IL-4 mAb (eBioscience) and analyzed by flow cytometry (Partec, Munster, Germany). All assays were performed in triplicate.

LPS binding assay

The methods for the LPS binding assay were as described previously (Yuan et al., 2009). In brief, immature DCs were treated at 37°C for 30 min with 1 μg/ml FITC-labeled LPS (FITC–LPS) and HNK at various concentrations (0, 10, and 20 μg/ml). They were then washed with HBSS for three times. The FITC–LPS binding assay was analyzed by flow cytometry.

Western blot

To investigate the phosphorylation levels of ERK1/2, JNK1/2, p38, IKKα, and IκBα, immature DCs were treated with LPS (1 μg/ml) in the presence or absence of 20 μg/ml HNK for 15, 30, 60, and 120 min. After stimulation, the cells were washed twice with PBS and lysed with lysis buffer. Whole cell lysates were separated by 10% SDS gel electrophoresis and electrotransferred to polyvinylidene difluoride membranes (Pall Corporation, East Hills, NY). The membranes were incubated in blocking solution (5% nonfat milk in PBS with 0.1% Tween 20) at room temperature for 1 h. They were then incubated with specific primary antibodies (1:1,000 diluted in blocking solution) for 2 h, washed, and incubated with a HRP-conjugated secondary antibody (1:2,000 diluted in blocking solution) to the primary antibody. The membranes were developed using an ECL Western blotting detection system (Pierce Corporation, Rockford, IL). The bands were quantified using AlphaImage software (Alpha Innotech Corporation, San Leandro, CA). The relative fold of phosphorylation activity each time was normalized to that of the unphosphorylated form and then compared to each untreated control group.

Statistical analysis

All assays were performed as at least three independent experiments using different donors. The results are presented as

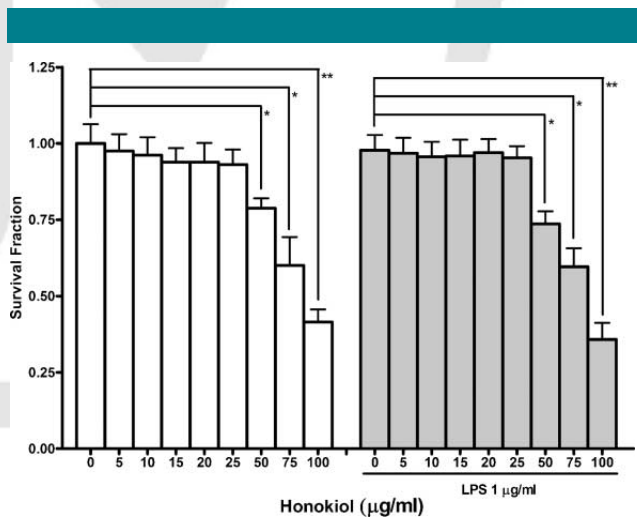

Fig. 1. HNK is not toxic at the experimental doses (≤20 μg/ml). Human immature DCs were treated with HNK at various concentrations (0, 5, 10, 15, and 20 μg/ml) in the absence or presence of LPS (1 μg/ml) for 2 days. Cell viability was assessed by the MTT assay. Data are presented as mean ± SD from three independent experiments. The statistical significance of the difference between two experimental measurements was assessed by Student's *t*-test and represented as follows: $^*P < 0.05$ and $^{**}P < 0.01$.

