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Fisetin Inhibits Lipopolysaccharide-Induced Macrophage Activation and Dendritic Cell Maturation

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Macrophages and dendritic cells are required for initiating innate immunity and adaptive immunity. Aberrant activation of macrophages and dendritic cells can cause detrimental immune responses; thus, agents effectively modulating their functions are of great clinical value. We herein investigated whether fisetin, a flavonoid prevalently present in fruits and vegetables, could inhibit macrophage activation and dendritic cell maturation. Fisetin suppressed LPS-induced NF-κB activation, expression of pro-inflammatory proteins (TNF-α and iNOS), MMP-9 activity, and phagocytic activity in macrophages. Furthermore, upon LPS-induced dendritic cell maturation, fisetin at nontoxic concentrations suppressed the expression of costimulatory molecules (CD80 and CD86), the production of cytokines (IL-12, IL-6, and TNF-α), and the endocytic activity of dendritic cells. Fisetin treatment significantly attenuated migration of dendritic cells into spleens and dendritic cell-mediated T cell activation in LPS-treated mice. Collectively, our data reveal that fisetin inhibits macrophage activation and impairs functional maturation of dendritic cells.

KEYWORDS: Fisetin; dendritic cell; macrophage; NF-κB; anti-inflammatory

INTRODUCTION

Macrophages and dendritic cells are important innate immune cells and are essential for induction of adaptive immunity. Macrophages are one of the principal cell types protecting the host from pathogen infection. Upon pathogen invasion, resting macrophages are attracted to infection sites and become activated (*1*). The activated macrophages exhibit higher phagocytic activity and elevated secretion of inflammatory mediators to facilitate pathogen elimination, secrete matrix metalloproteinases (MMPs) to facilitate cellular migration into the infection sites (*2*, *3*), capture and process pathogens into antigenic fragments, and present antigen-major histocompatibility complex class II (MHC II) complexes to native T cells to trigger adaptive immunity (*3*). Pathogen-derived products, such as lipopolysaccharide (LPS), have been shown to trigger macrophage activation via TLR4-dependent pathways (*4*). Aberrant activation of macrophages could result in damage of normal tissues and might cause inflammatory diseases. Therefore, therapeutic interventions targeting macrophage activation may open new avenues for better control of inflammatory diseases.

Dendritic cells are another important type of antigen-presenting cell in both innate and adaptive immunity (*5*, *6*). Tissue-residing immature dendritic cells are highly endocytic and either take up antigens or bind to them via pattern recognition receptors (PRRs) (*5*). Upon antigen processing, immature dendritic cells gradually lose endocytic activity and undergo maturation. Mature dendritic cells express increased levels of co-stimulatory molecules (e.g., CD80 and CD86), exhibit MHC II for presenting antigen to naïve T cells, and secrete IL-12 to promote the differentiation of naïve $CD4^+$ T cells into T_H1 cells as well as stimulate proliferation of T cells. These molecular events trigger subsequent adaptive immunity. It has been shown that some pathogen-derived products and pro-inflammatory cytokines can induce dendritic cell maturation (*7*). For example, LPS can induce dendritic cell maturation via activating TLR4-signaling pathways (*8*, *9*). Aberrant activation of dendritic cells is implicated in the pathogenesis of acute and chronic inflammation and autoimmune diseases (*10*, *11*). Therefore, identification and development of therapeutic agents that can modulate functions of dendritic cells are of great clinical relevance.

Flavonoids are a group of bioactive compounds found frequently in food. Fisetin (**Figure 1**), a flavonoid found in various types of fruits and vegetables, has many pharmacological activities including antioxidant (*12*), anticancer (*13*), neurotrophic (*14*), antiangiogenesis (*15*), and antimutagenic activities (*16*). Recent evidence has demonstrated the immunosuppressive potential of fisetin (*17*−*20*). Fisetin was shown to inhibit basophil-induced production of T_H2-type cytokines and nitric oxide (NO) (*17*, *20*), production of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-8) in human mast cells (HMC-1) (*18*), and production of

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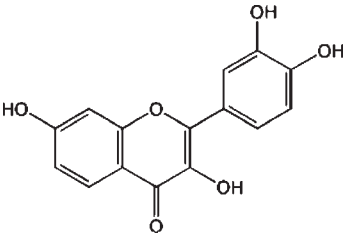

Figure 1. Structure of fisetin (3,3′,4′,7-tetrahydroxy-flavone).

pro-inflammatory cytokines and NO in RAW 264.7 macrophages and peripheral blood mononuclear cells (*18*−*20*). Fisetin was shown to suppress NF-κB activation triggered by many inflammatory agents in cancer cell lines (*13*). Although several reports demonstrated that fisetin can inhibit the production of inflammatory molecules (TNF-α and NO) in macrophages, its effect on aberrant macrophage activation has not been thoroughly evaluated. Furthermore, although the inhibitory effects of fisetin on several immune cell types have been demonstrated, its effect on dendritic cell functions has never been reported. To further explore the immuno-pharmacological potential of fisetin, we herein investigate the inhibitory effects of fisetin on the LPS-induced innate immune responses including macrophage activation and dendritic cell maturation.

MATERIALS AND METHODS

Chemicals and Antibodies. Our procedures required the following reagents: fisetin (Sigma-Aldrich, St. Louis, MO), ultrapure lipopolysaccharide (*Escherichia coli* 0111:B4; Invivogen, San Diego, CA), recombinant mouse GM-CSF (rmGM-CSF) and IL-4 (rmIL-4; Peprotech Inc., Rocky Hill, NJ), anti-iNOS, anti-β-tubulin, anti-mouse CD11c (HL3)-FITC, anti-mouse CD80 (16-10A1)-PE, anti-mouse CD86 (GL-1)-PE, 7-amino-actinomycin D (7-AAD; BD Biosciences, San Jose, CA), anti-actin and anti-BrdU antibody (Sigma-Aldrich), anti-IκB (Ser32) antibody (Cell Signaling, Danvers, MA), anti-mouse MHC Class II (I-A^b, I-A^q, I-A^d, I-E^d, and I-E^k [M5/114-PE]; Miltenyi Biotec, Auburn, CA), anti-mouse CD4 (RM4-5)-PE and isotype-matched control antibodies for CD11c, CD80, and CD86 (BioLegend, San Diego, CA), propidium iodide (Invitrogen, Carlsbad, CA), mouse IL-12p70, IL-6, and TNF-α ELISA kit (R&D Systems, Minneapolis, MN), and FlowCytomix kit (Bender MedSystems, Vienna, Austria).

