0026-895X/06/7003-1033–1044$20.00
MOLECULAR PHARMACOLOGY
Vol. 70, No. 3
24547/3135817
Mol Pharmacol 70:1033–1044, 2006
Printed in U.S.A.

Apigenin Inhibits Immunostimulatory Function of Dendritic Cells: Implication of Immunotherapeutic Adjuvant

Man-Soo Yoon, Jun Sik Lee, Byoung-Moon Choi, Young-Il Jeong, Chang-Min Lee, Jong-Hoon Park, Yuseok Moon, Si-Chan Sung, Sang Kwon Lee, Yun Hee Chang, Hae Young Chung, and Yeong-Min Park

College of Pharmacy, Pusan National University, Busan, Korea (J.S.L., H.Y.C.); and National Research Laboratory of Dendritic Cells Regulation and Medical Research Institute (Y.-I.J., C.-M.L., Y.M., Y.-M.P.) and Departments of Microbiology and Immunology (Y.-I.J., C.-M.L., Y.M., Y.-M.P.), Obstetrics and Gynecology (M.-S.Y., B.-M.C., J.-H.P.), and Thoracic Cardiovascular Surgery and Gynecology (S.-C.S., S.K.L., Y.H.C.), College of Medicine, Pusan National University, Pusan, Korea

Received March 15, 2006; accepted June 16, 2006

ABSTRACT

Apigenin, one of the most common flavonoids, has been shown to possess anti-inflammatory, anticarcinogenic, and free radical-scavenging properties. However, the influence of apigenin on the immunostimulatory effects and maturation of dendritic cells (DC) remains, for the most part, unknown. In this study, we have attempted to ascertain whether apigenin influences the expression of surface molecules, dextran uptake, cytokine production, and T-cell differentiation as well as the signaling pathways underlying these phenomena in murine bone marrow-derived DC. In the presence of apigenin, CD80, CD86, and major histocompatibility complex class I and II molecules, expressions on DC were significantly suppressed, and lipopolysaccharide (LPS)-induced interleukin (IL)-12 expression was impaired. The DC proved highly efficient at antigen capture, as evidenced by the observation of mannose receptor-mediated endocytosis in the presence of apigenin. The LPS-induced activation of mitogen-activated protein kinase, the nuclear translocation of its nuclear factor-κB p65 subunit, and the induction of the T-helper 1 response were all impaired in the presence of apigenin, whereas the cell-mediated immune response remained normal. These findings provide new insight into the immunopharmacological functions of apigenin and its effects on DC, and they may also prove useful in the development of adjuvant therapies for individuals suffering from acute or chronic DC-associated diseases.

Dendritic cells (DC) are antigen-presenting cells that are believed to possess immune sentinel properties. They are also believed to be capable of initiating T-cell responses to both microbial pathogens and tumors (Steinman, 1991; Banchereau and Steinman, 1998). Immature DC capture and process exogenous agents within peripheral tissues, in which they begin to mature. Once matured, they migrate into lymphoid organs, where they stimulate naive T cells via the signaling of both major antigen-presenting histocompatibility complex (MHC) molecules and costimulatory molecules (Austyn, 1998). DC have also been shown to be highly responsive to inflammatory cytokines and bacterial products, including tumor necrosis factor (TNF)-α and lipopolysaccharides (LPS). When encountered in the peripheral organs, these products induce a series of phenotypic and functional alterations in the DC (De Smedt et al., 1996; Cella et al., 1997). Similar maturation-indicative changes have also been reported after infections with *Mycoplasma* species, viruses, intracellular bacteria, and parasites (Kolb-Maurer, 2000; Salio et al., 2000). DC located in the peripheral tissues tend to be both phenotypically and functionally immature

This work was supported by the Korea Science and Engineering Foundation through National Research Laboratory Program grant M105-0000000805J000000810 and Medical Research Institute grant 2005-22, Pusan National University.

M.-S.Y., J.S.L., and B.-M.C. contributed equally to this work.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.106.024547.

ABBREVIATIONS: DC, dendritic cell(s); MHC, major histocompatibility complex; TNF, tumor necrosis factor; LPS, lipopolysaccharide; IL, interleukin; NF-κB, nuclear factor-κB; MAPK, mitogen-activated protein kinase; BM, bone marrow; BM-DC, bone marrow-dendritic cells; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; CHS, contact hypersensitivity; rm, recombinant mouse; ELISA, enzyme-linked immunosorbent assay; IFN, interferon; FITC, fluorescein isothiocyanate; PE, phycoerythrin; mAb, monoclonal antibody; Ab, antibody; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter; CFSE, 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester; p-, phospho-; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TNCB, 2,4,6-trinitrochlorobenzene; Th, T-helper; TNBS-DC, 2,4,6-trinitrobenzenesulfonic acid-dendritic cell; MFI, mean fluorescence intensity; 2-ME, 2-mercaptoethanol.

(Banchereau and Steinman, 1998); consequently, they are unable to induce primary immune responses, because they do express neither the requisite costimulatory molecules nor antigenic peptides with which they can form stable complexes with MHC molecules. Immature DC can effectively capture and process exogenous antigens within the peripheral tissues, in which their maturation has been associated with decreased or absent antigen uptake, the expression of high levels of MHC class II and accessory molecules, and the generation of interleukin (IL)-12 upon stimulation (Banchereau et al., 2000).

The flavonoids comprise a family of common phenolic plant pigments that have been identified as dietary anticarcinogens and antioxidants (Chen et al., 2002). We reported in a previous study that a variety of phytochemicals exhibit profound immunoregulatory activity, particularly in the DC (Ahn et al., 2004; Kim et al., 2004, 2005). Apigenin, one of the most common flavonoids, is found in a variety of fruits and vegetables, including onions, parsley, and oranges as well as chamomile tea, wheat sprouts, and certain seasonings (Duthie and Crozier, 2000). Apigenin has demonstrated anti-inflammatory, anticarcinogenic, and free radical-scavenging activities in a variety of in vitro systems (Kim et al., 1998). In a recent study, investigators identified apigenin as a potent inhibitor of the nuclear transcription factor nuclear factor-κB (NF-κB), which may perform a pivotal function in the regulation of cell growth, apoptosis, and the regulation of the cell cycle (Hastak et al., 2003). Studies using human leukemia cells as well as carcinoma cells in the breast, colon, and elsewhere have revealed that apigenin inhibits cell growth via the induction of cell cycle arrest and apoptosis (Wang et al., 2000). It also attenuates proinflammatory cytokine production in LPS-stimulated peripheral blood mononuclear cells via the selective elimination of monocytes and macrophages, inhibits TNF-α-induced intercellular adhesion molecule-1 up-regulation in vivo, and inhibits IL-1α-induced prostaglandin synthesis and TNF-α-induced IL-6 and IL-8 production (Sander Hougee et al., 2005). Moreover, it actively inhibits IκB kinase activity, IκBα degradation, NF-κB DNA protein-binding activity, NF-κB luciferase activity, and mitogen-activated protein kinase (MAPK) activity (Chen et al., 2004). Until now, the cellular targets of apigenin in the immune system have remained enigmatic, thereby leaving the role of apigenin in the cellular maturation and immunoregulatory activity of DC an open question.