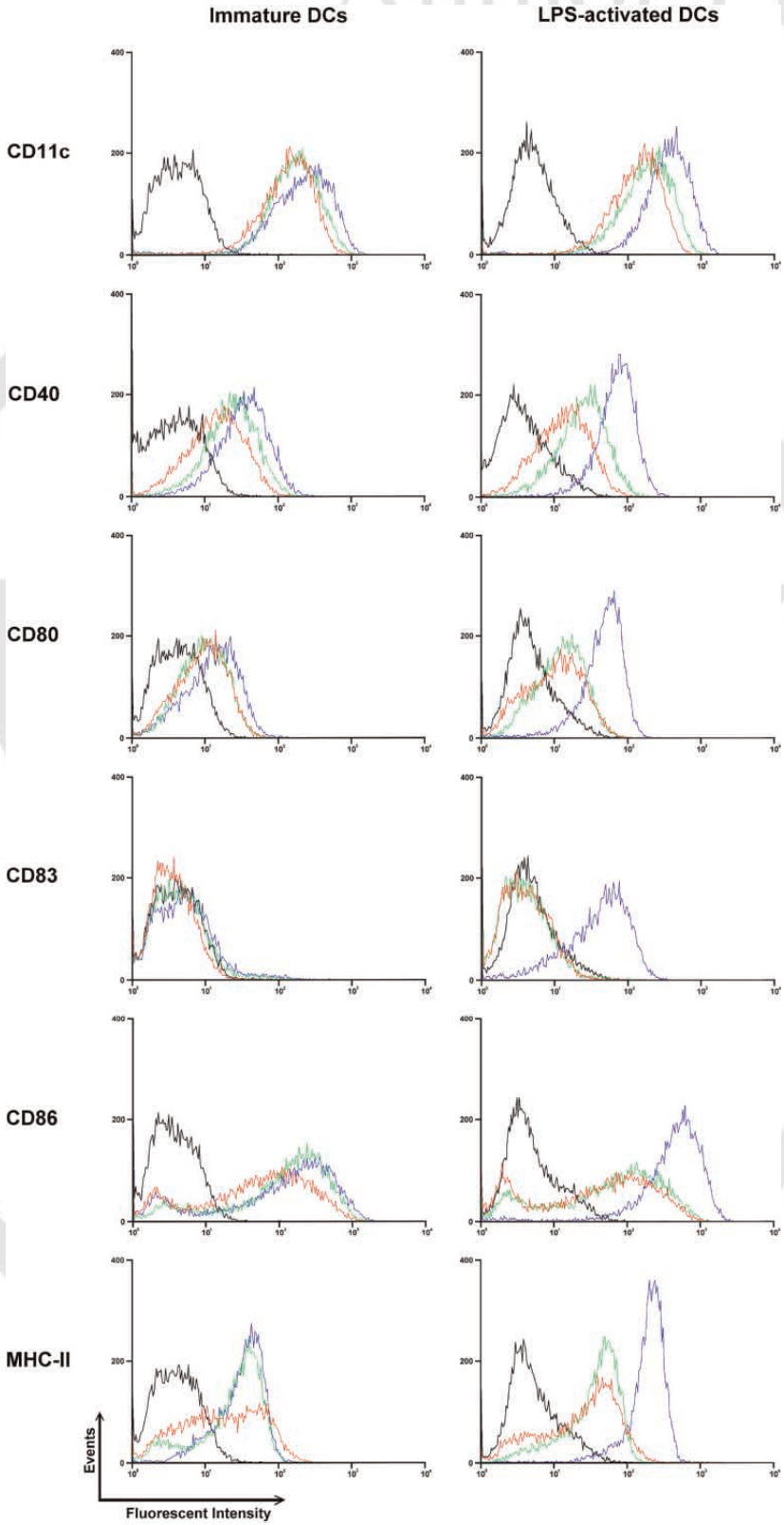

Fig. 2. Effects of HNK on the expression of the phenotypic characteristics of DCs. Expression of various surface markers on DCs was analyzed by flow cytometry after treatment with HNK at various concentrations (0 μg/ml (blue line), 10 μg/ml (green line), or 20 μg/ml (red line)) in the absence or presence of LPS (1 μg/ml) for 2 days. The black line represents staining with the corresponding isotype control mAbs. The results are representative of three independent experiments.

the mean ± SD of the repeated experiments and were analyzed using SPSS software (SPSS Inc., Chicago, IL). Data were compared between control and treatment groups by using Student's *t*-test. Differences were regarded as statistically significant for *P* values of less than 5% ($P < 0.05$) and 1% ($P < 0.01$).

Results

Dose (≤20 μg/ml) of HNK does not affect DC survival

The goal of this study was to investigate the effect of HNK on the immunomodulating functions of DCs. Considering that human monocyte-derived DCs are normal immune cells, the dosage of HNK used in this study should not affect their survival. To examine the toxicity of HNK, immature DCs were treated with HNK at various concentrations (0, 5, 10, 15, and 20 μg/ml) in the absence or presence of LPS (1 μg/ml) for 2 days. Cell viability was analyzed by the MTT assay. The results revealed that the cell survival rate did not differ significantly when the DCs were treated with ≤20 μg/ml HNK (Fig. 1). At these HNK concentrations, even the presence of LPS at 1 μg/ml did not significantly affect the DC survival rate (Fig. 1). However, HNK was toxic to the DCs at concentrations of ≥50 μg/ml (Fig. 1). The maximum dose used in the following experiments is 20 μg/ml HNK.

HNK inhibits phenotypic maturation of LPS-activated DCs

To investigate the effects of HNK on DC maturation, immature DCs were treated for 2 days with HNK at various concentrations (0, 10, and 20 μg/ml) in the absence or presence of LPS (1 μg/ml), which induces inflammation and promotes DC maturation (Roake et al., 1995; de Jong et al., 1999; Kelleher and Beverley, 2001). The expression of surface molecules (CD11c, CD40, CD80, CD83, CD86, and MHC-II) was examined by flow cytometry. The results showed that HNK suppressed the LPS-induced expression of CD11c, CD40, CD80, CD83, CD86, and MHC-II by DCs (Fig. 2 and Table 1). In the control group, HNK inhibited the expression of CD11c, MHC-II and costimulatory molecules (CD40, CD80, and CD86) by immature DCs, but its effects were less intense than those on LPS-stimulated DCs (Fig. 2 and Table 1).

HNK impairs endocytic activity in both immature DCs and LPS-activated DCs

Endocytosis is a process of antigen uptake, which is mediated by receptors such as the mannose receptor and Fc gamma receptor, whereby antigens and immune complexes are internalized (Lin et al., 2005; Huang et al., 2006). To examine whether HNK affects the endocytic activity of DCs, immature DCs generated from human monocytes were treated with HNK at various concentrations (0, 10, and 20 μg/ml) in the absence or presence of LPS (1 μg/ml) for 2 days. The endocytic activity was assessed by measuring uptake of FITC–dextran. We found that HNK decreased the FITC–dextran uptake of not only immature DCs (Fig. 3A,C) but also LPS-activated DCs (Fig. 3B,C). In addition, we also assessed the ability of FITC–*E. coli* and lucifer yellow uptake. These results were in close agreement to the result of FITC–dextran uptake (Supplementary Fig. S1).