Cells and Culture Medium. RAW 264.7/Luc-P1 cells, an LPS-responsive cell line with an integrated reporter gene (pELAM1-Luc), were generated from the RAW 264.7 cell line (Food Industry Research and Development Institute, Hsinchu, Taiwan) as described previously (*21*). Both the RAW 264.7 and RAW 264.7/Luc-P1 cells were cultured under conditions described previously (*21*). Dendritic cells isolated from mouse bone marrow were cultured in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (Biological Industries, Kibbutz, Israel), 1% nonessential amino acids, 20 mM HEPES (pH 7.4), 5×10^{-5} M β-mercaptoethanol (β-ME), 20 ng/mL rmGM-CSF, 20 ng/mL rmIL-4, 100 units/mL penicillin, 100 μg/mL streptomycin, 2 μM L-glutamine, and 1 mM sodium pyruvate in a 5% CO_2 incubator set at 37 °C.

Luciferase Reporter Assays. The RAW 264.7/Luc-P1 cells (4×10^5 cells in 24-well plates) were treated with fisetin or vehicle (0.1% DMSO) for 1 h and then LPS for 6 h, harvested, and analyzed using luciferase assays (Promega, Madison, WI) (*21*). The luminescence was measured with an AutoLumat LB953 (Berthold Technologies, Bad Wildbad, Germany).

Enzyme-Linked Immunosorbent Assay (ELISA). RAW 264.7 (2×10^5) or purified bone marrow-derived dendritic cells (10^6) were seeded and treated with fisetin or vehicle (0.1% DMSO) for 1 h and then LPS for various time periods. The supernatant was collected to determine cytokines (IL-12p70, IL-6, and TNF-α) using an ELISA kit. The A450 and A650 (reference absorbance) were measured using a microplate photometer (Multiskan RC, Model 351, Lab Systems, Stockholm, Sweden).

Isolation of Bone Marrow-Derived Dendritic Cells (BM-DCs). C3H/HeN mice were maintained in a specific pathogen-free area at the Animal Center of National Yang-Ming University (Taipei, Taiwan). To isolate dendritic cells, C3H/HeN mice were sacrificed under aseptic conditions, and the femurs were collected and cut open with scissors to expose the bone marrow. The bone marrow was flushed out with medium using a 10 mL syringe with a 25-gauge needle. After centrifugation at 500*g* for 5 min, the cell pellets were resuspended in growth medium using an 18-gauge needle to produce single-cell suspensions and transferred to 60 mm tissue culture dishes. Red blood cells were depleted by lysis with ammonium chloride (8.3 g/L). After incubation for 3 days, the suspended cells were removed and fresh growth medium was added to the adherent cells, which were maintained an additional 3 days. The suspension and loosely adherent cells were harvested and analyzed by flow cytometry. In general, over 70% of these cells expressed CD11c. For measuring cytokine production, purified dendritic cells were obtained using a miniMACS separation system (Bergisch Gladbach, Germany).

Western Blot. RAW 264.7 cells (10^6 cells in 60-mm dishes) were treated with fisetin or vehicle for 1 h and LPS for 30 min (for detecting phosphorylated IκB) or 24 h (for detecting iNOS) and then harvested in RIPA buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 5 mM EDTA [pH 8.0], and 1 mM EGTA [pH 8.0]) containing 0.5% protease inhibitor cocktail. Cell lysate (50 μg) was analyzed as described previously (*22*).

Gelatin Zymography. RAW 264.7 cells (10^6 cells in 60 mm dishes) grown in growth medium were treated with fisetin or vehicle for 1 h and LPS for 24 h and then grown in serum- and phenol-red-free medium for 8 h. After treatment, the culture media without phenol red/serum were collected and concentrated by using Amicon Ultra-4 filter units (Millipore). The proteins in the concentrated samples were quantified using the Bradford reagent (Sigma-Aldrich), and then 50−100 μg was separated by 7.5% SDS-PAGE containing 20 mg/mL gelatin at 4 °C. The gel was washed with renaturation buffer (2.5% Triton X-100), incubated overnight in development buffer (50 mM Tris-HCl, 13.3 mM $CaCl_2$, 50 mM NaCl [pH 7.6]) at 37 °C, and stained with Coomassie blue. The clear zones represent MMP-9 activity.

Phagocytosis Assay. The phagocytosis assay was described previously (*22*). In brief, RAW 264.7 cells (10^5 per well into 6-well plates) were treated with fisetin or vehicle for 1 h and lipopolysaccharide (LPS; 1 μg/mL) for 24 h, incubated with 50 μg of fluorescein-labeled bioparticles (Molecular Probes, Eugene, OR) in 2 mL of medium for 30 min, aspirated free of the bioparticle-containing medium, treated with trypan blue solution (0.25 mg/mL, 300 μL) immediately to quench the extracellular bioparticles, washed with phosphate buffered saline (PBS), trypsinized, and analyzed using a FACSCalibur flow cytometer (BD Biosciences).

Cell Viability of Dendritic Cells. BM-DCs (10^6 cells/well in 24-well plates) isolated from C3H/HeN mice as described above were treated with vehicle (0.1% DMSO) or various concentrations of fisetin for 24 h, stained with propidium iodide (PI; 5 μg/mL), and analyzed using a FACSCalibur flow cytometer.

Expression of Cell Surface Markers of Bone Marrow-Derived Dendritic Cells (BM-DCs). BM-DCs (10^6 cells) after various treatments were reacted with anti-mouse CD11c-FITC, CD80-PE, CD86-PE, or isotype-matched control antibody in 1× HBSS/1% BSA, stained with 7-AAD (0.5 μg/mL) to exclude dead cells, and analyzed using a FACSCalibur flow cytometer to determine the percentage of dendritic cells expressing various surface markers (CD80, CD86, and MHCII).

