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Immunomodulation of phloretin by impairing dendritic cell activation and function

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Dietary compounds in fruits and vegetables have been shown to exert many biological activities. In addition to antioxidant effects, a number of flavonoids are able to modulate inflammatory responses. Here, we demonstrated that phloretin (PT), a natural dihydrochalcone found in many fruits, suppressed the activation and function of mouse dendritic cells (DCs). Phloretin disturbed the multiple intracellular signaling pathways in DCs induced by the Toll-like receptor 4 (TLR4) agonist lipopolysaccharide (LPS), including ROS, MAPKs (ERK, JNK, p38 MAPK), and NF-κB, and thereby reducing the production of inflammatory cytokines and chemokines. Phloretin also effectively suppressed the activation of DCs treated with different dosages of LPS or various TLR agonists. The LPS-induced DC maturation was attenuated by phloretin because the expression levels of the MHC class II and the co-stimulatory molecules were down-regulated, which then inhibited the LPS-stimulating DCs and the subsequent naïve T cell activation in a mixed lymphocyte reaction. Moreover, *in vivo* administration of phloretin suppressed the phenotypic maturation of the LPS-challenged splenic DCs and decreased the IFN-γ production from the activated CD4 T cells. Thus, we suggest that phloretin may potentially be an immunomodulator by impairing the activation and function of DCs and phloretin-contained fruits may be helpful in the improvement of inflammation and autoimmune diseases.

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Introduction

Many components found in dietary plants have benefits for human health. In particular, the antioxidant effect of many compounds, derived from plant metabolites, is attractive.[1,2] In addition, researchers also focus on the regulatory effect of inflammation by natural compounds and their application in improving diseases,[3] such as asthma, rheumatoid arthritis, and cardiovascular diseases.[4–7] More and more dietary compounds with health benefits are continuously being identified.

Phloretin, a naturally occurring phytochemical found in the Rosaceae family, is a dihydrochalcone of bicyclic flavonoids.[8] It widely exists in the bark, leaves, and fruit of apple trees.[4] Phloretin has many biological activities, such as antioxidation, the regulation of glucose transporters, and anticancer.[8–13] With regards to the study of its anti-inflammatory effect, phloretin significantly inhibits proinflammatory gene expressions, such as TNF-α, CXCL-10, IL-8 and represses NF-κB-, IP-10-, and IL-8-promoter driven reporter gene expressions found in the LPS-stimulated, human acute, monocytic leukemia-derived cell line.[14] Recently, Chang *et al.* showed that the levels of proinflammatory cytokines and mediators, such as NO, PGE2, IL-6, TNF-α, iNOS and COX-2, are reduced by phloretin *via* the suppression of NF-κB and MAPK activation in LPS-stimulated murine RAW264.7 macrophages.[15] However, the effect of phloretin on the regulation of immune responses is unknown.

Dendritic cells (DCs) are professional antigen-presenting cells which link innate and adaptive immunity.[16] DCs mature as they meet various stimuli in peripheral tissues, such as pro-inflammatory cytokines and microbial products. Specialized pattern recognition receptors (PRRs), like the Toll-like receptor (TLR) family, sense the microbes, and then the DCs translate the microbial signals to adaptive immunity by presenting antigens to the T cells.[17] Mature DCs reduce antigen-loading, produce cytokines and chemokines, and enhance the expression of major histocompatibility complexes (MHC) and accessory molecules, such as CD86, CD80 and CD40.[18] In addition to their role in controlling infections, DCs are also involved in the pathogenesis of immune disorders, such as chronic

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inflammation and autoimmunity.[19,20] Thus, the modulation of DC activation and function may be a potential way of preventing or curing these kinds of disease.

In the present study, we evaluate the immunomodulatory effect of phloretin on DCs. We found that the LPS-induced activation and the functions of DCs, such as the production of proinflammatory cytokines and chemokines, the expression level of MHC II and co-stimulatory molecules, and the induction of T cell activation, were impaired by phloretin. It is suggested that phloretin may possess the ability to modulate immune responses.

Materials and methods

Mice and generation of DCs

C57BL/6 mice were obtained from the National Laboratory Animal Center (Taipei, Taiwan). All of the mice were housed in the barrier facility at Taichung Veterans General Hospital (Taichung, Taiwan) and all of the procedures were performed in accordance with the Institutional Animal Care and Use Committee guidelines for animal experimentation. DCs were generated from mouse bone marrow, as described previously.[21] Bone marrow (BM) cells were flushed from the femurs and tibias of the C57BL/6 mice and cultured in a RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U mL^{-1} penicillin G, 100 μg mL^{-1} streptomycin, 2 mM L-glutamine, 20 ng mL^{-1} recombinant mouse IL-4, and 10 ng mL^{-1} recombinant mouse granulocyte–monocyte colony-stimulating factor (GM-CSF, Peprotech). A fresh medium was supplied every 2 days, and nonadherent cells were harvested on day 7. $CD11c^+$ cells were enriched from nonadherent BM cells by positive selection using an anti-CD11 kit (Miltenyi Biotec) according to the manufacturer's instructions. The purity of the $CD11c^+$ cells was >90% which were used for the subsequent experiments.

Phloretin cytotoxicity assay

As previously described,[22] the DCs were treated with phloretin (Sigma-Aldrich, dissolved in DMSO) at the concentrations of 3.125, 6.25, 12.5, 25, 50 and 100 μM for 24 h. Less than 0.1% (v/v) of DMSO was used in all of the experiments. The viability of the cultured cells was assessed using the colorimetric assay kit Cell Counting Kit-8 (CCK-8) according to the manufacturer's instructions (Sigma-Aldrich).

Measurement of cytokine and chemokine production

Supernatants were collected from the DCs (1×10^6 per mL) treated with the conditions reported previously.[23] The DCs were pretreated with 0.1% DMSO or the indicated dose of phloretin for 1 h before TLR stimulation. The TLR ligands, including the LPS (100 ng mL^{-1}, TLR4), Poly I:C (250 μg mL^{-1}, TLR3), PGN (1 μg mL^{-1}, TLR2), CpG (200 nM. TLR9), and Imiquimod (3 μg mL^{-1}, TLR7) (which were all from Invivogen) were used to stimulate the DCs. After 18 h (4 h for TNF-alpha and RANTES), the cytokines (TNF-α, IL-6, IL-12, and IL-10) and chemokines (MIP-1β, and RANTES) were produced in a culture supernatant and were measured using ELISA (Peprotech).