HNK decreases the production of TNF-α, IL-1β, IL-6, and IL-12p70 but increases the production of IL-10 and TGF-β1 by LPS-activated DCs

DC-secreted cytokines play a pivotal role in the immune response and the interaction between DCs and T cells (Banchereau et al., 2000; Guermonprez et al., 2002). IL-12 release by DCs drives the differentiation of naïve T cells toward the IFN-γ-producing Th1 phenotype (Gately et al., 1998). Proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 play a key role in inducing innate and acquired immune responses (Dinarello, 1996). In this study, the cytokine levels in the supernatant of DCs cultured with HNK at various concentrations were measured by ELISA. The experimental results indicated that HNK treatment did not alter the background levels of TNF-α, IL-1β, IL-6, and IL-12p70 in the immature DC population, but it decreased the production of these cytokines by LPS-activated DCs in a dose-dependent manner (Fig. 4). In addition, we also examined whether HNK affects the production of anti-inflammatory cytokines, IL-10, and TGF-β. Our experimental results showed that HNK treatment did not alter the background levels of IL-10 and TGF-β1 in the immature DC population, but increased the production of these cytokines by LPS-activated DCs in a dose-dependent manner (Fig. 4).

HNK suppresses the LPS-induced allogeneic T cell proliferation

The above-mentioned results showed that HNK inhibited the expression of MHC-II and costimulatory molecules by LPS-activated DCs (Fig. 2). These surface molecules are involved in T-cell costimulation. Therefore, we further examined whether HNK affects LPS-induced DC-elicited T-cell proliferation. Immature DCs generated from human monocytes were treated with HNK at various concentrations (0, 5, 10, 15, and 20 μg/ml) in the absence or presence of LPS (1 μg/ml) for 2 days. The DCs were then cocultured with allogeneic T cells in an MLR assay. We found that HNK treatment of LPS-activated DCs significantly inhibited the proliferation of allogeneic T cells in the MLR assay in a dose-dependent manner (Fig. 5). When immature DCs were treated with HNK at various concentrations in the absence of LPS, the proliferation of the allogeneic T cells remained unaffected (data not shown).

HNK alters the LPS-induced Th1 response toward the Th2 response

In the initial stage of the DC-T cell interaction, the cytokine microenvironment plays a key role in Th cell differentiation toward the Th1 or Th2 cell types. Effector Th1 cells predominantly secrete IFN-γ and IL-2 and regulate cell-mediated immunity against intracellular pathogens, whereas differentiated Th2 cells produce IL-4, IL-10, and IL-13 and

TABLE 1. Effects of HNK on the expression of the phenotypic characteristics of DCs

	CD11c	CD40	CD80	CD83	CD86	MHC-II
Immature DCs						
Immature DCs only	200 ± 11	33 ± 5	14 ± 2	7 ± 2	86 ± 9	37 ± 6
Immature DCs + 10 μg/ml HNK	172 ± 10[a]	23 ± 2[a]	11 ± 2[a]	6 ± 1	78 ± 17	30 ± 8[a]
Immature DCs + 20 μg/ml HNK	147 ± 16[a]	17 ± 3[a]	9 ± 1[a]	5 ± 2	52 ± 8[a]	23 ± 6[b]
LPS-activated DCs						
LPS-activated DCs only	336 ± 30	68 ± 6	54 ± 13	45 ± 9	389 ± 21	204 ± 23
LPS-activated DCs + 10 μg/ml HNK	171 ± 6[a]	26 ± 5[b]	18 ± 6[a]	6 ± 2[a]	62 ± 12[b]	43 ± 12[b]
LPS-activated DCs + 20 μg/ml HNK	140 ± 13[a]	19 ± 6[b]	14 ± 4[a]	6 ± 2[a]	47 ± 14[b]	33 ± 12[b]

Value are mean fluorescent intensity ± SD, n = 3. The statistical significance was set at [a]$P < 0.05$; [b]$P < 0.01$ compared to DCs only group.