FlowCytomix. Purified bone-marrow-derived dendritic cells (10^6 per well in 24-well plates) were treated with fisetin or vehicle for 1 h and then LPS for 24 h. The supernatants were collected to measure cytokine expression using the FlowCytomix system (Bender MedSystems). In brief, samples were first reacted with beads coated with 10 different primary antibodies (specific for GM-CSF, IFN-γ, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-17, and TNF-α). The mixture was incubated first with a biotin-conjugated secondary antibody then with streptavidin-PE, and finally analyzed using a FACSCalibur flow cytometer.

Endocytosis Assay. Immature dendritic cells (2×10^5) under various treatments were incubated with growth medium (37 °C, 10 min), treated with FITC-dextran (40 000 molecular mass; 1 mg/mL for 1 h; Sigma-Aldrich), washed three times with Hanks' balanced salt solution containing 1% BSA, and then analyzed using a FACSCalibur flow cytometer.

Animal Experiments and Immunohistochemistry. C3H/HeN mice ($n = 3$) were injected (i.p.) on days 1, 2, 3, and 4 with fisetin 3 mg/kg/day or vehicle (100 μL of PBS containing 3% Tween-80 and 2% DMSO). On day 4, 1 h after fisetin treatment, mice were injected (i.p.) with LPS (50 μg/mice) and sacrificed 12 h later. Spleens were removed for frozen sections. Immunohistochemistry was used to evaluate colocalization of dendritic cells and T cells in spleen sections. In brief, spleen sections (10 μm) were fixed in cold methanol for 10 min, incubated with blocking solution (3% BSA/PBS) for 1 h, stained with CD4-PE (T helper cell marker) and CD-11c (dendritic cell marker) in 1% BSA/PBS overnight, washed in 0.1% Tween 20/PBS, and mounted with ProLong Gold antifade reagent (Invitrogen) for histological examination. All animal protocols were performed according to the instructions issued by the Institutional Animal Care and Use Committee of National Yang-Ming University.

BrdU Incorporation and Immunostaining. C3H/HeN mice ($n = 3$) were injected with fisetin 3 mg/kg (i.p.) or vehicle (100 μL of PBS containing 3% Tween-80 and 2% DMSO) on days 1, 2, 3, and 4 twice per day. One hour after the last fisetin treatment on day 4, these mice were injected (i.p.) with LPS (5 mg/kg), then injected (i.p.) with 100 mg/kg 5-bromo-2′-deoxyuridine (BrdU) 24 h later (on day 5) every 3 h for a total of three injections, and sacrificed 3 h after the last BrdU injection. The spleens were removed for frozen sections. The spleen sections (10 μm) were fixed with cold methanol (10 min), incubated with in 2 N HCl (30 min, 37 °C), treated with 0.1 M $Na_2B_4O_7$ at pH 8.5 (10 min), blocked with 3% BSA/PBS (1 h), stained with anti-BrdU in 1% BSA/PBS overnight, incubated with FITC-conjugated anti-mouse IgG, washed with 0.1% Tween 20/PBS, and mounted with ProLong Gold antifade reagent (Invitrogen) for subsequent microscopic examination. The proliferation status of T cells in spleen sections was evaluated by counting $BrdU^+$ cells in $CD4^+$ areas.

Image Acquisition and Quantification. The fluorescent images from each spleen section were captured by fluorescence microscopy (Olympus BX61) and quantified using Image-Pro Plus software (version 6.0, MediaCybernetics, Bethesda, MD). Five independent microscopic fields from spleen sections of each mouse were analyzed (3 mice per group). To quantify dendritic cells migrating into spleens of mice under various treatments, three areas for each microscopic field were selected and the percentage of these chosen fluorescent areas ($CD11c^+$ areas) versus total fluorescent areas were calculated. The data from the LPS treatment group served as a reference for comparison (set as 100%). In the BrdU incorporation assay, the proliferation status of T cells was measured by counting $BrdU^+$ cells (green fluorescence) in the $CD4^+$ area (red fluorescence). For each microscopic field, the numbers of proliferating T cells from three selected areas were counted.

Statistical Analysis. Data are shown as mean ± SEM of three independent experiments. Statistical analysis was performed using Student's t test to compare between-group differences. Differences were considered as statistically significant when $p < 0.05$.

RESULTS

Fisetin Inhibits Activation of RAW 264.7 Macrophages. Many inflammatory diseases involve the activation of NF-κB, which is a major transcription factor regulating diverse functions. Using an NF-κB activity-based reporter assay in RAW 264.7 macrophages, we evaluated the effects of fisetin on NF-κB activity. LPS, a bacterial glycolipid known to activate macrophages, was used as the inducer. As shown in **Figure 2**, fisetin inhibited NF-κB activity in a concentration-dependent manner and decreased the protein level of phosphorylated IκB (p-IκB; the negative regulator of NF-κB). Furthermore, fisetin treatment reduced LPS-induced production of TNF-α and inducible nitric oxide synthase (iNOS), which are NF-κB downstream pro-inflammatory molecules (**Figure 3A** and **B**). The inhibition of NO production by fisetin was also validated (Supporting Information Figure 1). Since activated macrophages secrete more matrix metalloproteinases (MMPs), gelatin zymography was used to measure MMP-9 activity in fisetin-treated macrophages and demonstrated a concentration-dependent decrease in MMP-9 activity (**Figure 3C**). Given that activated macrophages usually display a distinct