Western blotting

The DCs were pretreated with 0.1% DMSO or phloretin (25 and 50 μM) for 1 h and were immediately stimulated with the LPS (100 ng mL^{-1}). After 30 min, the cells were harvested and lysed, and then SDS-PAGE and western blotting were performed. The primary antibodies against phospho-p38, protein p38, phospho-p42/44, protein p42/44, phospho-JNK, NF-κB p65, histone, β-actin (all from Cell Signaling Technology), and protein JNK (Santa Cruz Biotechnology) were used and subsequently detected by HRP-conjugated secondary antibodies (Jackson ImmunoResearch). The protein signals were then developed with enhanced chemiluminescence (GE Healthcare) and analyzed using the LAS3000 system (Fujifilm). Densitometric analysis was performed with Image J software (National Institute of Health, MD, USA). Phosphorylated Erk, p38, and JNK were normalized with their total protein.

Assay of NF-κB activation

The DCs were pretreated with 0.1% DMSO or phloretin (25 and 50 μM) for 1 h and were immediately stimulated with the LPS (100 ng mL^{-1}). After 30 min, the nuclear extracts of the DCs were prepared using the NE-PER Nuclear and Cytoplasmic Extraction system (Pierce) according to the manufacturer's instructions. The protein concentrations were determined using a BCA protein assay kit (Pierce). The activated NF-κB in the nuclear extracts (5 μg mL^{-1} for each assay) was measured using a TransAM NF-κB p65 ELISA kit (Active Motif) according to the manufacturer's instructions.

Measurement of Reactive Oxygen Species (ROS)

To detect the intracellular ROS level, the DCs (3×10^5 cells mL^{-1}) were cultured in the presence of 0.1% DMSO (control) or phloretin (25 and 50 μM) for 1 h and then stimulated with the LPS (100 ng mL^{-1}) for 3 h. After stimulation, the media were replaced by a fresh medium containing 10 μM 5-(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCFDA, Molecular Probes) and the cultures were kept in the dark for 30 min at 37 °C before being thoroughly washed with PBS. The fluorescent signal was detected and analyzed by flow cytometry.

Assay for the effect of specific inhibitors

To confirm the molecular mechanism of phloretin reducing the TLR4 signaling pathway, the DCs were pretreated with various specific inhibitors, including SB203580 (for p38 MAPK, 10 μM), JNKI (for JNK, 10 μM), PD98059 (for ERK, 10 μM), BAY 11-7082 (for NF-kB, 10 μM) (all from Calbiochem), *N*-acetyl-L-cysteine (NAC for ROS, 10 μM), or cobalt protoporphyrin-IX (CoPP-IX for ROS, 50 μM) (all from Sigma-Aldrich) for 1 h and then incubated with the LPS for 30 min. The production of cytokine from the LPS-stimulated DCs was determined by ELISA, as described previously.

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Analysis of the DC maturation

The DC maturation was determined by the upregulation of the MHC class II and costimulatory molecule expression, as described previously.[24] The cells were pretreated with 0.1% DMSO or phloretin (25 and 50 μM) for 1 h and stimulated with the LPS for 18 h, and then stained with mAbs which are specific for mouse CD11c, I-A^b, CD40 and CD80 (Biolegend) as well as with isotype-matched control antibodies. The expression levels of each molecule were analyzed by flow cytometry and the results were analyzed by WINMDI software (Scripps, La Jolla, CA, USA).

Allogenic mixed lymphocyte reaction

The DC-induced T cell activation was assayed by an allogenic mixed lymphocyte reaction, as described previously.[25] The T cells were isolated from the spleen of Balb/c mice using an Easy-Sep Mouse T Cell Enrichment Kit according to the