In this study, we have attempted to characterize the effects of a noncytotoxic concentration of apigenin on the maturation and functional properties of murine bone marrow (BM)-derived DC (BM-DC). Our findings demonstrated, for the first time, that apigenin induces the phenotypical and functional maturation of DC and suppresses the LPS-induced activation of ERK1/2, JNK, and p38 MAPK as well as the nuclear translocation of the NF-κB p65 subunit in DC. In vivo data reveal that although apigenin-treated DC have been shown to migrate to the T-cell areas of secondary lymphoid tissue, they do not induce normal cell-mediated contact hypersensitivity (CHS). Moreover, this readily available agent may provide a simple, inexpensive, and highly effective means for the manipulation of the immunostimulatory properties of DC. Considering, then, the critical role of antigen-presenting cells in the initiation and regulation of immune responses as well as the ready availability of apigenin, our findings may bear important implications for the manipulation of the functions of DC in potential therapeutic applications.

Materials and Methods

Animals and Chemicals. Male 8- to 12-week-old C57BL/6 (H-2Kb and I-Ab) and BALB/c (H-2Kd and I-Ad) mice were purchased from the Korean Institute of Chemistry Technology (Daejeon, Korea). They were housed in a specific pathogen-free environment within our animal facility for at least 1 week before use. Apigenin was purchased from Sigma-Aldrich (St. Louis, MO).

Reagents and Antibodies. Recombinant mouse (rm)granulocyte macrophage–colony-stimulating factor (GM-CSF) and rmIL-4 were purchased from R&D Systems (Minneapolis, MN). Apigenin, dextran-fluorescein isothiocyanate (FITC) (mol. wt. 40,000), and LPS (from *Escherichia coli* 055:B5) were obtained from Sigma-Aldrich. An endotoxin filter (END-X) and an endotoxin removal resin (END-X B15) were acquired from Associates of Cape Cod, Inc. (East Falmouth, MA). Cytokine ELISA kits for murine IL-12 p70, IL-4, and IFN-γ were purchased from BD Biosciences PharMingen (San Diego, CA). FITC- or phycoerythrin (PE)-conjugated mAbs used to detect the expression of CD11c (HL3), CD80 (16-10A1), CD86 (GL1), CD40 (1C10), IA^b β-chain (AF-120.1), $H2K^b$ (AF6-88.5), and CD4 (L3T4), or the intracellular expression of IL-12 p40/p70 (C15.6) and IL-10 (JESS-16E3) by flow cytometry, as well as isotype-matched control mAbs and biotinylated anti-CD11c (N418) mAb were purchased from BD Biosciences PharMingen. To detect protein levels, we purchased anti-phospho-ERK, anti-ERK, anti-phospho-p38, anti-p38, anti-p-IκB, anti-IκB, anti-phospho-JNK, and anti-JNK from Cell Signaling Technology Inc. (Beverly, MA) and anti-p65 Ab from Abcam (Cambridge, MA).

Isolation and Culture of DC. DC were generated from murine BM cells, as described by Inaba et al. (1992) and Porgador and Gilboa (1995) with modifications. In brief, BM were flushed from the tibiae and femurs of C57BL/6 and depleted of red cells with ammonium chloride. The cells were plated in six-well culture plates (10^6 cells/ml; 3 ml/well) in Opti-MEM (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM *l*-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5×10^{-5} M 2-ME, 10 mM HEPES, pH 7.4, 20 ng/ml rmGM-CSF, and rmIL-4 at 37°C, 5% CO_2. On day 3 of the culture, floating cells were gently removed, and fresh medium was added. On day 6 or 7 of the culture, nonadherent cells and loosely adherent proliferating DC aggregates were harvested for analysis or stimulation, or, in some experiments, replated in 60-mm dishes (106 cells/ml; 5 ml/dish). On day 6, 80% or more of the nonadherent cells expressed CD11c. In certain experiments, to obtain highly purified populations for subsequent analyses, the DC were labeled with bead-conjugated anti-CD11c mAb (Miltenyi Biotec, Gladbach, Germany) followed by positive selection through paramagnetic columns (LS columns; Miltenyi Biotec) according to the manufacturer's instructions. The purity of the selected cell fraction was >90%.

Stimulation of DC by Apigenin. Apigenin was dissolved in DMSO, or DMSO alone [0.01% (v/v)] was added to cultures of isolated DC in six-well plates (10^6 cells/ml; 3 ml/well). DMSO (0.1%) alone was used as control, because of no cytotoxicity in DC. For the analysis of apoptosis, DC were stimulated with LPS or left without any stimuli, and apoptosis was analyzed over time by staining of phosphatidylserine translocation with FITC-annexin-V in combination with propidium iodine kit (BD Biosciences PharMingen) according to the manufacturer's instructions.

Flow Cytometric Analysis. On day 7, BM-DC were harvested, washed with phosphate-buffered saline (PBS), and resuspended in fluorescence-activated cell sorter (FACS) washing buffer (2% fetal bovine serum and 0.1% sodium azide in PBS). Cells were first blocked with 10% (v/v) normal goat serum for 15 min at 4°C and stained with PE-conjugated anti-$H2K^b$ (MHC class I), anti-I-Ab (MHC class II), anti-CD80, and anti-CD86 with FITC-conjugated

anti-CD11c (BD Biosciences PharMingen) for 30 min at 4°C. The stained cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

Quantitation of Antigen Uptake. Endocytosis was quantitated, as described by Lutz et al. (1996) and Sallusto et al. (1995). In brief, 2×10^5 cells were equilibrated at 37 or 4°C for 45 min and then pulsed with fluorescein-conjugated dextran (mol. wt. 40,000; Sigma-Aldrich) at a concentration of 1 mg/ml. Ice-cold staining buffer was added to stop the reaction. The cells were washed three times and stained with PE-conjugated anti-CD11c Abs and then analyzed by FACSCalibur. Nonspecific binding of dextran to DC, determined by incubation of DC with FITC-conjugated dextran at 4°C, was subtracted. The medium used in the culture, to stimulate DC with apigenin, was supplemented with GM-CSF, because the ability of DC to capture antigen is lost if DC are cultured without GM-CSF (Rescigno et al., 1998).