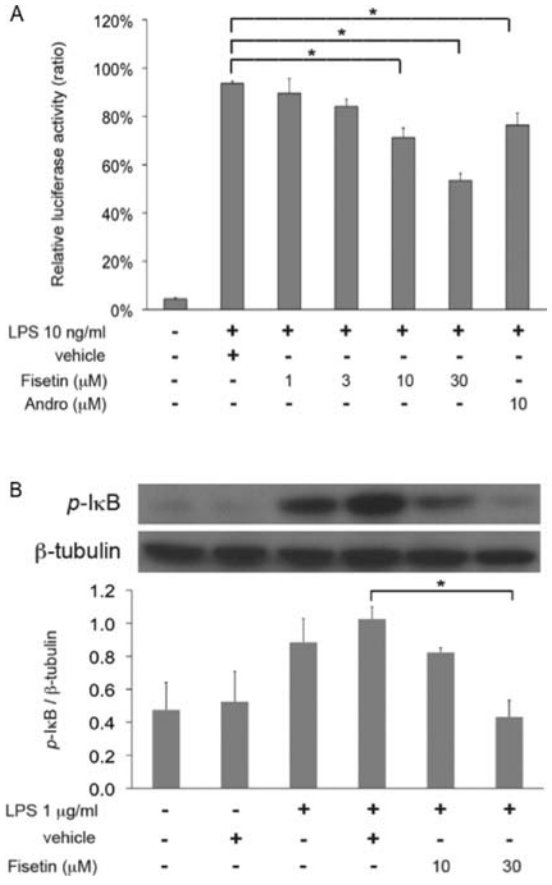

Figure 2. Fisetin inhibits NF-κB activity in LPS-stimulated RAW 264.7 macrophages (**A**). Fisetin inhibits NF-κB activity in a concentration-dependent manner. Andro (andrographolide) is the positive control. Asterisk (*) indicates $p < 0.05$ versus LPS-treated group. (**B**) Fisetin inhibits LPS-induced IκB phosphorylation. IκB phosphorylation and β-tubulin expression were detected by Western blot. β-Tubulin served as the internal control. Quantification data from three independent experiments are shown in the bottom panel, and asterisk (*) indicates $p < 0.05$ versus LPS-treated group.

morphology and acquire higher phagocytotic activity, we next examined whether fisetin shows any effect on morphology and the phagocytic activity of the LPS-treated RAW 264.7 cells. As shown in **Figure 3D**, fisetin significantly inhibited phagocytic ability of the LPS-treated macrophages. In addition, RAW 264.7 cells exposed to LPS exhibited morphological alteration and became polygonal with pseudopodi, as compared with those treated with vehicle. Notably, fisetin at 10 or 30 μM significantly blocked LPS-induced morphological alteration (**Figure 3E**). The effect of fisetin on the cell viability of RAW 264.7 cells was examined by MTT assays, and the results showed that fisetin treatment for 48 h, up to 30 μM, did not cause any cytotoxicity (data not shown). In conclusion, fisetin suppresses NF-κB-signaling, expression/activity of pro-inflammatory molecules (TNF-α, iNOS, and MMP-9), phagocytic function, and morphological features of activated macrophages.

Fisetin Treatment Decreases the Expression of Surface Markers Characteristic of Mature Dendritic Cells. Bacterial LPS is a known inducer of dendritic cell maturation. To determine whether fisetin can modulate dendritic cell maturation, we evaluated the effects of fisetin on LPS-induced maturation of bone-marrow-derived $CD11c^+$ dendritic cells (BM-DCs). The effect of fisetin on viability of BM-DCs was first investigated. Since no cytotoxicity by treatment for 24 h (at 3 or 10 μM) was found (**Figure 4A**), all