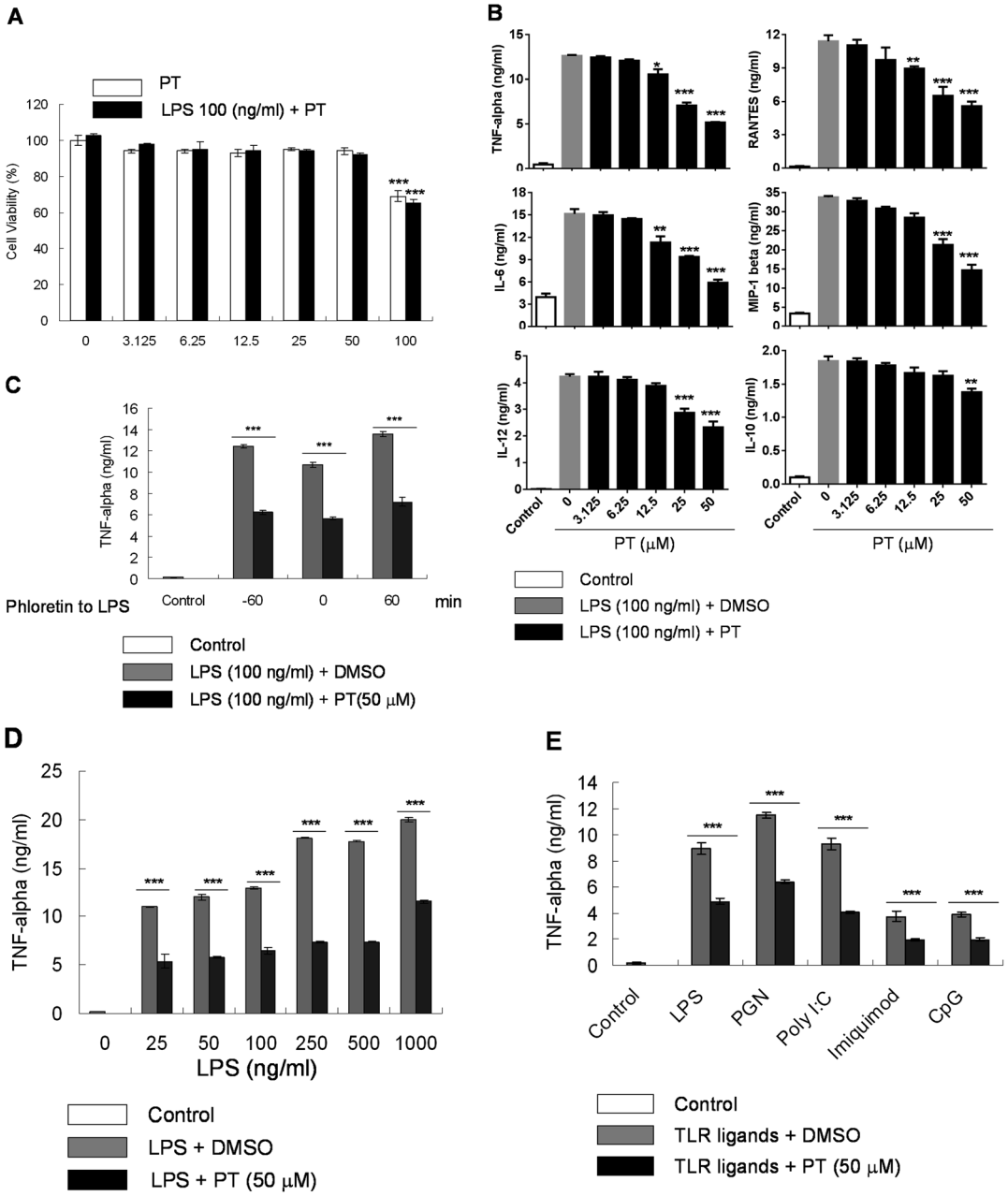

Fig. 1 Phloretin efficiently inhibited DC activation. (A) The cytotoxicity of phloretin (PT) on immature (without the LPS) and mature (with the LPS) DCs were analyzed by a CCK-8 assay kit. (B) The production of cytokines and chemokines were determined by ELISA. The supernatants were collected from the phloretin-treated LPS-stimulated DCs after 18 h (4 h for TNF-α and RANTES). (C) The inhibitory effect of phloretin on the LPS-stimulated DCs at different time points. The DCs were treated with phloretin (50 μM) for 1 h before or after the LPS stimulation and the supernatants were harvested to determine the TNF-α production. (D) The inhibitory effect of phloretin (50 μM) on the DCs stimulated with different doses of the LPS. (E) The inhibitory effect of phloretin (50 μM) on the DCs stimulated with various TLR ligands. The data are expressed as the mean ± SD of triplicate repeats. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; similar results were obtained from the three independent experiments.

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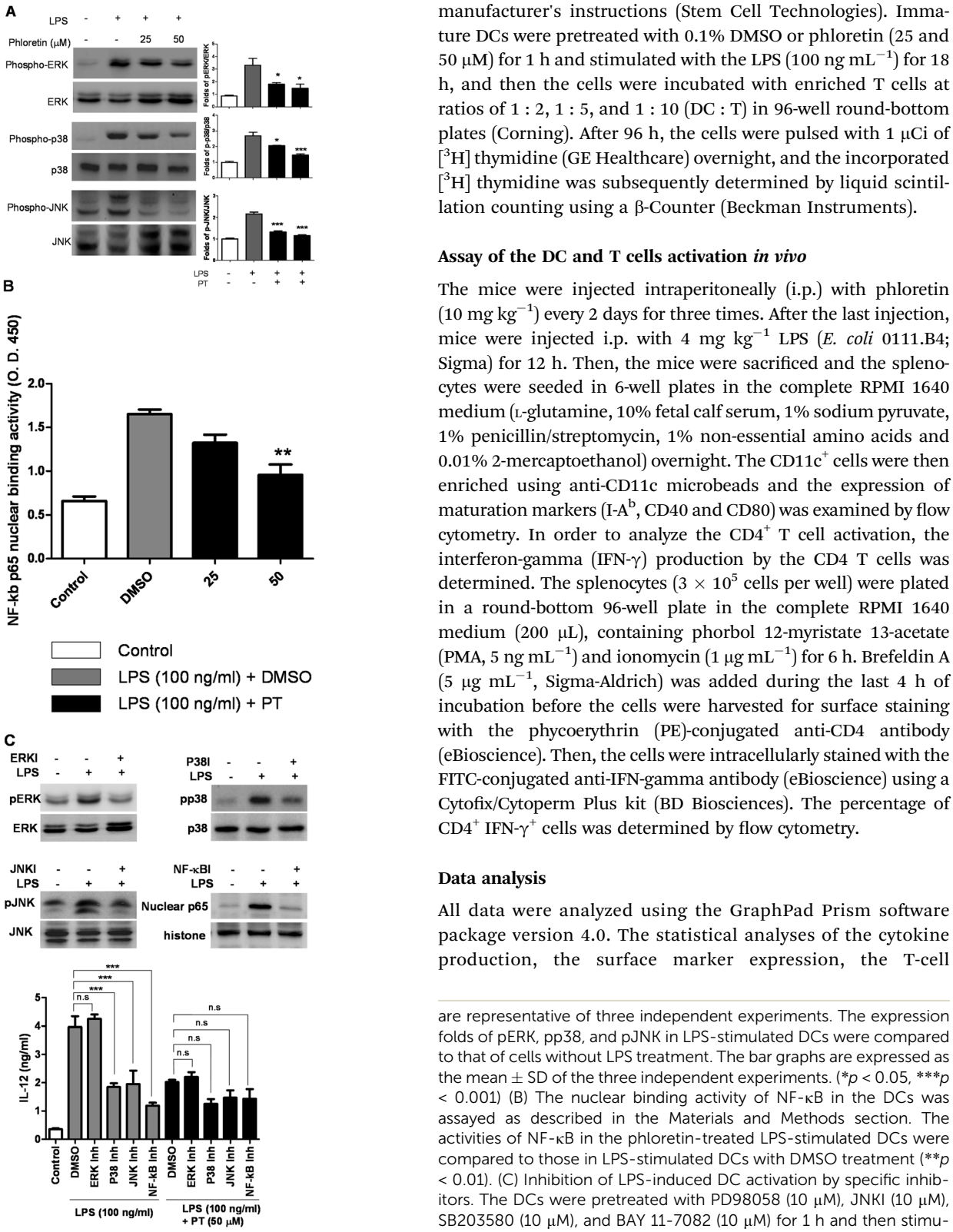