Cytokines Assay. Cells were first blocked with 10% (v/v) normal goat serum for 15 min at 4°C and then stained with FITC-conjugated $CD11c^+$ antibody for 30 min at 4°C. Cells stained with the appropriate isotype-matched Ig were used as negative controls. The cells were fixed and permeated with the Cytofix/Cytoperm kit (BD Biosciences PharMingen) according to manufacturer's instructions. Intracellular IL-12p40/p70, IL-10, and IFN-γ were stained with fluorescein *R*-PE-conjugated antibodies (BD Biosciences PharMingen) in a permeation buffer. The cells were analyzed on a FACSCalibur flow cytometer with the CellQuest program. Furthermore, murine IL-12p70, IL-4, and IFN-γ from DC were measured using an ELISA kit (BD Biosciences PharMingen), according to manufacturer's instructions.

Mixed Lymphocyte Reaction. Responder T cells, which participate in allogeneic T-cell reactions, were isolated when passed through mononuclear cells from BALB/c mice in a MACS column (Miltenyi Biotec). Staining with FITC-conjugated anti-CD3 antibodies (BD Biosciences PharMingen) revealed that they consisted mainly of $CD3^+$ cells (>95%). The lymphocyte population (95% of $CD3^+$ cells) was then washed twice in PBS and labeled with CFSE, as described previously (Lyons, 2000). The cells were resuspended in 1 μM CFSE in PBS. After being shaken for 8 min at room temperature, the cells were washed once in pure FBS and twice in PBS with 10% FBS. DC (1×10^4) or DC exposed to 20 μM/ml apigenin or 100 ng/ml LPS for 24 h were cocultured with 1×10^5 allogenic CFSE-labeled T lymphocytes in 96-well, U-bottomed plates (Nalge Nunc International, Rochester, NY). A negative control ($CD3^+$ lymphocytes alone) and a positive control ($CD3^+$ lymphocytes in 5 μg of concanavalin A) were created for each experiment. After 4 days, the cells were harvested and washed in PBS. CFSE dilution optically gated lymphocytes were assessed by flow cytometry.

Nuclear and Cytoplasmic Extracts and Western Blot. The cells were exposed to 200 ng/ml LPS in the absence or presence of 20 μM apigenin pretreatment. Then, after 15 or 30 min of incubation at 37°C, cells were washed twice with ice-cold PBS and lased with modified radioimmunoprecipitation assay buffer (1.0% Nonidet-40, 1.0% sodium deoxycholate, 150 nM NaCl, 10 mM Tris-HCl, pH 7.5, 5.0 mM sodium pyrophosphate, 1.0 mM $NaVO_4$, 5.0 mM NaF, 10 μM/ml leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride) for 15 min at 4°C. Lysates were cleared by centrifuging at 14,000*g* for 20 min at 4°C. The protein content of cell lysates was determined using the Micro BCA assay kit (Pierce Chemical, Rockford, IL). Equivalent amounts of proteins were separated by SDS-10% polyacrylamide gel electrophoresis and analyzed by Western blotting using an anti-phospho-ERK1/2 (p-ERK; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-phospho-JNK (p-JNK; Santa Cruz Biotechnology, Inc.), or anti-phospho-p38 (p-p38; Santa Cruz Biotechnology, Inc.) MAPK mAb for 2 h, as described by the manufacturer of the antibodies. After washing three times with Tris-buffered saline/Tween 20, membranes were incubated with secondary horseradish peroxidase-conjugated anti-mouse IgG for 1 h. After washing, the blots were developed using the ECL system (GE Healthcare, Little Chalfont, Buckinghamshire, UK), by following the manufacturer's instructions. DC nuclear extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Chemical), according to manufacturer's instructions. NF-κB p65 subunits in the nuclear extracts were determined by Western blot analysis with anti-NF-κB p65 subunit Ab (Santa Cruz Biotechnology, Inc.).

Generation of DC from Spleen and Culture. Mice were injected intraperitoneally with 5 mg/kg apigenin every 3 days before the administration of 1 mg/kg LPS in a lateral vein of the tail. Twenty-four hours after LPS challenge, mice were scarified; their spleens were disrupted; and the cells were centrifuged at 400*g* for 5 min, resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, *l*-glutamine, nonessential amino acids, sodium pyruvate, penicillin-streptomycin, HEPES, and 2-ME (all from Sigma-Aldrich) for 2 h; and then nonadherent cells were washed out. The residual adherent cells were maintained in the culture medium and incubated overnight at 37°C in a 5% CO_2 atmosphere. After incubation, DC (which exhibit adherence capacity in the first hours of culture) become nonadherent and floats in the medium. The cells were gated on $CD11c^+$ for DC.

Generation of $CD4^+$ T Cells from Spleen and Culture. Mice were injected intraperitoneally with 5 mg/kg apigenin every 3 days before the administration of 1 mg/kg LPS in a lateral vein of the tail. Twenty-four hours after LPS challenge, mice were scarified; their spleens were disrupted; and the cells were centrifuged at 400*g* for 5 min and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, *l*-glutamine, nonessential amino acids, sodium pyruvate, penicillin-streptomycin, HEPES, and 2-ME (all from Sigma-Aldrich) for 4 h. The residual adherent cells were maintained in the culture medium and incubated overnight at 37°C in a 5% CO_2 atmosphere. The cells were fixed and permeated with the Cytofix/Cytoperm kit (BD Biosciences PharMingen) according to manufacturer's instructions. Intracellular IFN-γ was stained with fluorescein *R*-PE-conjugated antibodies (BD Biosciences PharMingen) in a permeation buffer. The cells were gated on $CD4^+$ T cell for T cells.

2,4,6-Trinitrobenzenesulfonic Acid-Induced CHS. In the sensitization phase, bead-sorted $CD11c^+$ DC were pulsed with 0.1% (w/v) TNBS (Sigma Aldrich) in PBS for 15 min at 37°C. After three washing in PBS, the cells were counted, and their viability was assessed by trypan blue exclusion. One million cells were injected s.c. into the abdomen of animals shaved and painted with 7% (w/v) 2,4,6-trinitrochlorobenzene (TNCB; Sigma-Aldrich) diluted in acetone/olive oil [4/1 (v/v)]; vehicle). Negative controls included animals injected with unpulsed DC (without hapten) and animals treated with the vehicle alone. Seven days after sensitization, the mice were painted on the dorsal and ventral side of the left ear with 10 μl of 1% TNCB in vehicle. The thickness of the left (challenged) and the right (control) ear was measured after 48 h by using an engineer's spring-loaded micrometer (Mitutoyo, Kawasaki, Japan). The percentage of increase in ear thickness was calculated using the following formula: $100 \times$ [(thickness of challenged ear − thickness of unchallenged ear)/thickness of unchallenged ear].