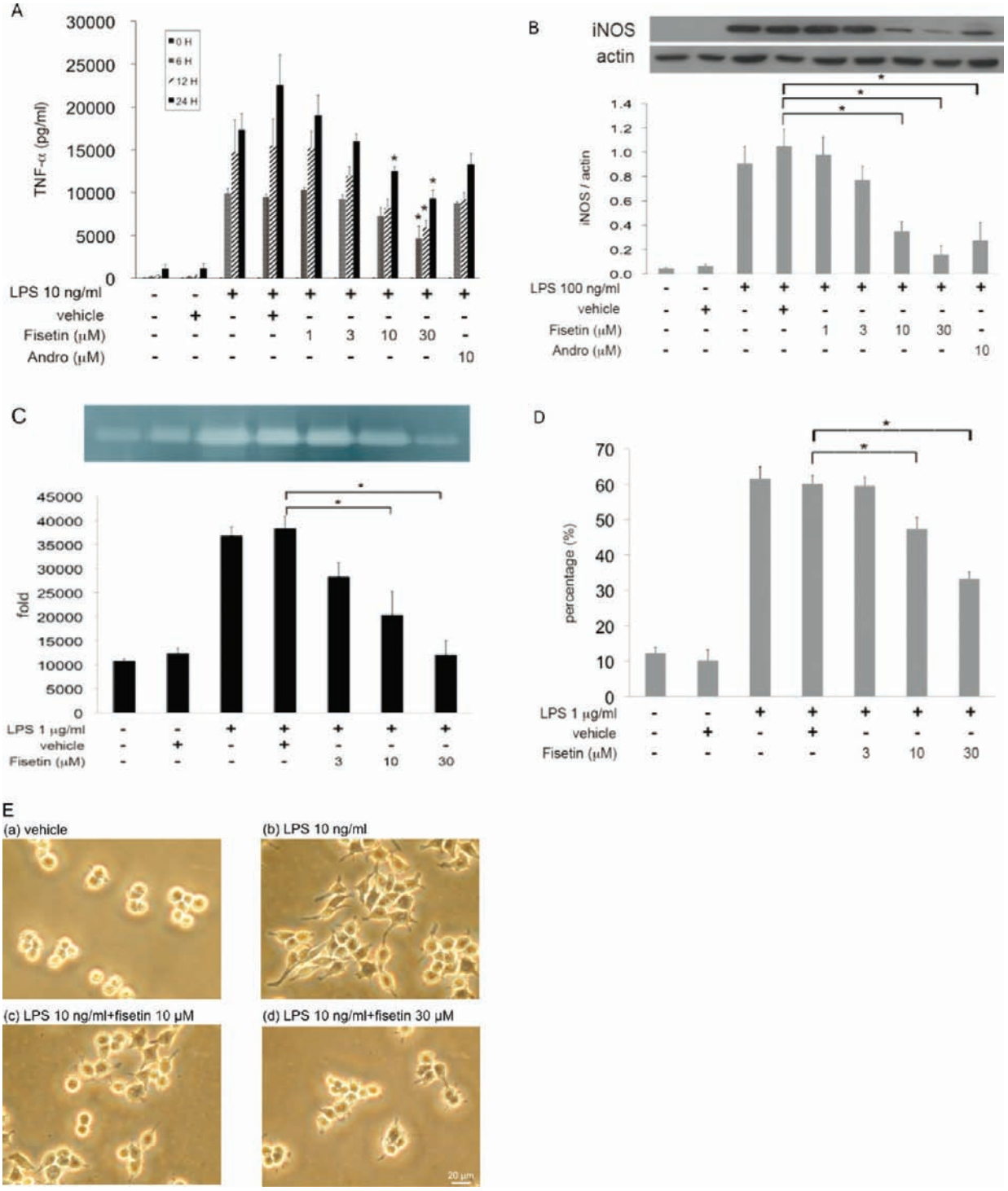

Figure 3. Fisetin inhibits the production of proinflammatory molecules and the phagocytotic activity of LPS-stimulated RAW 264.7 macrophages (**A**). Fisetin suppresses production of TNF-α in a concentration-dependent manner. The RAW 264.7 macrophages were treated with various concentrations of fisetin or vehicle (0.1% DMSO) for 1 h, followed by LPS treatment. Culture medium was then assayed for TNF-α production using ELISA assays. Andro (andrographolide) is the positive control. Asterisk (∗) indicates $p < 0.05$ versus LPS-treated groups. (**B**) Fisetin suppresses production of iNOS in a concentration-dependent manner. The expression of iNOS and actin was detected by Western blot. Actin expression served as the internal control. Asterisk (∗) indicates $p < 0.05$ versus LPS-treated group. (**C**) Fisetin suppresses MMP-9 activity in a concentration-dependent manner. MMP-9 activity was measured by gelatin zymography. Quantification data from three independent experiments are shown in the bottom panel, and asterisk (∗) indicates $p < 0.05$ versus LPS-treated group. (**D**) Fisetin suppressed the phagocytic ability of RAW 264.7 cells in a concentration-dependent manner. Cells under various treatments were incubated with fluorescein-labeled bioparticles and measured using flow cytometry. Quantification data from three independent experiments are shown. Asterisk (∗) indicates $p < 0.05$ versus LPS-treated group. (**E**) Fisetin attenuates morphological alteration induced by LPS. RAW 264.7 cells, treated with vehicle (0.1% DMSO), LPS, or LPS + fisetin for 24 h, were photographed under a phase-contrast microscope.

subsequent fisetin treatments were carried out at these concentrations. We measured the expression of co-stimulatory molecules (CD80 and CD86) and major histocompatibility complex II (MHCII) in the LPS-stimulated dendritic cells with or without