Fig. 2 Phloretin disturbed MAPK activation and NF-κB translocation in the LPS-stimulated DCs. The DCs were pretreated with DMSO or phloretin (25 and 50 μM) for 1 h and then stimulated with 100 ng mL^{-1} LPS for 30 min, and lysed immediately for assaying protein phosphorylation. (A) The phosphorylation of ERK, JNK, and p38 MAPK in the DCs was determined by western blotting. The total ERK, JNK, and p38 MAPK proteins were used for loading control, respectively. The results are representative of three independent experiments. The expression folds of pERK, pp38, and pJNK in LPS-stimulated DCs were compared to that of cells without LPS treatment. The bar graphs are expressed as the mean ± SD of the three independent experiments. (*$p < 0.05$, ***$p < 0.001$) (B) The nuclear binding activity of NF-κB in the DCs was assayed as described in the Materials and Methods section. The activities of NF-κB in the phloretin-treated LPS-stimulated DCs were compared to those in LPS-stimulated DCs with DMSO treatment (**$p < 0.01$). (C) Inhibition of LPS-induced DC activation by specific inhibitors. The DCs were pretreated with PD98058 (10 μM), JNKI (10 μM), SB203580 (10 μM), and BAY 11-7082 (10 μM) for 1 h and then stimulated with LPS for 30 min. The whole or nuclear extracts were assayed for the phosphorylation of ERK/JNK/p38 MAPK and NF-κB p65 by western blotting, respectively (upper panels). For cytokine production, the groups of DCs shown were stimulated by LPS for 18 h and the supernatants were collected for the detection of IL-12 by ELISA. The differences between the inhibitor-treated and the DMSO-treated LPS-stimulated DCs are shown (***$p < 0.001$). All data are representative of three independent experiments.

manufacturer's instructions (Stem Cell Technologies). Immature DCs were pretreated with 0.1% DMSO or phloretin (25 and 50 μM) for 1 h and stimulated with the LPS (100 ng mL^{-1}) for 18 h, and then the cells were incubated with enriched T cells at ratios of 1 : 2, 1 : 5, and 1 : 10 (DC : T) in 96-well round-bottom plates (Corning). After 96 h, the cells were pulsed with 1 μCi of [^{3}H] thymidine (GE Healthcare) overnight, and the incorporated [^{3}H] thymidine was subsequently determined by liquid scintillation counting using a β-Counter (Beckman Instruments).

Assay of the DC and T cells activation *in vivo*

The mice were injected intraperitoneally (i.p.) with phloretin (10 mg kg^{-1}) every 2 days for three times. After the last injection, mice were injected i.p. with 4 mg kg^{-1} LPS (*E. coli* 0111.B4; Sigma) for 12 h. Then, the mice were sacrificed and the splenocytes were seeded in 6-well plates in the complete RPMI 1640 medium (L-glutamine, 10% fetal calf serum, 1% sodium pyruvate, 1% penicillin/streptomycin, 1% non-essential amino acids and 0.01% 2-mercaptoethanol) overnight. The $CD11c^+$ cells were then enriched using anti-CD11c microbeads and the expression of maturation markers (I-A^b, CD40 and CD80) was examined by flow cytometry. In order to analyze the $CD4^+$ T cell activation, the interferon-gamma (IFN-γ) production by the CD4 T cells was determined. The splenocytes (3×10^5 cells per well) were plated in a round-bottom 96-well plate in the complete RPMI 1640 medium (200 μL), containing phorbol 12-myristate 13-acetate (PMA, 5 ng mL^{-1}) and ionomycin (1 μg mL^{-1}) for 6 h. Brefeldin A (5 μg mL^{-1}, Sigma-Aldrich) was added during the last 4 h of incubation before the cells were harvested for surface staining with the phycoerythrin (PE)-conjugated anti-CD4 antibody (eBioscience). Then, the cells were intracellularly stained with the FITC-conjugated anti-IFN-gamma antibody (eBioscience) using a Cytofix/Cytoperm Plus kit (BD Biosciences). The percentage of $CD4^+$ IFN-γ^+ cells was determined by flow cytometry.

Data analysis

All data were analyzed using the GraphPad Prism software package version 4.0. The statistical analyses of the cytokine production, the surface marker expression, the T-cell

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proliferation, the percentage of $CD4^+$/IFN-gamma$^+$ T cells, and the western blotting, were performed using one-way ANOVA followed by Tukey's post hoc test. The values of $p < 0.05$ were considered to be statistically significant.

Results

Cytotoxicity of phloretin on mouse bone marrow derived DCs

To study the effect of phloretin on DCs, we determined the cytotoxicity of phloretin on mouse bone marrow-derived DCs (BMDCs) by CCK-8 assay first. No significant cell death was observed after the treatment of phloretin (<100 μM) alone or when combined with the LPS (100 ng mL^{-1}) (Fig. 1A). Thus, the concentrations of phloretin used in all of the experiments were always less than 50 μM.

Phloretin inhibited production of cytokines and chemokines by LPS-stimulated BMDCs

When DCs are activated, they can produce cytokines and chemokines. Thus, we evaluated the effects of phloretin on BMDCs by detecting the amount of cytokines and chemokines produced. We did not see any activation of the BMDCs by phloretin treatment alone (data not shown), suggesting that the phloretin may not be a stimulant for DCs. We then treated the BMDCs with various concentrations of phloretin (3.125, 6.25, 12.5, 25 or 50 μM) together with the LPS (100 ng mL^{-1}). The amount of proinflammatory cytokines (TNF-α, IL-6, IL-12), anti-inflammatory cytokine (IL-10), and chemokines (RANTES and MIP-1β) was reduced in comparison to the treatment of the LPS alone (Fig. 1B). In addition, this inhibitory effect of phloretin on TNF-α production, secreted by the LPS-stimulated BMDCs, was significant when adding phloretin both before and after the LPS stimulation (Fig. 1C). These results indicate that phloretin has a suppressive effect on the production of pro-inflammatory cytokines and chemokines by the LPS-stimulated DCs.