Statistics. All results were expressed as the means ± S.D. of the indicated number of experiments. Statistical significance was estimated using a Student's *t* test for unpaired observations, and the differences were compared with regard to statistical significance by one-way analysis of variance, followed by Bonferroni's post hoc test. The categorical data from the fertility test were subjected to statistical analysis via chi square test. A p of <0.05 was considered significant.

Results

Apigenin Inhibits Phenotypic Maturation of Murine DC. In the initial series of experiments, we attempted to determine whether apigenin influences the maturation of DC. BM-DC were cultured for 6 days in Opti-MEM supple-

mented with GM-CSF at concentrations of 20 ng/ml and IL-4 at 20 ng/ml. Different apigenin concentrations were added to the cultures on day 6, with or without 200 ng/ml LPS. Apigenin was determined to be cytotoxic to BM-DC at concentrations in excess of 50 μM. There were no marked differences in the percentage of dead cells, as evidenced by $CD11c^+$ cell and annexin-V/propidium iodide (PI) staining (Fig. 1A); for this reason, the apigenin concentration was raised to >50 μM. We then evaluated the effects of a range of apigenin concentrations on the maturation of DC. BM-DC were cultured for 24 h in the presence of 0 to 20 μM apigenin, as was described under *Materials and Methods*. As is shown in Fig. 1A, 20 μM apigenin significantly attenuated the expression of CD80, CD86, and MHC class I and II on the surfaces of the $CD11c^+$ cells. These inhibitory effects occurred in a dose-dependent manner and were most notable with regard to the expression of CD80, CD86, CD40, MHC class I, and MHC class II molecules (Table 1). These molecules were also up-regulated within 24 h of exposure to LPS (Fig. 1C, thick lines). Exposure to 20 μM apigenin in the presence of LPS was accompanied by an impaired expression of the costimulatory molecules CD80 and CD86. It is noteworthy that a significant down-regulation of these two molecules as well as the MHC class I and class II molecules was also observed under these conditions (Fig. 1C, thin lines).

Apigenin Impairs the Secretion of IL-12 and Does Not Influence IL-10 Production during the LPS-Induced Maturation of DC. It has been hypothesized that DC as well as macrophages and monocytes function as sources of proinflammatory molecules (Lapointe et al., 2000; Mosca et al., 2000). Thus, we assessed the ability of BM-DC to generate proinflammatory cytokines. IL-12 expression has previously been identified as a specific marker for DC activity (An et al., 2002). It is also an important marker for DC maturation and can be used in the selection of Th1-dominant adjuvants. The secretion of bioactive IL-12 p70 requires the coordinated expression of two of its subunits, p35 and p40, which are encoded for by two separate genes and are independently regulated (Lutz et al., 1996). We analyzed the production of both intracellular IL-12p40/p70 and bioactive IL-12p70 in the apigenin-treated DC. As was shown in Fig. 2A, the intracellular staining of FITC-labeled anti-$CD11c^+$ DC with PE-labeled anti-IL-12 p40/p70 or anti-IL-10 mAbs showed that DC stimulated with 20 μM apigenin expressed small amounts of IL-12 p40/p70, compared with unstimulated DC, whereas IL-10 was not detectable. When the supernatants were analyzed using ELISA, IL-10 was also undetectable 24 h after stimulation with 200 ng/ml LPS. As is shown in Fig. 2B, ELISA analyses revealed high levels of IL-12 p70 when the DC were stimulated for 24 h with LPS (92.3 $\pm$ 4.2 ng/ml). Apigenin (64.1 $\pm$ 3.4 ng/ml) alleviated the effects of LPS. These results indicate that exposure to apigenin impairs the ability of DC to generate large quantities of IL-12 p70 and proinflammatory cytokines. The results also suggest that apigenin suppresses the functional maturation of LPS-stimulated DC.

Apigenin Enhances the Immature State of DC with High Endocytotic Activity. The expression of surface molecules on DC and the observed changes in IL-12 production reveal that apigenin exposure results in a profound inhibition of the phenotypic and functional maturation of DC in vitro. However, these results did not allow us to dismiss the