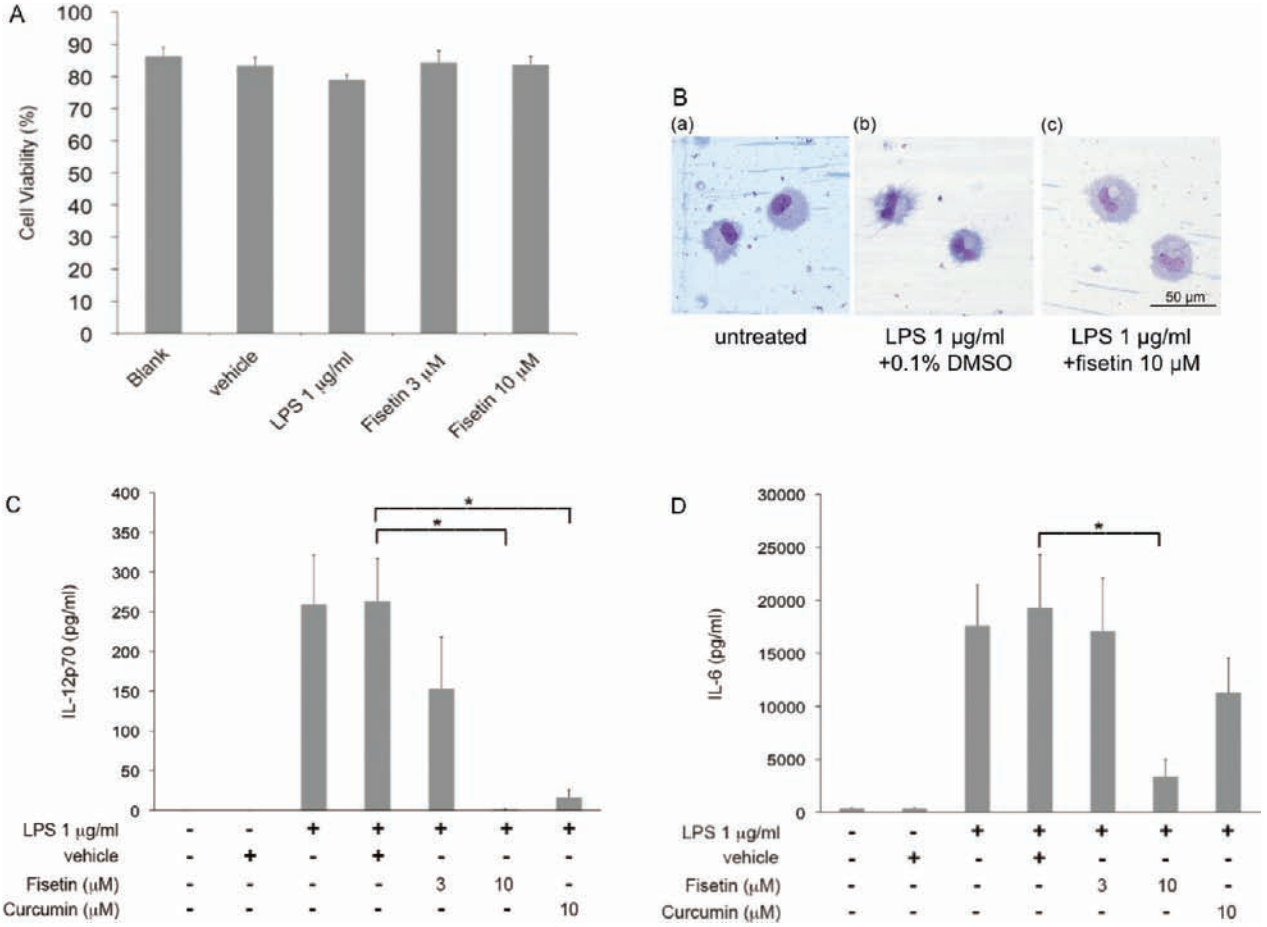

Figure 4. Fisetin inhibits maturation of bone-marrow-derived dendritic cells. (**A**) Fisetin (up to 10 μM) is not cytotoxic to BM-DCs. The effects of fisetin on cell viability of dendritic cells were measured by staining with propidium iodide and analyzing using flow cytometry. (**B**) Morphology of BM-DCs under various treatments. Dendritic cells ($CD11c^+$) were isolated from bone marrow and cultured with RPMI growth medium supplemented with GM-CSF and IL-4 for 6 days. The cells were treated with or without fisetin, followed by incubation with LPS (1 μg/mL) for 24 h. After treatment, cytospin technique was performed and the cells were stained with Giemsa-Wright stain. (a) Dendritic cells in the absence of LPS; (b) dendritic cells in the presence of LPS (1 μg/mL); (c) fisetin-treated dendritic cells in the presence of LPS (1 μg/mL). (**C**, **D**) Fisetin inhibits LPS-stimulated IL-12 and IL-6 secretion ,respectively. The BM-DCs were treated with various concentrations of fisetin or vehicle (0.1% DMSO) for 1 h, followed by LPS treatment for 24 h. Culture medium was then assayed using ELISA assays. Curcumin is the positive control. Asterisk (*) indicates $p < 0.05$ versus LPS-treated group ($p < 0.05$).

fisetin treatment. Fisetin clearly decreased the expression level of CD80 and CD86 but did not significantly affect MHCII expression (**Table 1**). Furthermore, upon LPS stimulation, fisetin-treated dendritic cells showed fewer dendrites than vehicle-treated cells, indicating that the maturation process of dendritic cells was impaired by fisetin treatment (**Figure 4**B).

Fisetin Reduces the Expression of IL-12, IL-1α, TNF-α, and IL-6 of Mature Dendritic Cells. Dendritic cells secrete diverse cytokines to activate adaptive immunity. We investigated the expression pattern of cytokines. The $CD11c^+$ dendritic cells were purified using miniMACs and analyzed for their cytokine production using ELISA and FlowCytomix. We found that fisetin suppressed LPS-stimulated secretion of IL-12 in dendritic cells (**Figure 4C**) and decreased the production of pro-inflammatory cytokines IL-1α, TNF-α, and IL-6 in dendritic cells (**Figure 4D** and **Table 2**).

Fisetin Enhances Endocytic Capacity of Dendritic Cells. Immature dendritic cells exhibit higher antigen-uptake ability, and their maturation is associated with decreased antigen-uptake ability. To further examine the effect of fisetin on the antigen capture of dendritic cells, we measured the endocytic activity of dendritic cells using FITC-conjugated dextran. As shown in **Figure 5A**, fisetin enhanced endocytotic ability of LPS-stimulated BM-DCs, suggesting that fisetin renders dendritic cells to remain immature forms.

Fisetin Attenuates Migration Activity and T-Cell Stimulatory Capacity of Dendritic Cells in Vivo. After maturation, DCs migrate to the secondary lymphoid tissues where they encounter and interact with native T cells. We next examined whether fisetin affected the migration of dendritic cells into the periarteriolar lymphoid sheath (PALS) of spleens in LPS-administered mice. Immunohistochemistry was utilized to detect the presence of dendritic cells and T cells, and demonstrated that fisetin treatment suppressed the migration of dendritic cells, which presumably attenuated the priming of T cells in the PALS of spleens (**Figure 5B** and **C**). As expected, T cell activation induced by LPS-induced dendritic cells was also attenuated by fisetin, as demonstrated by the decreased T cell proliferation after fisetin treatment (**Figure 5D** and **E**). Notably, oral administration of fisetin could also impair the DC migration induced by LPS treatment (Supporting Information Figure 2).

DISCUSSION

Macrophages and dendritic cells are key players in innate immunity and are essential for induction of adaptive immunity. However, aberrant activation of these two cell types could lead to

Table 1. Effect of Fisetin on the Expression of Cell Surface Markers in LPS-Stimulated CD11c$^+$ Dendritic Cells

	MFI[a] (% positive cells)[b]					
	blank (medium)	0.1% DMSO	LPS 1 μg/mL	LPS + 0.1% DMSO	LPS + fisetin 3 μM	LPS + fisetin 10 μM
CD80	221 ± 38 (69 ± 4)	168 ± 27 (69 ± 4)	585 ± 78 (62 ± 2)	600 ± 52 (64 ± 3)	594 ± 49 (62 ± 2)	369 ± 6[c] (63 ± 3)
CD86	129 ± 24 (61 ± 3)	80 ± 6 (60 ± 3)	556 ± 112 (63 ± 1)	616 ± 49 (65 ± 3)	582 ± 39 (63 ± 2)	300 ± 27[c] (61 ± 3)
MHCII	31 ± 6 (48 ± 4)	29 ± 4 (49 ± 3)	58 ± 16 (53 ± 2)	78 ± 12 (57 ± 5)	73 ± 10 (55 ± 2)	63 ± 6 (53 ± 5)

[a] MFI = mean fluorescence intensity. [b] Data are shown as mean ± SEM of three independent experiments. [c] $p < 0.05$ versus LPS plus vehicle-treated group.