Phloretin blocked TNF-α production by BMDCs stimulated with various TLR agonists

Phloretin also blocked the TNF-α production by BMDCs stimulated with the LPS at a high concentration (1000 ng mL^{-1}) (Fig. 1D). Furthermore, phloretin exerted a similar inhibitory

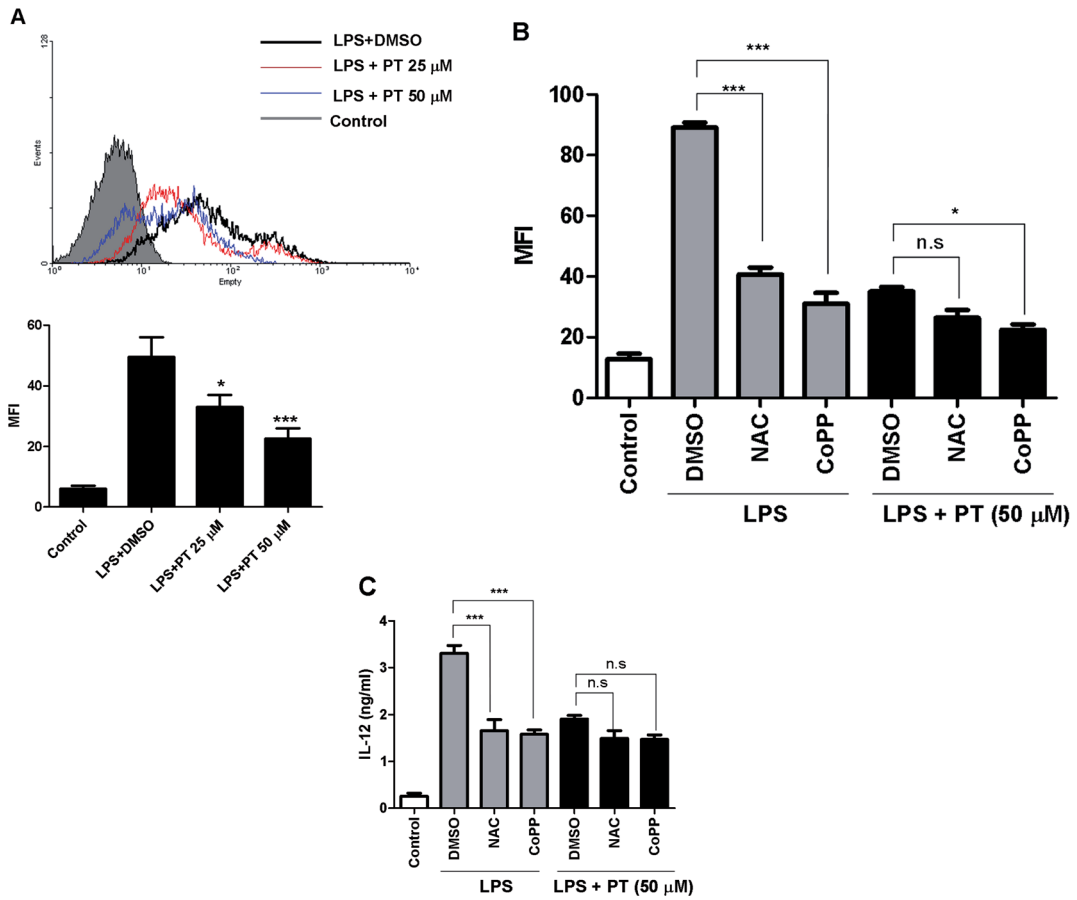

Fig. 3 ROS production in LPS-stimulated DCs was impaired by phloretin. (A) DCFDA-loaded DCs were pretreated with phloretin (25, 50 μM) for 1 h and then stimulated with 100 ng mL^{-1} LPS for 6 h. The ROS generation within the cells was determined by flow cytometry as represented by the mean fluorescence intensity (MFI). The data are expressed as the mean ± SD of the triplicate; *$p < 0.05$, ***$p < 0.001$. (B) Inhibition of ROS production in the LPS-stimulated DCs by specific inhibitors. The DCs were pretreated with NAC or CoPP-IX for 1 h before DMSO or phloretin treatment, and then stimulated with LPS for 6 h. ROS production was measured by flow cytometry with DCFDA and was shown by the MFI as described above. The data are expressed as the mean ± SD of the triplicate; *$p < 0.05$, ***$p < 0.001$, n.s $p > 0.05$. (C) The DCs were treated as described in (B) and stimulated with LPS for 18 h. The supernatants were collected for the determination of IL-12 production by ELISA. The differences between the inhibitor-treated and the DMSO-treated LPS-activated DCs are shown (***$p < 0.001$, n.s $p > 0.05$). All data are representative of three independent experiments.

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effect on BMDCs activated by various TLR agonists, such as PGN (for TLR2), poly I:C (for TLR3), Imiquimod (for TLR7), and CpG (for TLR9) (Fig. 1E). These data show that phloretin efficiently blocks the TNF-α production by the activation of DCs *via* all of the TLR signaling pathways.

Activation of MAPKs and NF-κB was disturbed by phloretin in LPS-stimulated BMDCs

The activation of MAPKs and NF-κB in TLR signaling was critical for regulating the release of proinflammatory cytokines and chemokines in DCs. We subsequently investigated the involvement of these signaling pathways in the suppressive effect of phloretin on DCs. MAPKs, including ERK 1/2, p38, and JNK, were activated by BMDCs after the LPS stimulation; however, the phloretin decreased the phosphorylation of these MAPKs but not the amounts of them (Fig. 2A). In addition, the nuclear binding activity of NF-κB (p65) was also effectively attenuated by the phloretin in a dose-dependent manner (Fig. 2B). Next, we used specific inhibitors to confirm that the reduction of the MAPK and NF-κB activation might be the mechanism for the suppressive effect of phloretin on the DCs. The DCs were pretreated with SB203580 (for p38 MAPK), JNK inhibitor II (for JNK), PD98059 (for ERK), and BAY 11-7082 (for NF-κB) for 1 h and then stimulated with the LPS. These inhibitors significantly reduced the activation of MAPKs and NF-κB (Fig. 2C, upper panels). The production of IL-12 decreased when assayed with the DCs stimulated with the LPS in the presence of specific inhibitors, including SB203580, JNK I and BAY 11-7082, or inhibitors plus phloretin, but did not decrease with PD98059. However, co-treatment with SB203580, JNK I or BAY 11-7082 did not significantly increase the suppressive effect of phloretin (Fig. 2C, lower panel), indicating that the inhibitory effect of phloretin on the LPS-stimulated DCs is overlapped with the inhibitors used. Thus, we concluded that phloretin inhibits the cytokine and chemokine production of the LPS-stimulated DCs by disturbing the MAPKs and NF-κB signaling pathways.