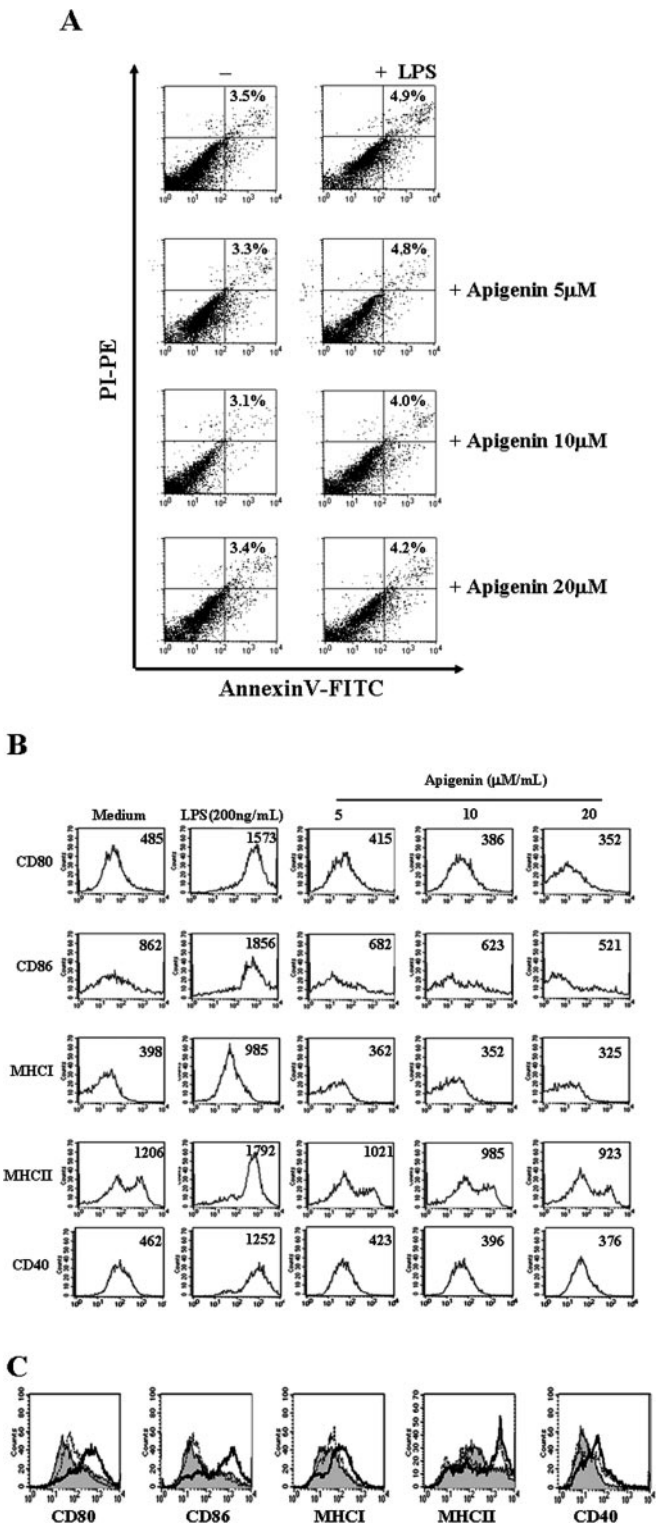


Fig. 1. Apigenin suppresses the expression of costimulatory (CD80 and CD86) and MHC class I and II molecules in a dose-dependent manner during the maturation of DC. DC were generated as described under *Materials and Methods*. On day 6, the cells were harvested and analyzed using two-color flow cytometry. A, apigenin exerts no influence on growth and no cytotoxicity in $CD11c^+$ DC. The cells were gated on $CD11c^+$. Apigenin was added to the DC for 24 h at concentrations of 5, 10, and 20 μM. B, expression of surface molecules was then analyzed. C, DC were left untreated (control) or were stimulated for 24 h with 200 ng/ml LPS in the absence or presence (gray line, control; thin line, apigenin + LPS; and thick line, LPS) of 20 μM apigenin on day 6. UN, chemically untreated control group. The histogram is from a single experiment that is representative of three.

possibility that apigenin may induce a general inhibition of the physiological functions of DC. We thus attempted to ascertain whether the stimulation of DC with apigenin alters their antigen capture ability. We included the DC with apigenin, with or without LPS, and added dextran-FITC to the culture media. The percentage of double-positive cells ($CD11c^+$ + dextran-FITC) was identical for the apigenin-treated and nontreated DC. The percentage of LPS-stimulated DC was less than the percentage of the untreated DC. The apigenin-treated DC exhibited a higher degree of endocytotic capacity for dextran-FITC than did the LPS-stimulated DC (Fig. 3). This again shows that the apigenin-treated DC were phenotypically and functionally immature. A set of experiments identical to these was also conducted at 4°C, and the results showed that the uptake of dextran-FITC by DC is inhibited at low temperatures. The results also indicate that apigenin induces immaturity in the DC.

Apigenin Impairs the Allostimulatory Capacity of DC. The fluorescein-based dye CFSE has biochemical properties that render it particularly appropriate for this appli-

TABLE 1

Apigenin markedly inhibits the expression of costimulatory molecules (CD80, CD86, and CD40) and MHC class I and II molecules on LPS-stimulated CD11c + DC

BM-derived DC were cultured in the absence or presence of 20 μM apigenin following the 200 ng/ml LPS stimulation for 24 h. The expression of surface molecules was analyzed by FACSCalibur. Two-color flow cytometry was used to determine the level of antigen expression on CD11c + DC. The results are from one experiment of three performed.

Surface Antigen	Positive Cells (MFI)			
	Medium	Apigenin	LPS	Apigenin + LPS
		%		
CD80	72 ± 3 (480 ± 25)	73 ± 1 (372 ± 15)*	87 ± 3 (1604 ± 86)	75 ± 2 (642 ± 48)**
CD86	70 ± 4 (872 ± 42)	68 ± 1 (510 ± 27)	85 ± 1 (1853 ± 107)	70 ± 3 (902 ± 86)**
MHC I	71 ± 5 (392 ± 13)	70 ± 2 (317 ± 8)*	87 ± 3 (983 ± 103)	74 ± 1 (562 ± 107)**
MHC II	83 ± 4 (1250 ± 107)	79 ± 1 (951 ± 102)*	91 ± 2 (1802 ± 125)	84 ± 4 (1379 ± 95)*
CD40	75 ± 3 (451 ± 12)	73 ± 3 (370 ± 7)*	86 ± 3 (1302 ± 97)	77 ± 2 (570 ± 43)**

>Statistical significance between samples with and without apigenin is indicated [*, $p < 0.01$ vs. unstimulated DC (medium); **, $p < 0.01$ vs. LPS-stimulated DC].