Table 2. Effect of Fisetin on the Secretion of Various Cytokines in LPS-Stimulated CD11c$^+$ Dendritic Cells[a]

	pg/mL							
sample	IL-1α	IL-2	IL-5	IL-6	IL-10	IFN-γ	TNF-α	IL-17
blank	29.18 ± 5.44	ND	ND	397.6 ± 143.53	ND	ND	ND	ND
0.1% DMSO	35.58 ± 9.32	ND	ND	346.89 ± 106.08	ND	ND	ND	ND
LPS 1 μg/mL	181.59 ± 25.42	ND	ND	27115.62 ± 8902.38	ND	ND	1125.26 ± 126.94	18.29 ± 6.01
LPS + 0.1% DMSO	214.77 ± 36.3	ND	ND	31462.15 ± 8384.72	ND	ND	1233.66 ± 171.35	21.13 ± 5.10
LPS + fisetin 3 μM	221.62 ± 6.11	ND	ND	24969.56 ± 10072.27	ND	ND	757.79 ± 162.60	15.04 ± 5.14
LPS + fisetin 10 μM	107.97 ± 15.80[b]	ND	ND	4894.61 ± 2471.54[b]	ND	ND	55.47 ± 24.26[b]	1.91 ± 1.91[b]
LPS + curcumin 3 μM	208.00 ± 19.23	ND	ND	21350.78 ± 6593.04	ND	ND	880.61 ± 134.92	15.19 ± 4.62
LPS + curcumin 10 μM	174.13 ± 16.07	ND	ND	12819.03 ± 1695.82	ND	ND	509.32 ± 48.30[b]	6.07 ± 4.82

[a] Data are shown as mean ± SEM of three independent experiments. ND = nondetectable. [b] $p < 0.05$ versus LPS plus vehicle-treated group.

detrimental immune responses. In this study, we demonstrated that fisetin could inhibit macrophage activation via suppressing NF-κB activity, TNF-α production, iNOS expression, MMP-9 activity, and phagocytic activity (**Figures 2** and **3**). These observations support the conclusion reported previously that fisetin is anti-inflammatory (*19*, *20*, *23*). More importantly, we showed that fisetin could inhibit dendritic cell maturation by reducing costimulatory molecules (CD80 and CD86) and cytokine expression (IL-12, TNF-α, and IL-6) (**Figure 4** and **Tables 1** and **2**). The functions of LPS-stimulated dendritic cells, such as endocytosis, migration, and T cell activation, were also impaired by fisetin treatment (**Figure 5**). Our study is the first to report the suppressive role of fisetin in phenotypic and functional maturation of dendritic cells, revealing a new immunopharmacologic activity of this compound.

The immunosuppressive activity of andrographolide and curcumin has been well documented (*24*, *25*), and we thus chose them as positive controls in this study. Notably, the extent of inhibition on various characters of LPS-induced macrophages (including NF-κB activity, TNF-α secretion, iNOS expression, and NO production) by 10 μM fisetin was comparable to that of treatment by 10 μM andrographolide (**Figures 2**, **3** and Supporting Information Figure 1). Furthermore, when compared with curcumin, fisetin showed stronger inhibition on DC maturation based on data from cytokine production (e.g., TNF-α, IL-6, IL-12, and IL-1α) and endocytotic activity (**Figures 4**, **5** and **Table 2**). These data support that fisetin is a potent immunosuppressive compound.

Aberrant macrophage activation is known to cause inflammatory disease. In this report, fisetin inhibited NF-κB signaling pathways and its downstream molecules in activated macrophages, which is consistent with previous observations (*19*, *20*, *23*). Notably, we demonstrated for the first time that fisetin has inhibitory effects on MMP-9 activity and LPS-induced phagocytosis in macrophages. The suppressive effect of fisetin on MMP-9 activity indicates that this compound may interfere with recruitment of macrophages to the inflammatory sites. Furthermore, the inhibitory effect of fisetin on phagocytic capacity of activated macrophages implies that fisetin may suppress antigen engulfment by macrophages. Thus, fisetin could impair multiple characteristic features and functions of activated macrophages.

Dendritic cells (as the most important antigen-presenting cells) are central to various strategies of therapeutic manipulation. Using in vitro and in vivo assays, we demonstrated that fisetin at nontoxic concentration impairs LPS-induced phenotypic and functional maturation of dendritic cells. LPS treatment is known to promote functional maturation of BM-DCs via the TLR4 receptor, which in turn leads to induction of both NF-κB- and MAPK-signaling pathways. Activation of both NF-κB- and MAPK-signaling pathways are important events in DC maturation (*26*). Accumulating evidence, including data presented in this study, has demonstrated that fisetin can inhibit the NF-κB-signaling pathway in diverse cell types responding to different stimuli. It is highly possible that fisetin suppresses the phenotypic and functional maturation of dendritic cells via targeting the NF-κB pathway. Notably, several flavonoids, such as apigenin, luteolin, silibilin, and EGCG have been shown to suppress maturation of DCs via inhibiting both the NF-κB- and MAPK-signaling pathways (*27*−*30*). Since fisetin was reported to inhibit MAPK-pathways in various cell types (*31*, *32*), it is also possible that fisetin mediates DC maturation via modulation of MAPK activity. Certainly, the molecular mechanism of fisetin-inhibited dendritic cell maturation merits further investigation.

IFN-γ production by dendritic cells could be detected under certain conditions. For example, IL-12 could induce IFN-γ production in dendritic cells, especially when combined with IL-18 (*33*−*35*). In response to allogeneic CD4$^+$ T cells, dendritic cell production of IFN-γ can also be up-regulated by IL-12 (*33*). Conversely, the presence of IL-4 or IL-10 during dendritic cell maturation could suppress the expression of IFN-γ in mature dendritic cells (*34*). In our in vitro system, the possible reasons that IFN-γ production was undetectable (**Table 2**) may have been the absence of allogeneic CD4$^+$ T cells and the presence of IL-4.

Recently, the immunosuppressive effects of fisetin have been demonstrated in vitro on several cell types, such as mast cells, basophils, and macrophages (*17*−*19*, *36*−*38*). Our data further provide evidence supporting the inhibitory effect of fisetin on macrophage activation and reveal its hitherto unknown inhibitory effects on functional maturation of dendritic cells. Collectively, current data indicate that fisetin attenuates aberrant activation of immune cells and thereby has therapeutic potential on inflammatory and autoimmune diseases. Indeed, two recent