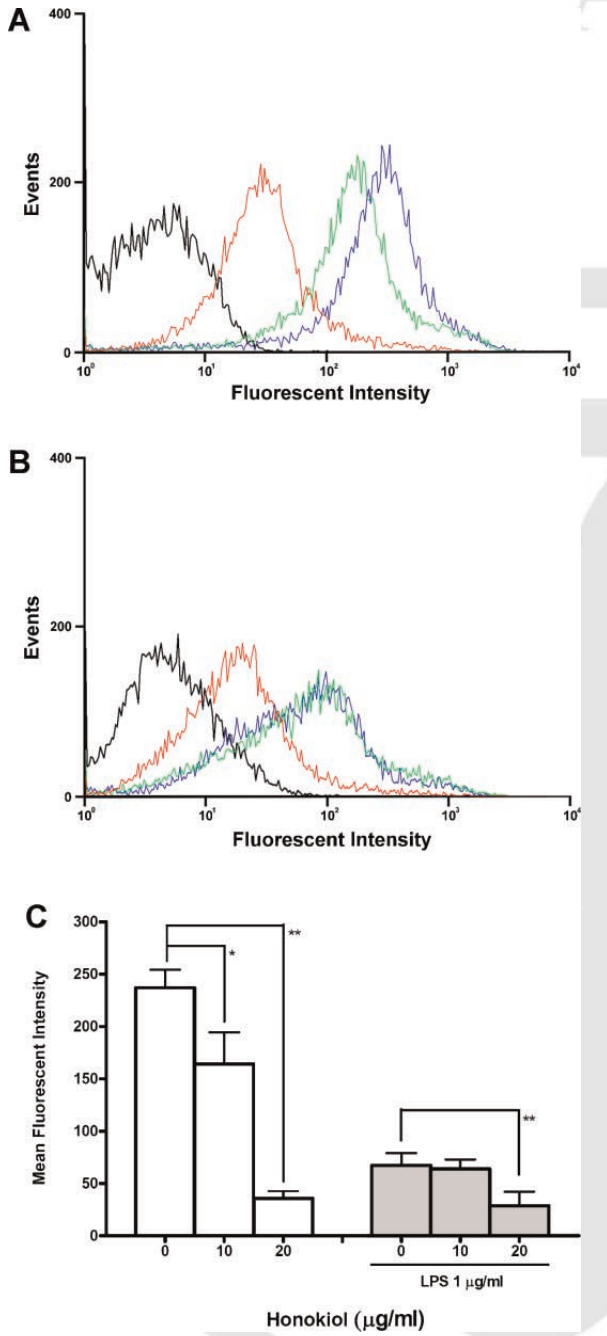

Fig. 3. Effects of HNK on the endocytic activity of DCs. Human immature DCs were treated with HNK at various concentrations (0 μg/ml (blue line), 10 μg/ml (green line), or 20 μg/ml (red line)) of HNK in the (A) absence or (B) presence of LPS (1 μg/ml) for 2 days. The cells were then incubated with FITC–dextran either at 37°C for 30 min or at 4°C as a control (black line). The endocytic capacity was determined by assessing the endocytosis of FITC–dextran by flow cytometry. The results are representative of three independent experiments. C: Data are presented as mean fluorescent intensity ± SD from three independent experiments. The statistical significance of the difference between two experimental measurements was assessed by Student's *t*-test and represented as follows: $^{*}P < 0.05$ and $^{**}P < 0.01$.

promote antibody-mediated humoral immune responses (Abbas et al., 1996). We investigated the T-cell differentiation induced by HNK-treated DCs. For this, culture supernatants from allogeneic MLR experiments were analyzed by ELISA. The results showed that the HNK-treated immature DCs did not alter the background levels of IFN-γ, IL-4, IL-10, and IL-13 produced by T cells (Fig. 6A). However, IFN-γ production by T cells cultured with HNK-treated LPS-activated DCs was lower than that by T cells cultured with DCs treated with LPS alone (Fig. 6A). Further, IL-4, IL-10, and IL-13 production by T cells cultured with HNK-treated LPS-activated DCs was greater than that by T cells cultured with DCs treated with LPS alone (Fig. 6A). We further confirmed the cytokine produced by T cells cultured with HNK-treated LPS-activated DCs using intracellular staining assay. Our experimental results indicated that culturing with HNK-treated LPS-activated DCs had a lower proportion of IFN-γ producing T cells and had a greater proportion of IL-4 producing T cells than culturing with LPS-activated DCs alone (Fig. 6B). The proportions of IFN-γ or IL-4 producing T cells had no significant change between HNK-treated immature DCs and immature DCs alone (data not shown).

HNK inhibits the activation of NF-κB and MAPK in LPS-activated DCs

LPS potently induces DC maturation and the production of proinflammatory cytokines by the activation of TLR4 through the NF-κB and MAPK signaling pathways (Medzhitov, 2001; Dabbagh et al., 2002). To examine whether the effects of HNK on LPS-induced DCs are associated with the NF-κB and MAPK signaling cascades, immature DCs were treated with LPS in the presence or absence of HNK. The phosphorylation levels of MAPK, including ERK1/2, JNK1/2, and p38, were analyzed by Western blot analysis. The experimental results showed that HNK inhibits the phosphorylation levels of ERK1/2 significantly, p38 modestly, and JNK1/2 slightly on LPS-activated DCs (Fig. 7). We also found that HNK inhibits the phosphorylation of IκBα significantly and IKKα modestly on LPS-activated DCs (Fig. 7). These results indicate that HNK inhibits the activation of the MAPK and NF-κB signaling cascades in LPS-activated DCs.

Discussion

DCs play an important role in both the innate and adaptive immune responses. LPS can trigger an acute inflammatory response, leading to a series of pathological events such as septic shock (Martich et al., 1993). DCs are extremely sensitive to LPS and respond by releasing inflammatory mediators, maturing, and triggering an antigen-specific T-cell response (Verhasselt et al., 1997; Rieser et al., 1998; Lee et al., 2007). During DC maturation, the increased expression of MHC-II and costimulatory molecules leads to T-cell response priming and proinflammatory cytokine secretion (Banchereau and Steinman, 1998; Banchereau et al., 2000). Several characteristics of DCs change during DC maturation, for example, CD83 expression is induced (Reddy et al., 1997), the expression of MHC-II and costimulatory molecules increases, and endocytic ability is lost (Banchereau et al., 2000).

Our experimental results indicate that HNK suppresses the LPS-induced expression of the CD11c, CD40, CD80, CD83, CD86, and MHC-II molecules by DCs, reduces the LPS-induced inflammatory cytokine (Th1-type cytokines) production while increasing anti-inflammatory cytokine (Th2- and regulatory-type cytokines) production by DCs, suppresses the LPS-induced DC-elicited allogeneic T-cell proliferation, and Thus, HNK effectively prevents the LPS-induced inflammatory response and DC maturation. Recent study also demonstrated that HNK treatment decreased levels of proinflammatory cytokines and increased levels of the Th2- and regulatory-type cytokines IL-13, IL-10, and TGF-β in lung homogenates from