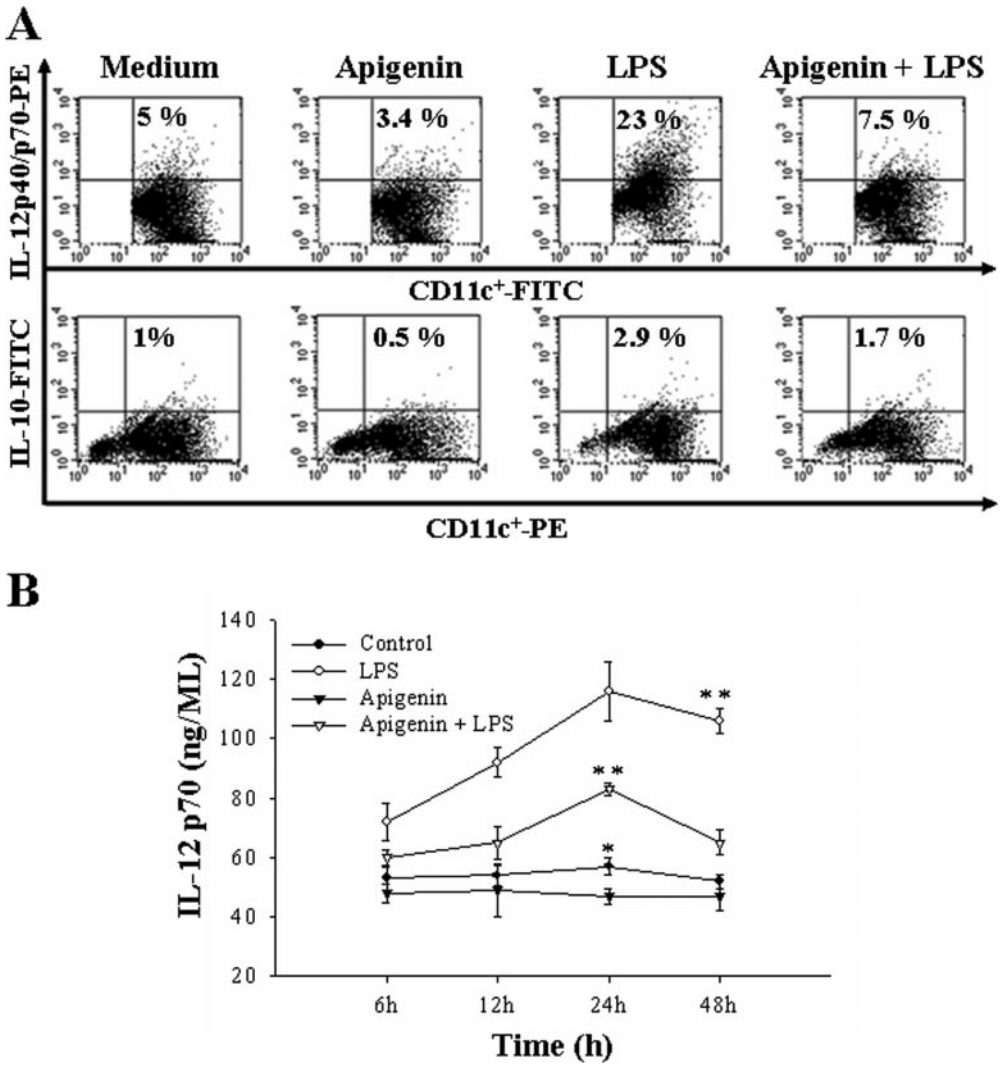
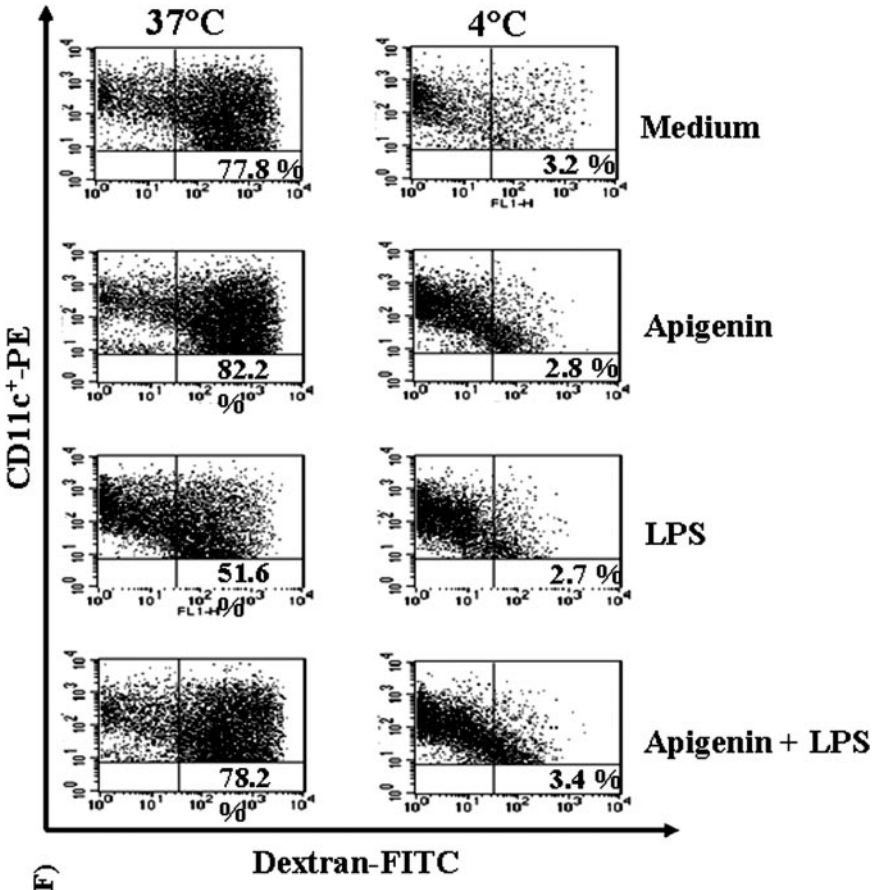


Fig. 2. Apigenin impairs the secretion of IL-12 and does not influence IL-10 in LPS-induced maturation DC. Murine DC was stimulated by 20 μM apigenin for 24 h with or without LPS. A, $CD11c^+$ DC were subsequently analyzed via intracellular cytokine staining. B, cells were gated on $CD11c^+$. The DC (5×10^5 cells/ml) were cultured for 24 h, and the production of bioactive IL-12 p70 in the culture supernatants was analyzed using ELISA. The results shown are from one representative experiment of three [*, $p < 0.01$ versus unstimulated DC (control); **, $p < 0.01$ versus LPS-stimulated DC].

cation. Specifically, CFSE dye is loaded into cells in vitro, and the CFSE in a given cell is monitored over time. Upon division, the CFSE segregates equally between the daughter cells, such that the intensity of cellular fluorescence decreases 2-fold with each successive generation. This property of CFSE allows for the accurate tracking of the number of divisions that a given cell has undergone, either in vitro or after transfer in vivo (Lyons, 2000). To determine whether apigenin exerts a detectable effect on allogeneic T-cell stimulation, we treated DC with apigenin for 24 h. As shown in Fig. 4B, the LPS-treated DC exhibited a more profound proliferation rate than did the controls, whereas apigenin seemed to impair the proliferation response in allogeneic T cells elicited by LPS-activated DC. It is noteworthy that the maturation induced by LPS stimulation (24 h at 200 ng/ml) profoundly promoted the allostimulatory capacity of the untreated DC, whereas apigenin exposure impaired their allostimulatory capacity significantly.

We also attempted to determine the potency of DC with regard to their ability to adhere to T cells and, thus, to form clusters. The size of the DC/T-cell clusters decreased in the presence of apigenin, compared with the LPS-treated group. In the presence of apigenin, the LPS-treated DC formed clusters 57% of the size of the clusters formed by the LPS-stimulated DC in the absence of apigenin (Fig. 4A). Considering the established inhibitory effects of apigenin on the production of IL-12 (a Th1-inducing cytokine) in DC, we attempted to characterize the quality of the primary T-cell response in DC matured in the presence of apigenin. Naive allogeneic T cells primed with mature DC differentiated into



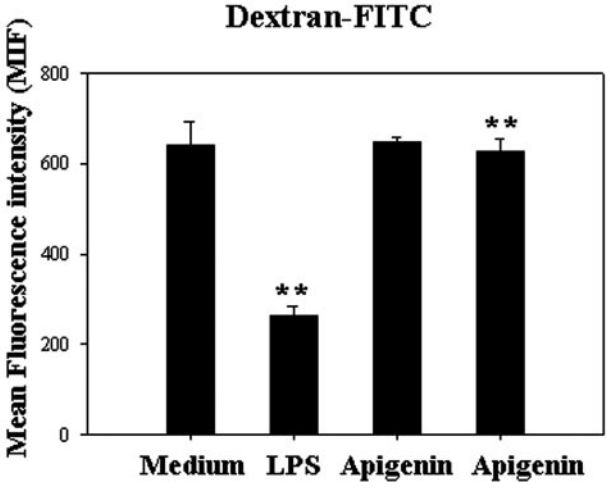


Fig. 3. Apigenin-stimulated DC exhibited increased antigen uptake. DC (1×10^5 cells) were treated with 20 μM apigenin with or without 200 ng/ml LPS for 24 h. The endocytic activity of the DC was evaluated using flow cytometry after the administration of treatment with FITC-dextran. The cells were then washed twice in ice-cold Hanks' balanced salt solution and stained with PE-conjugated anti-CD11c antibody. The endocytic activity of the controls was determined after exposure to FITC-dextran at 4°C. The numbers represent the percentages of cells. Medium designates the chemically untreated control group. To confirm these results, we repeated these experiments three times. The cells were gated on $CD11c^+$. The numbers indicate the percentage of $CD11c^+$ cells. The results represent two separate experiments that yielded similar results.