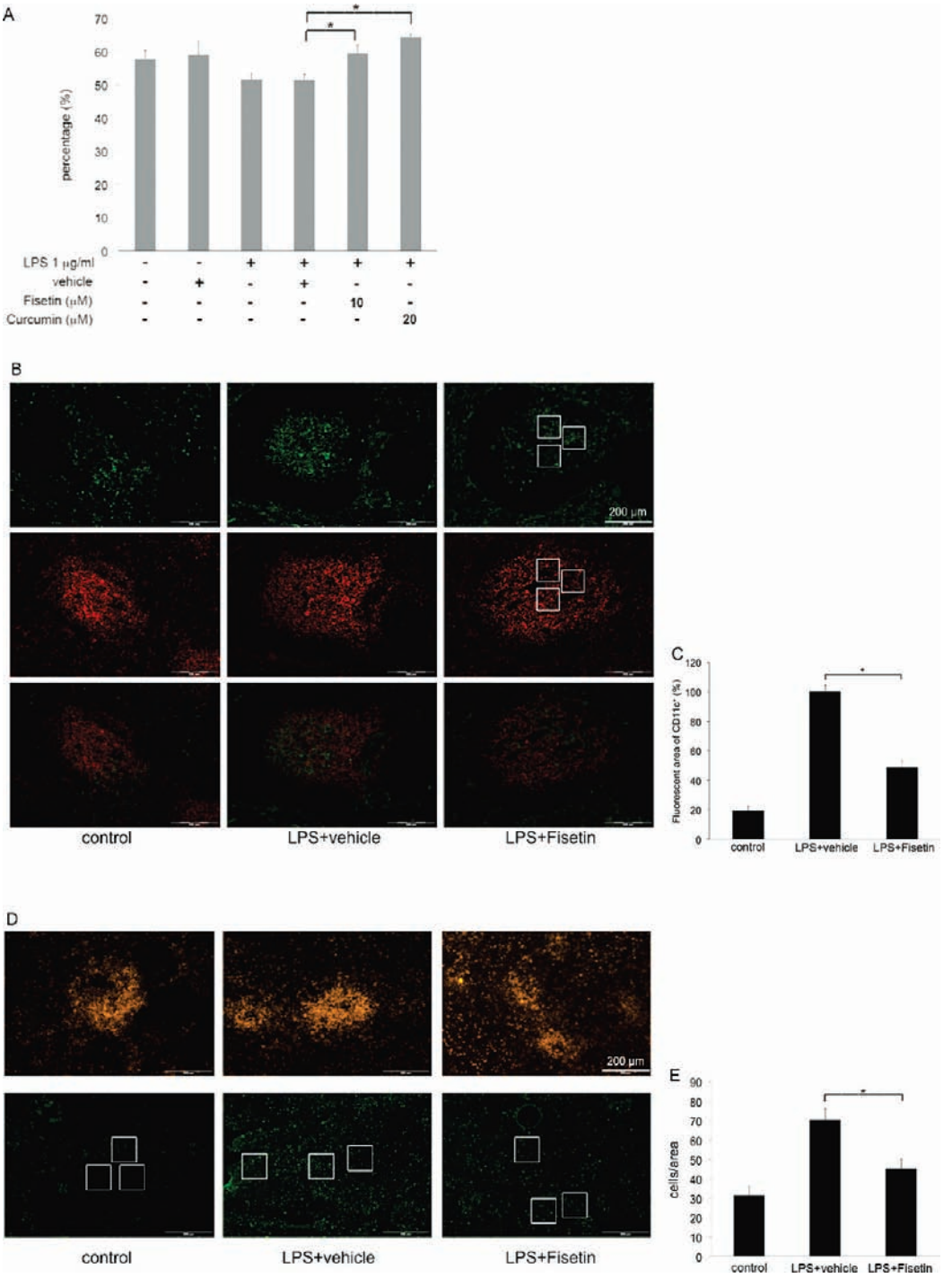

Figure 5. Fisetin inhibits functions of dendritic cells. (**A**) Fisetin increases endocytosis ability of mature BM-DCs. Dendritic cells were treated with fisetin for 1 h, followed by LPS treatment for 24 h. The treated-DCs were assessed by FITC-dextran endocytosis assay. Curcumin is the positive control. Asterisk (∗) indicates $p < 0.05$ versus LPS-treated group. (**B**) Fisetin inhibits migration ability of DCs into spleens of LPS-administrated mice. The colocalization of dendritic cells and T cells in spleen sections of mice ($n = 3$) in fisetin- and control-treated groups was evaluated by immunohistochemistry. The spleen sections were stained with CD4 antibody for detecting T cells (red) and with CD11c antibody for detecting dendritic cells (green). (**C**) Quantification of dendritic cells migrating into spleens of mice under various treatments. For each microscopic field, three different fluorescent areas (shown as white boxes in **B**) were selected for quantification. For each group of mice, a total of 15 fields were analyzed. (**D**) Fisetin inhibits proliferation of T cells in spleens of LPS-administrated mice. The proliferating T cells in spleen sections were identified by BrdU incorporation (green) in the $CD4^+$ area (red). (**E**) Quantification of proliferating T cells in spleens of mice under various treatments. For each microscopic field, the $BrdU^+$ cells from different areas (shown as white boxes in **D**) were counted. For each group of mice, a total of 15 fields were analyzed. Control group: PBS plus 0.2% DMSO. LPS + vehicle group: LPS plus 0.2% DMSO. Asterisk (∗) indicates $p < 0.05$ versus LPS-treated group.

animal studies have demonstrated that LPS-induced acute pulmonary inflammation and collagen-induced arthritis were both improved by fisetin treatment (*32*, *39*). Another ex vivo study also demonstrated that fisetin were able to attenuate LPS-induced cytokine release from leukocytes of patients with chronic obstructive pulmonary disease or type 2 diabetes (*40*).

Many immunosuppressive drugs, such as corticosteroids, salicylates, mycophenolate mofetil, and cytokines (TGF-β and IL-10), have been developed to attenuate inflammatory and autoimmune diseases (*41*−*45*), but most compounds exhibit significant side effects. Emerging evidence, including data described in this study, have demonstrated that fisetin is a potent immunosuppressor. Since fisetin is commonly present in dietary fruits and vegetables, this compound presumably is a safer immunosuppressive agent and thus a promising candidate for immunotherapy. Certainly, the molecular mechanisms and clinical implications of fisetin-mediated immunosuppressive activity warrant further investigation.

Supporting Information Available: Additional experimental results. This material is available free of charge via the Internet at http://pubs.acs.org.

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