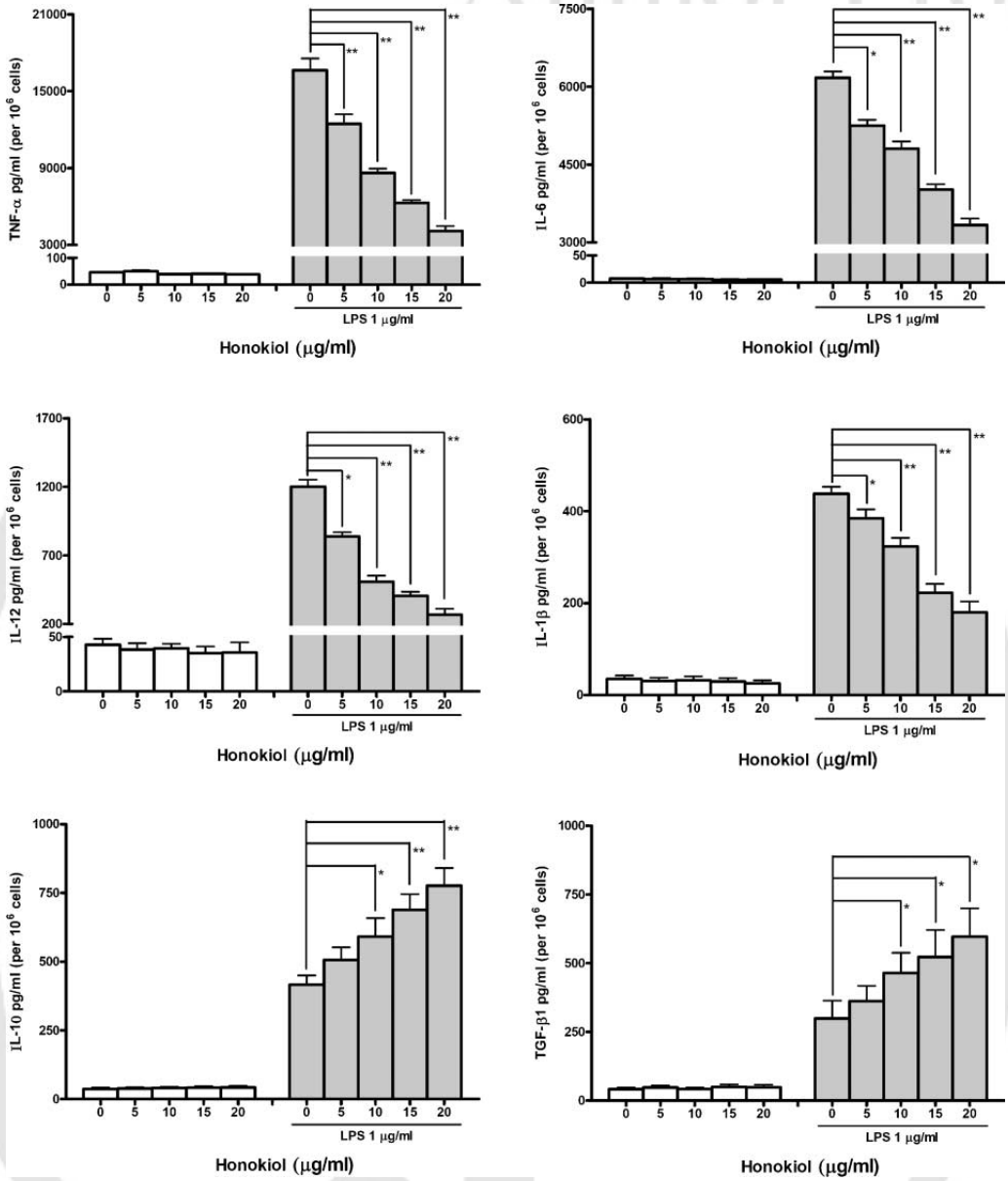

Fig. 4. Effects of HNK on cytokine production by DCs. Human immature DCs were treated with HNK at various concentrations (0, 5, 10, 15, and 20 μg/ml) in the absence or presence of LPS (1 μg/ml) for 2 days. The amounts of TNF-α, IL-6, IL-12p70, IL-1β IL-10, and TGF-β1 in the culture supernatants were measured by ELISA. Data are presented as mean ± SD from three independent experiments. The statistical significance of the difference between two experimental measurements was assessed by Student's *t*-test and represented as follows: $^{*}P < 0.05$ and $^{**}P < 0.01$.

mice in acute and chronic asthma models (Munroe et al., 2010). In addition, HNK decreased the mannose receptor-mediated endocytic ability of LPS-stimulated DCs (Fig. 3B). Although endocytic ability is a characteristic of mature DCs, previous studies have shown that mannose receptor-mediated endocytosis does not affect the expression of MHC-II and costimulatory molecules, and antigen cross-presentation by mannose receptor-deficient DCs (Burgdorf et al., 2006). The mannose receptor plays a homeostatic role in the clearance of microorganisms and serum glycoproteins (Burgdorf et al., 2006). Therefore, we suggest that HNK not only inhibits LPS-induced DC maturation but also decreases the mannose receptor-mediated endocytic ability of DCs.