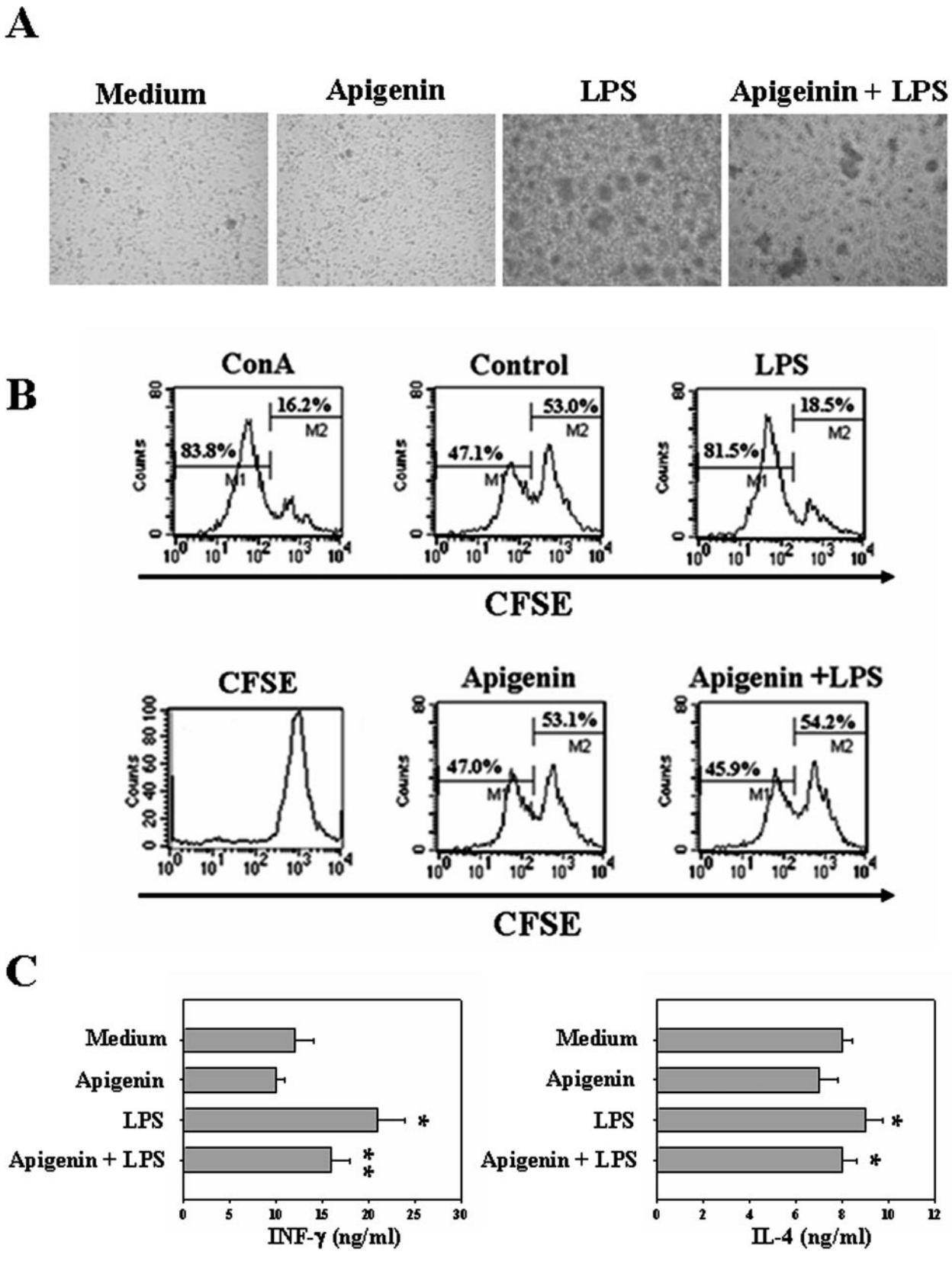


Fig. 4. DC exposed to apigenin display an impaired ability to induce the proliferation of allogenic T cells and to initiate Th1 responses in vitro. The DC were incubated for 24 h in medium alone, 20 μM apigenin, 200 ng/ml LPS, or apigenin with LPS. The DC were washed and then cocultured with T cells. A, clustering was assessed after 64 h. B, DC were cultured in medium with or without apigenin for 24 h. The treated DC were harvested and washed thoroughly to remove the apigenin. A mixed lymphocyte reaction was allowed to proceed for 4 days, as described under *Materials and Methods*. C, cells were then examined after 48 h for cytokine release using ELISA. The data are expressed as nanograms per milliliter/10^6 cells $\pm$ S.D. of triplicate cultures (**, $p < 0.005$ versus T cells primed with immature DC). Medium represents the chemically untreated control group. Similar results were obtained in three separate experiments.

Th1 lymphocytes when they generated high levels of INF-γ and low levels of IL-4 (Fig. 4C). By contrast, T lymphocytes primed with DC that had matured in the presence of apigenin inhibited INF-γ production. These results show that the majority of the effects of apigenin on the T-cell-differentiating properties of DC are a consequence of the inhibition of IL-12 production.

Apigenin Suppresses the LPS-Induced Phosphorylation of MAPK and the Nuclear Translocation of the NF-κB p65 Subunit in DC. LPS stimulation has been shown to affect the activation of MAPK and NF-κB signal pathways in DC (Rescigno et al., 1998). The activations of MAPK and NF-κB are important events in DC maturation (Rescigno et al., 1998). LPS activates p-p38 kinase, p-ERK1/2, and p-JNK (Fig. 5A). To characterize the effects of apigenin on p-p38 kinase, p-ERK1/2, and p-JNK expression in DC, we exposed immature DC to apigenin before the application of LPS stimulation. Pretreatment with 20 μM apigenin resulted in a marked inhibition of the LPS-induced up-regulation of p-p38, p-ERK1/2, and p-JNK. The total ERK1/2 proteins were constitutively expressed (Fig. 5A). Furthermore, LPS signal transduction has been shown by other researchers to activate a variety of signal pathways, including the NF-κB pathway (Rescigno et al., 1998), which performs a critical function in the regulation of gene expression. These results show that apigenin inhibits MAPK expression, which is relevant to the regulation of LPS-induced DC maturation. To determine the role of NF-κB translocation, we stimulated immature DC with LPS before exposing them to apigenin. To determine whether apigenin can affect the blockade of the LPS-induced translocation of NF-κB, we prepared nuclear extracts from DC that had been treated with both LPS and apigenin. The nuclear translocation of the NF-κB p65 subunit was then detected via Western blotting. LPS was shown to enhance the nuclear translocation of the NF-κB p65 subunit within 30 min of exposure. Conversely, pretreatment with 20 μM apigenin resulted in the suppression of the LPS-enhanced nuclear translocation of NF-κB p65 (Fig. 5B).