Phloretin modulated ROS production in LPS-stimulated DCs

Reactive oxygen species (ROS) have been shown to participate in the activation of DCs.[26] To assess the effect of phloretin on ROS production, we analyzed the ROS levels in the DCs after LPS stimulation with or without phloretin pretreatment. As expected, phloretin reduced LPS-mediated ROS production in the DCs (Fig. 3A). In order to confirm this suppressive mechanism, LPS-stimulated DCs were pretreated with the antioxidant *N*-acetyl-L-cysteine (NAC) or cobalt protoporphyrin-IX (CoPP). As shown in Fig. 3B and C, NAC or CoPP (with or without phloretin) significantly reduced the ROS level and IL-12 production in the

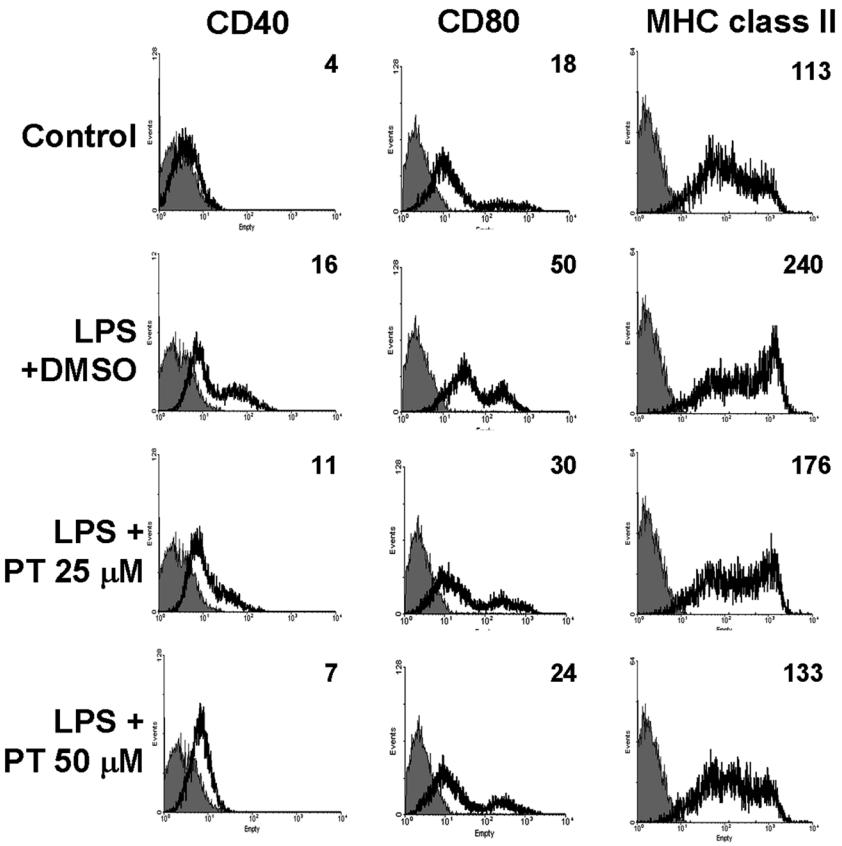

Fig. 4 Phloretin attenuated LPS-induced DC maturation. The DCs were pretreated with phloretin (25 and 50 μM) for 1 h and then stimulated with 100 ng mL^{-1} LPS for 18 h. The control group was pretreated with 0.1% DMSO. The expressions of MHC class II, CD40 and CD80, were determined by flow cytometry. The MFI is indicated in each graph. The gray area represents the isotype-matched mAb control. Similar results were obtained from three independent experiments.

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LPS-stimulated DCs. Therefore, these results indicate that phloretin suppresses cytokine production in LPS-stimulated DCs *via* the inhibition of ROS production.

Attenuation of LPS-induced DC maturation and the subsequent induction of T cell activation by phloretin *in vitro*

After activation the DCs undergo a maturation process and then initiate the adaptive immune responses. To investigate the effect of phloretin on DC maturation, we examined the expressions of CD40, MHC class II and costimulatory molecule CD80 in BMDCs, which represent the key phenotypes of DC maturation. The expression levels of CD40, MHC class II, and CD80 on BMDCs stimulated by LPS were increased (mean fluorescence intensity (MFI) = 4 to 16, 113 to 240 and 18 to 50, respectively), but were dose-dependently reduced (MFI = 16 to 11 and 7, 240 to 176 and 133, 50 to 30 and 24, respectively) by phloretin (Fig. 4).

The primary function of mature DCs is to induce the activation and proliferation of naive T-cells. The treatment of BMDCs with phloretin effectively reduced their T cell priming function in a mixed lymphocyte reaction (MLR) as the proliferation of the cocultured naive T cells was suppressed (Fig. 5). Collectively, we suggested that phloretin can attenuate LPS-stimulated DC maturation and the subsequent induction of the T cell activation by the activated DCs.