Uncommitted $CD4^{+}$ T helper cells can develop into four types of T cells: Th1, Th2, Th17, and regulatory T cells, based on the local cytokine milieu. The balance between these four types of helper T cells is important for the regulation of immune responses (Bettelli et al., 2006; Korn et al., 2009). Th1 cells are involved in cellular immunity, Th2 cells are involved in humoral immunity, Th17 cells are characterized as IL-17-producing CD4+ T cells and are critical for the initiation and maintenance of inflammation, and regulatory T cells are important in the maintenance of self-tolerance and in regulation of inflammation (Saito, 2010). Previous studies have indicated that the balance between Th17 and regulatory T cells as a key factor that regulates helper T-cell function relating to Th1/Th2 shift (Bettelli et al., 2006; Afzali et al., 2007). Our results indicate that HNK shifts the LPS-activated DC-driven Th1 response toward the Th2 response which may associate with the balance between Th17 and regulatory T cells. We further examined

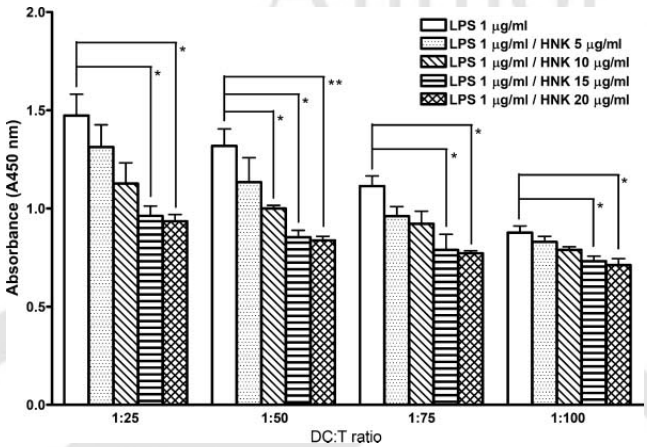

Fig. 5. Effects of HNK on the induction of allogeneic T-cells proliferation by LPS-activated DCs. Human immature DCs were treated with HNK at various concentrations (0, 5, 10, 15, and 20 μg/ml) and LPS (1 μg/ml) for 2 days. The results of allogeneic T-cell proliferation were obtained with different DC:T cell ratios and various dose responses. Allogeneic T-cell proliferation was measured by BrdU ELISA. Data are presented as mean ± SD from three independent experiments. The statistical significance of the difference between two experimental measurements was assessed by Student's *t*-test and represented as follows: $^{*}P < 0.05$ and $^{**}P < 0.01$.

whether HNK-treated LPS-activated DCs affected the balance between Th17 and regulatory T cells. Our experimental results showed that IL-17 production by T cells cultured with HNK-treated LPS-activated DCs was lower than that by T cells cultured with DCs treated with LPS alone (Supplementary Fig. S2). In addition, culturing with HNK-treated LPS-activated DCs had a lower proportion of $CD4^{+}CD25^{+}FoxP3^{+}$ regulatory T cells than culturing with LPS-activated DCs alone (Supplementary Fig. S3). These results indicated that HNK shifting the LPS-activated DC-driven Th1 response toward the Th2 response was not due to promoting regulatory T cells differentiation.

CD14 is essential for the LPS binding through the TLR4 receptor. Although monocyte-derived DCs do not express CD14 (data not shown; Coutant et al., 1999; Yang et al., 2008), soluble CD14 can also mediate LPS binding on DCs, although with lower efficiency (Verhasselt et al., 1997; Fenton and Golenbock, 1998; Scott and Billiar, 2008). In addition, LPS uptake can also be mediated by the β2-integrin receptor, independent of CD14 (Fenton and Golenbock, 1998; Ammon et al., 2000; Scott and Billiar, 2008). A question derived appears that HNK may compete with DCs for LPS binding and uptake leading to the inhibition of LPS-induced DC maturation and the subsequent inflammatory response. The results of the LPS-binding assay in this study showed that HNK does not affect LPS binding on DCs (Supplementary Fig. S4), which suggests that the inhibition of the LPS-induced inflammatory response by HNK is not due to the direct interaction between LPS and HNK. Moreover, we also examined whether HNK affects sCD40L- and CPG ODN-induced DC maturation. Our results showed that HNK inhibits sCD40L- and CPG ODN-induced CD80 and MHC-II expression by DCs, indicating that HNK not only specifically affects LPS-induced DC maturation but also affects sCD40L- and CPG ODN-induced DC maturation (Supplementary Fig. S5). In addition, our results showed that HNK decreases the expression of CD11c, MHC-II, and costimulatory molecules by immature DCs and reduces their endocytic activity. Although these effects may be associated with DC maturation and the function of antigen uptake as well as antigen presentation, the precise mechanisms underlying these results remain unclear at present.

The transcription factor NF-κB plays an important role in the regulation of multiple signaling pathways that control the activation of many immune cells (Li and Verma, 2002; Kure et al., 2010). Inhibition of NF-κB signaling pathway blocks LPS-mediated maturation of DCs and up-regulation of MHC and costimulatory molecules expression (Rescigno et al., 1998). LPS activates NF-κB via a cascade of events leading to the activation of IKKs, which in turn phosphorylate IκB and lead to the activation of NF-κB-mediated gene expression (Karin and Ben-Neriah, 2000). In the present study, we found that HNK treatment inhibited the phosphorylation of IKKα and IκBα, which indicates that the NF-κB pathway may also be involved in the anti-inflammatory effects of HNK on LPS-activated human monocyte-derived DCs. A recent report indicated that HNK completely inhibits TNF-induced NF-κB activation in embryonic kidney cells (A293) and T-cell leukemia (Jurkat) cells (Ahn et al., 2006). Taken together, the results suggest a pharmacological potential of HNK in NF-κB-associated inflammatory disorders. Similar results were reported regarding the action of HNK on exercise-induced muscle damage in rats (Chiang et al., 2009). HNK was reported to suppress high glucose content-induced cyclooxygenase (COX)-2 up-regulation and prostaglandin E_2 production by human umbilical vein endothelial cells (Sheu et al., 2008), the CD40- and LMP1-mediated inflammatory signaling pathway in mouse B-cell activation (Munroe et al., 2007), and the production of IL-8, COX-2, and TNF-α by *Propionibacterium acnes*-activated THP-1 cells (Lee et al., 2005). In addition, the phosphorylation levels of ERK1/2, p38, and JNK1/2 were decreased in HNK-treated DCs, indicating the role of MAPK pathways in HNK-associated anti-inflammatory reactions. MAPK activation is a downstream target of TLR4 signaling, which controls proinflammatory cytokine expression (Robinson and Cobb, 1997). Previous studies have shown that MAPK activation is also important for regulation of the maturation and survival of and cytokine secretion by DCs (Arrighi et al., 2001; Aiba et al., 2003; Nakagawa et al., 2004; Yu et al., 2004). Collectively, the findings indicate that HNK inhibits the LPS-induced maturation and inflammatory responses of DCs and that these changes are associated with the NF-κB and MAPK pathways.