Suppression of LPS-induced DC maturation and T cell activation by phloretin *in vivo*

The inhibitory effect of phloretin on the DC activation and maturation has been demonstrated *in vitro*. Therefore, we further examined its inhibitory effect *in vivo*. The B6 mice were administered intraperitoneally with phloretin (10 mg kg^{-1}) for 3 times, as described in the Materials and Methods section, and then were challenged with LPS (4 mg kg^{-1}). The splenic DCs were harvested and analyzed for the expression of the MHC class II and CD80 maturation markers. The expression levels of MHC class II and CD80 were lower in the phloretin-treated DCs than in DMSO-treated ones (Fig. 6). Next, we determined the effect of phloretin on DC-induced T cell activation by detecting the IFN-γ produced by the CD4 T cells. Consistently, the percentage of IFN-γ-producing CD4 T cells was reduced in the phloretin-treated mice compared to DMSO control mice (from 10% to 3.5%) (Fig. 7). Thus, we concluded that phloretin can suppress the phenotypic maturation of the DCs and impair the T cell activation after LPS treatment *in vivo*.

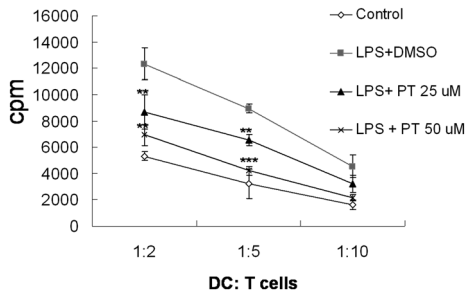

Fig. 5 Phloretin impaired the T cell activation by LPS-stimulated DCs. The DCs were pretreated with DMSO or phloretin for 1 h and then stimulated with 100 ng mL^{-1} LPS for 18 h. The $CD4^+$ T cells isolated from Balb/c mice were mixed with the DCs at the ratios of 1 : 2, 1 : 5 and 1 : 10 and incubated for 96 h. The proliferation of the cells was determined by $[^3H]$-thymidine incorporation assay. The data are expressed as the mean ± SD of the triplicate. $^{**}p < 0.01$, $^{***}p < 0.001$; similar results were obtained from three independent experiments.

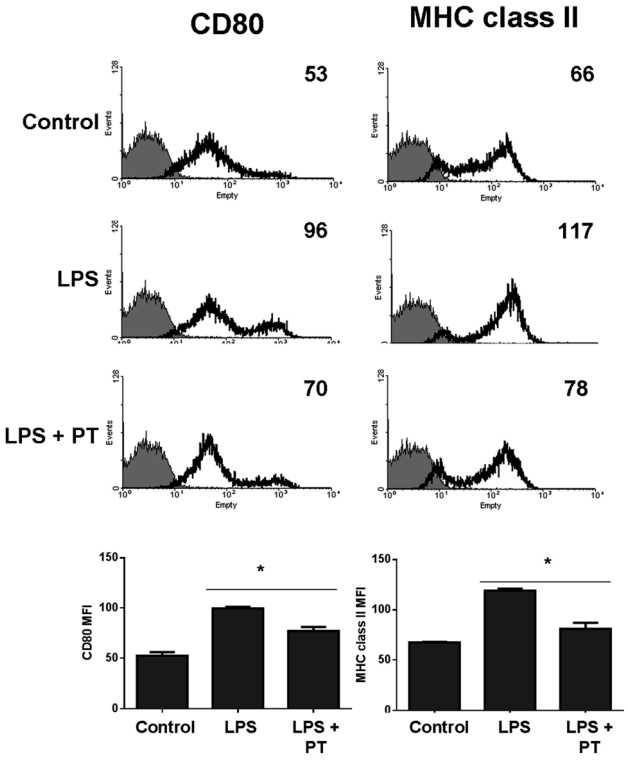

Fig. 6 Suppression of the phenotypic maturation of the LPS-challenged splenic DCs by phloretin *in vivo*. The mice were injected i.p. with phloretin (10 mg kg^{-1}) for three times at two-day intervals and then injected i.p. with the LPS (4 mg kg^{-1}). After 12 h, the splenocytes were isolated and the $CD11c^+$ DCs were assayed for the expression levels of CD80 and $I\text{-}A^b$ (MHC class II), as described in the Materials and Methods section. The MFI is indicated in each graph. The gray-filled histograms represent the isotype-matched mAb control. The expression levels (MFI) of CD80 and $I\text{-}A^b$ are presented as the mean ± SD of the three samples. $^*p < 0.05$; the data are representative of two independent experiments ($n = 6$ in each experiments).

Discussion

We identified the suppressive effect of phloretin on DC activation and function in this study and provided both *in vitro* and *in vivo* evidence. Phloretin has been shown to exert various biological activities; however, here we reported for the first time that phloretin is an immunomodulator which can inhibit DC function. This information may suggest that fruits containing phloretin, especially apple, can promote health by reducing harmful immunity.

Phloretin is a flavonoid, a group of polyphenols with a large spectrum of biological effects.[27,28] Many bioactivities of

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phloretin have been reported, including antioxidant, anti-inflammation, and anti-cancer.[15,29,30] Although several studies have shown the suppressive effect of phloretin on immune cells, such as macrophages,[15,31] osteoclasts,[32] neutrophils,[33] basophils,[34] and T cells,[35,36] there is no report on DCs. It will be interesting to explore the inhibitory effect of phloretin on other types of immune cell. These studies will provide cell-specific information which is necessary for the development of powerful immunosuppressants derived from phloretin.

Here we showed that phloretin disturbed multiple intracellular signaling pathways in DCs, including MAPKs (ERK, JNK, p38 MAPK) and NF-κB (Fig. 2), and these observations agree with the effects of phloretin on mouse skin[37] and macrophages.[15] NF-κB is critical for LPS-induced DC activation and maturation, including cytokine and chemokine production, and the up-regulation of costimulatory molecules.[38] Furthermore, NF-κB subunits c-Rel and p50 control the expression of the costimulatory molecule CD40 and IL-12 production by LPS-stimulated DCs.[39] Thus, the inhibition of NF-κB activation might be an important mechanism for the suppressive effect of phloretin on DC activation.

We reported that phloretin can inhibit the LPS-induced MAPK signaling pathways in the DCs. In contrast, recent studies showed that the phloretin activated MAPK pathways in HEI-OC1 auditory cells[40] and enhanced p38 MAPK activation in PINK1–KO astrocytes.[41] There may be two possible explanations for the different effects of phloretin on MAPK activities. One may be due to differences in the species or the cell-type. Another explanation could be that phloretin may regulate the upstream of MAPK pathways but not MAPKs directly. Therefore, the identification of the exact mechanisms responsible for these different effects may be helpful for the application of phloretin in the treatment of clinical diseases in the future.