Natural products are a source of many medically beneficial drugs, and their importance in preventing and treating diseases is increasingly being recognized. HNK has the potential to be a novel therapeutic agent. It was found to be safe and effective in

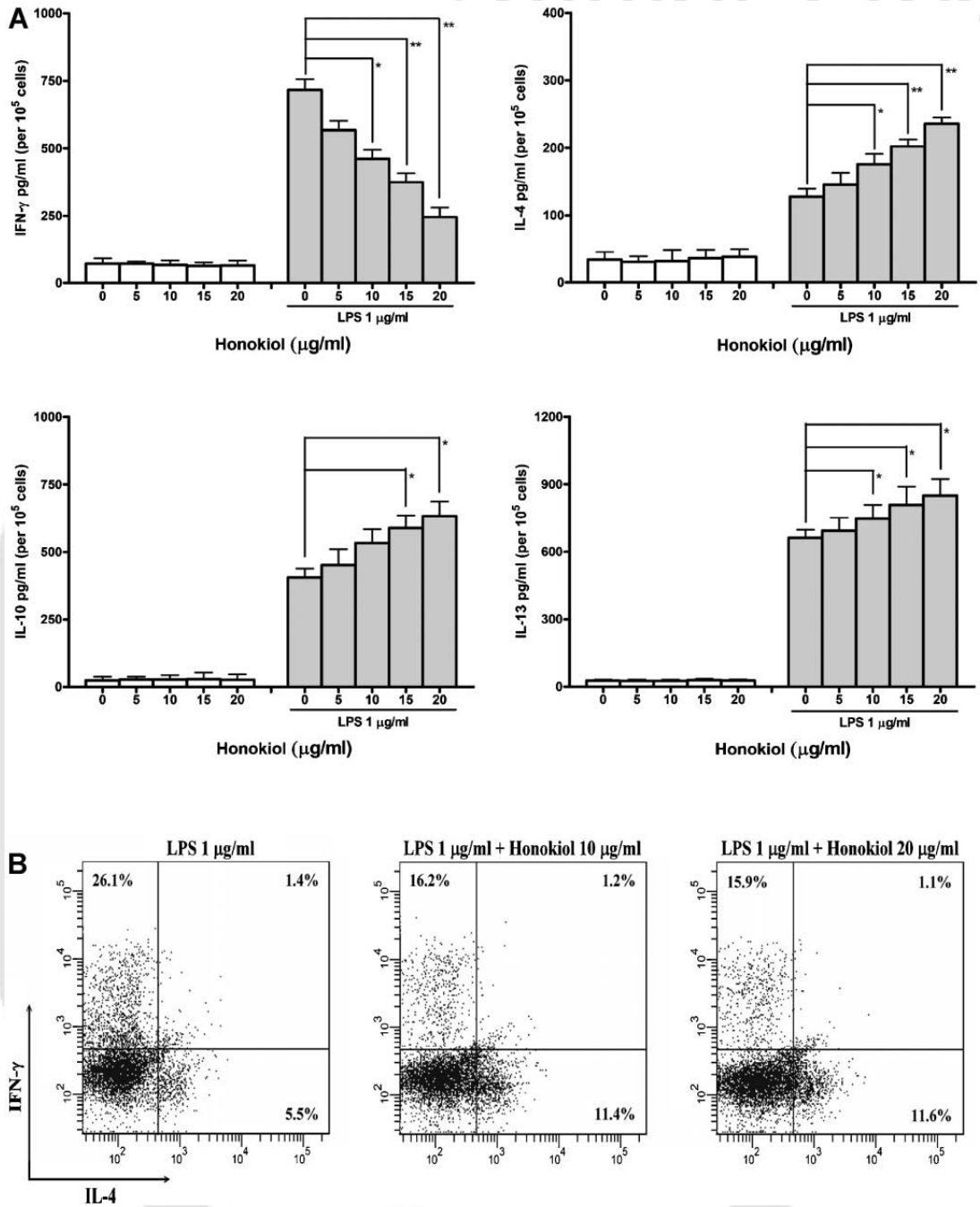

Fig. 6. Effects of HNK on the induction of allogeneic T-cells differentiation by DCs. A: The amounts of IFN-γ, IL-4, IL-10, and IL-13 in the cultured supernatants from allogeneic MLR experiments were measured by ELISA. B: Analysis of intracellular cytokine production by T cells cocultured with LPS- or LPS/HNK-activated DCs. Data are presented as mean ± SD from three independent experiments. The statistical significance of the difference between two experimental measurements was assessed by Student's *t*-test and represented as follows: $^{*}P < 0.05$ and $^{**}P < 0.01$.

treating inflammatory arthritis in a mouse model (Munroe et al., 2007) and as an anti-angiogenic agent in the treatment of cancer, both in vitro and in vivo (Bai et al., 2003; Shigemura et al., 2007). HNK has poor water solubility and pharmacokinetics that limit its clinical application in immune and cancer therapy (Liu et al., 2008). However, as a solution to overcoming these problems, it can be administered by encapsulation in liposomes (Wen et al., 2009) or micelles (Gong et al., 2009).