In the specific inhibitor study, the IL-12 production was decreased by LPS-stimulated DCs with SB203580 and JNKI, but not PD98059 (Fig. 2C). It has been shown that LPS can activate all three MAPKs in DCs; however, the inhibition of ERK1/2 by PD98059 does not affect the DC maturation but rather regulates DC survival. In contrast, the inhibition of p38 MAPK by SB203580 profoundly reduces the phenotypic changes during LPS-induced DC maturation.[42,43] Thus, our data are consistent with the different regulation of MAPKs with regards to DC activation and function. We suggest that p38 MAPK and JNK pathways could be involved in the suppressive effect of phloretin on LPS-induced DCs maturation in this study. In addition to the signaling pathways examined in this study, other

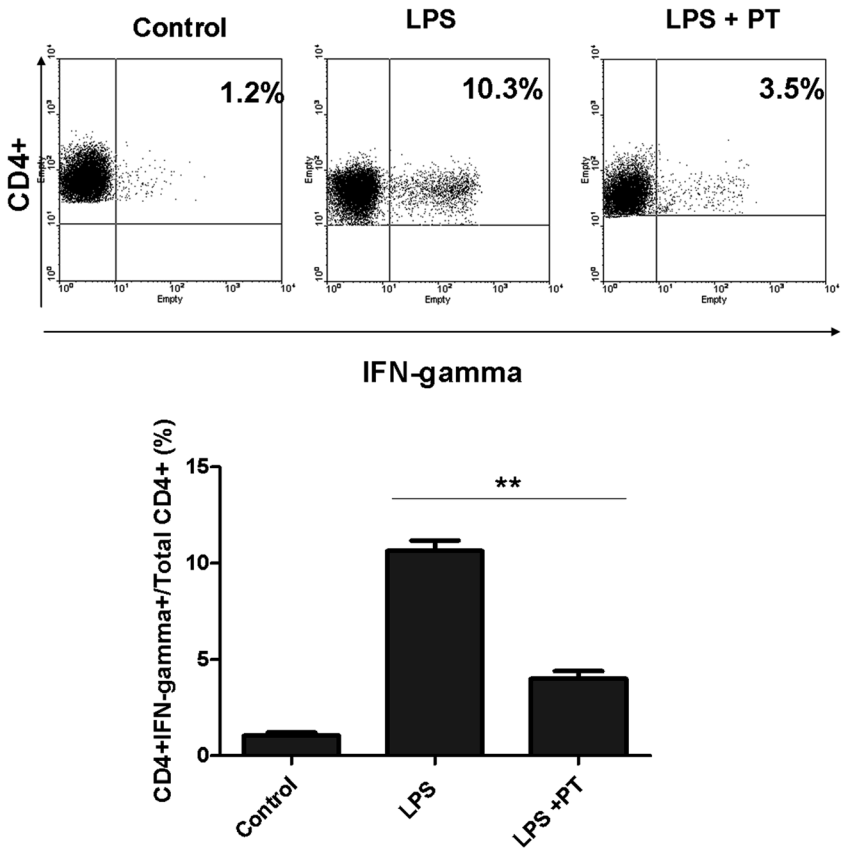

Fig. 7 Phloretin impaired the IFN-γ production by the $CD4^+$ T cells in the LPS-challenged mice. The mice were injected i.p. with phloretin (10 mg kg^{-1}) for three times at two-day intervals and then injected i.p. with LPS (4 mg kg^{-1}). After 12 h, the splenocytes were isolated and stimulated with PMA and ionomycin for 6 h, and then the production of IFN-γ by the $CD4^+$ T cells was analyzed using flow cytometry as described in the Materials and Methods section. The percentages of the $CD4^+$IFN-γ^+ cells are indicated in the dot plots. The bar graphs represent the mean ± SD of four to six mice per group from two independent experiments (**$p < 0.01$).

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mechanisms such as the blocking of protein kinase C,[44] inhibition of Akt activation,[34] and the induction of PPARgamma transcriptional activity[45] have been reported. It will be important to examine whether these pathways are also related to the suppressive effect of phloretin on DCs.

We also observed that phloretin, as well as the antioxidants NAC and CoPP, attenuated ROS production in LPS-stimulated DCs in this study (Fig. 3). ROS are known to influence the secretion of cytokine by the DCs and NAC inhibits the antigen presentation of the DCs to the T cells.[46] Another study showed that CoPP reduces LPS-induced phenotypic maturation and the secretion of proinflammatory cytokines in human and rat DCs, resulting in the inhibition of alloreactive T-cell proliferation.[47] In addition, it has been reported that the LPS-induced ROS generation and the related decline in glutathione (GSH)/oxidized glutathione (GSSG) occur in human monocyte-derived DCs and that the former is involved in cytokine production, while the latter is involved in the up-regulation of cell surface molecules and the allostimulatory capacity.[48] Collectively, these results suggest that phloretin may suppress ROS production through its antioxidant activity in LPS-stimulated DCs.

We have proved the immunomodulatory effect of phloretin *in vivo* (Fig. 6 and 7); however, phloretin from fruits needs to be absorbed in the gastrointestinal tract in order to exert its functions. It has been shown that phloretin is found in the urine of rats who have been fed apple juice[49] and in the ileostomy effluent of humans after apple juice consumption.[50] Thus, these reports suggest that direct ingestion of phloretin-containing fruits may have immunomodulatory functions when ingested directly. The strategies for promoting the function of phloretin-containing fruits would be valuable to study.

In summary, our study illustrates that phloretin can suppress DC activation and DC-induced T cell responses, prompting us to speculate that phloretin could potentially be used for the prevention and treatment of inflammation, autoimmunity, and transplantation. In addition, we suggest that fruits containing phloretin can promote health by impairing inflammation-related diseases, especially in the elderly. Although our results showed that phloretin affects DC function *in vitro* and *in vivo*, it remains to be revealed how this modulation happens *via* oral administration. Analyzing more compositions and immune functions in fruits will be important issues in food research.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

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