In summary, to our knowledge, this investigation is the first to demonstrate the anti-inflammatory activity of HNK in LPS-activated DCs. Our results show that HNK inhibits LPS-induced DC maturation, suppresses the LPS-induced overactive inflammatory response, inhibits LPS-induced T-cells proliferation, alters the LPS-activated DC-driven Th1 response toward a Th2 response, and inhibits phosphorylation in the NF-κB and MAPK signaling cascades. The findings suggest that HNK inhibits the LPS-induced maturation and the inflammatory response of human monocyte-derived DCs via the MAPK and NF-κB signaling pathways. Finally, this study provides new insights into the anti-inflammatory properties of HNK and

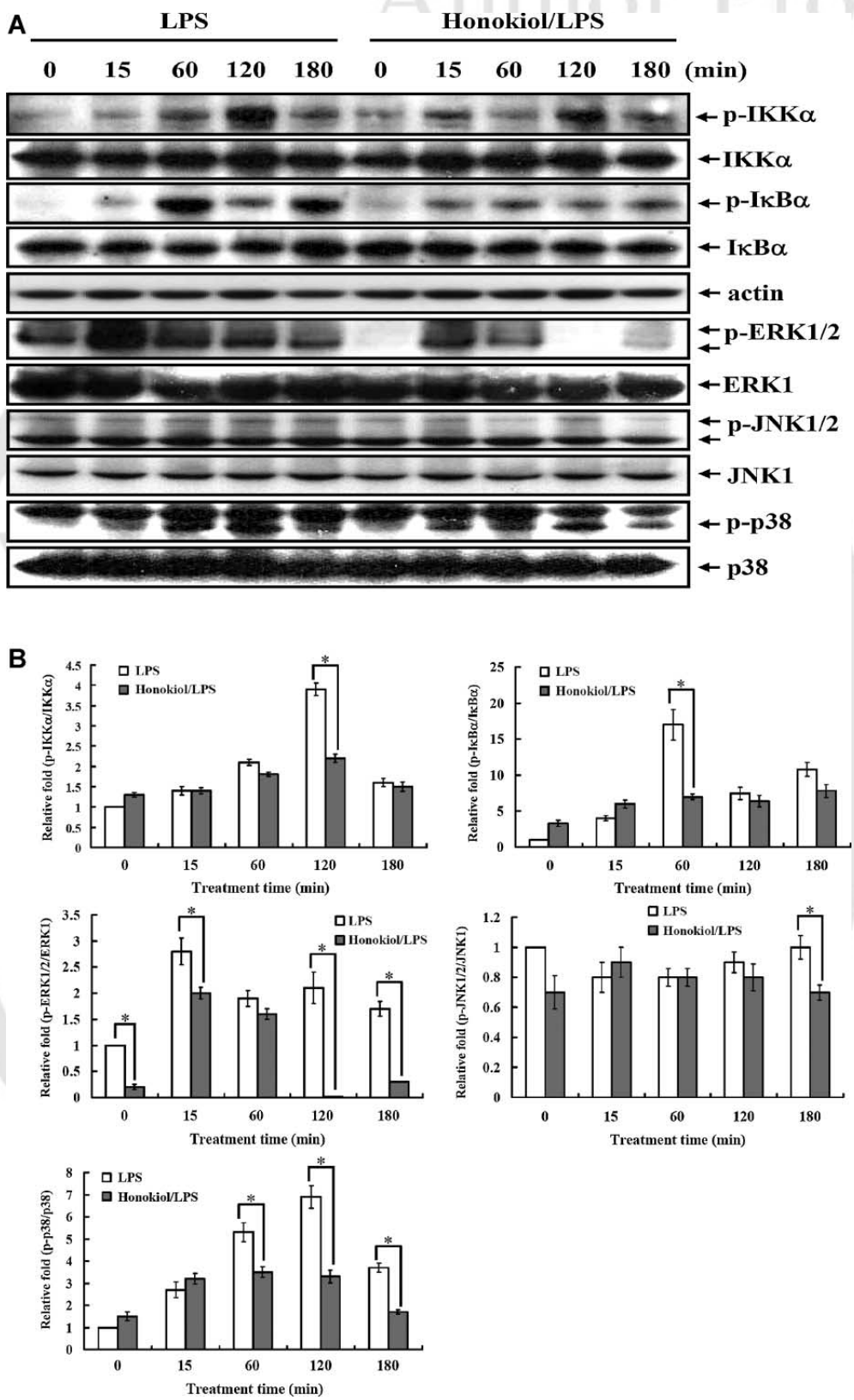

Fig. 7. Effects of HNK on the activation of the NF-κB and MAPK signaling cascades in LPS-activated DCs. Human immature DCs were treated with LPS (1 μg/ml) in the absence or presence of HNK (20 μg/ml) for 0, 15, 60, 120, and 180 min. A: The phosphorylation levels of ERK1/2, JNK1/2, p38, IKKα, and IκBα were measured by Western blot analysis. The figure is a representative of three independent experiments. B: The relative fold of phosphorylation activity each time was quantified using AlphaImage software and normalized to that of the unphosphorylated form and compared to untreated control group (LPS alone).

further supports the role of HNK as a candidate medicine for treating inflammation-related diseases.

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