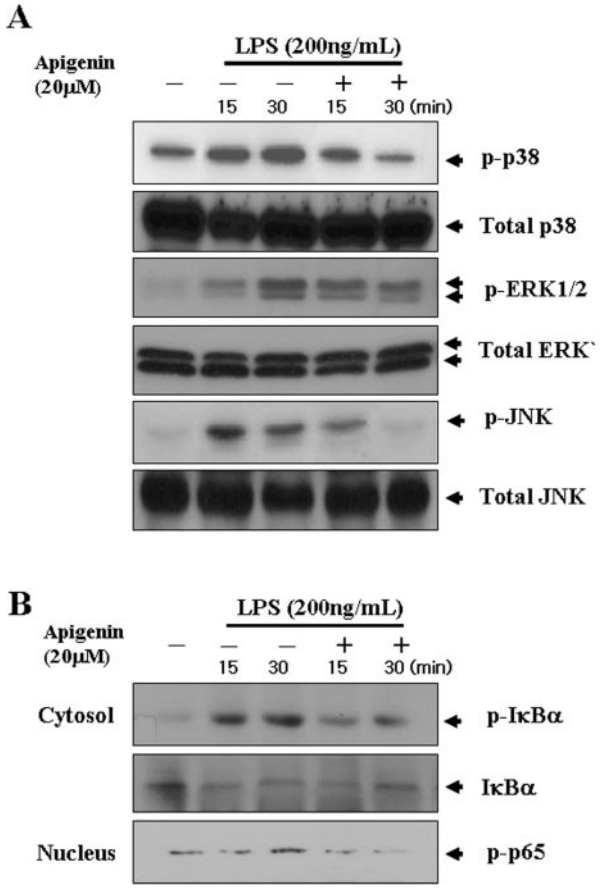


Fig. 5. Apigenin decreased MAPK and NF-κB translocation in LPS-stimulated DC. The DC were pretreated with 20 μM for 30 min before 200 ng/ml LPS stimulation. A, cell lysates were prepared and blotted with anti-phospho-ERK1/2 (p-p44/42), anti-ERK1/2 (p44/42), anti-phospho-p38 (p-p38), anti-p38 (p38), and anti-phospho-IκBα Abs. B, LPS-induced nuclear translocation of the NF-κB p65 subunit was inhibited by apigenin. The DC were pretreated with apigenin for 30 min and stimulated with LPS 200 ng/ml for the indicated times. Nuclear extracts were blotted with anti-p65 Ab. The bound antibodies were visualized using biotinylated goat anti-rabbit IgG. The results shown represent three independent experiments. UN, chemically untreated control group.

Intraperitoneal Administration of Apigenin Inhibits LPS-Induced DC Maturation. To determine whether the apparent inhibitory effects of apigenin on splenic DC maturation in vivo was mediated by drug toxicity alone, or by interference with DC production, we investigated the effects of apigenin on the phenotypic characteristics of LPS-stimulated mice. We isolated spleen-derived DC from all experimental groups and confirmed their phenotypic characteristics using flow cytometry. We determined that 95% of the evaluated DC expressed $CD11c^+$ molecules (Fig. 6). Representative FACS histograms showed that only the splenic DC that had been exposed to LPS expressed detectable levels of costimulatory and MHC molecules. However, in cells that had been pretreated for 3 days with apigenin, CD80, CD86, and MHC class I and II molecules were markedly down-regulated 24 h after LPS challenge. These in vivo results indicate that apigenin pretreatment inhibits the phenotypic maturation of LPS-exposed DC.

Apigenin Impairs INF-γ Production by $CD4^+$ T Cells in LPS-Treated Mice. Naive allogeneic T cells that had been primed with mature DC differentiated into Th1 lymphocytes when they produced high levels of INF-γ, and high levels of IFN-γ in $CD4^+$ T cells induce IL-12 production in DC. We attempted to characterize the effects of apigenin on INF-γ production in $CD4^+$ T cells in mice exposed to LPS. We isolated spleen-derived $CD4^+$ T cells from all of the experimental mice and then measured INF-γ production via flow cytometry. We determined that 95% of the evaluated T cells were expressing $CD4^+$ molecules (Fig. 7). Representative FACS histograms revealed that only the LPS-exposed splenic $CD4^+$ T cells expressed detectable INF-γ levels. However, in cells pretreated for 3 days with apigenin, INF-γ production was markedly down-regulated 24 h after LPS challenge. These in vivo data show that apigenin pretreatment impairs INF-γ production in splenic $CD4^+$ T cells that had been stimulated with LPS. These results show that the majority of the effects of apigenin on the T cell-differentiating properties of DC occur via INF-γ inhibition.

Apigenin-Treated DC Fail to Induce Normal Cell-Mediated Immune Responses. A single s.c. injection of 10^6 TNBS-pulsed purified DC was shown to induce a significant CHS response, which was visualized 7 days after the injection, when the animals were challenged with a hapten. By contrast, the apigenin-treated DC failed to elicit a significant immune response under identical conditions (Fig. 8). In ad-

dition, the responses of animals that had been sensitized with TNBS-pulsed, apigenin-treated DC were similar to those of the unsensitized animals. The results for the control group, which were injected with unpulsed DC (negative control), and for the group that was sensitized by means of an epicutaneous application of hapten (positive control) confirmed that the immune responses were antigen-specific.

Discussion

The activation and maturational states of DC are regulated by a variety of extracellular stimuli, including cytokines and bacterial products (Thomas Luft et al., 2002). These events are closely related to changes in the phenotypic and functional properties of DC in draining lymph nodes and activating antigen-specific T cells. DC located in an inflammatory site or at a portal of entry for various pathogens undergo maturation via migration to the T-cell area; this is a critical component of antigen presentation. DC are thought to perform an important role in establishing hypersensitivity and transplantation tolerance (Thomson et al., 1995; Starzl and Zinkernagel, 1998). In mice, the role of thymic DC in negative selection has been verified through the targeted expression of MHC class II molecules in DC (Starzl and Zinkernagel, 1998). However, the role of DC in establishing peripheral T-cell tolerance has yet to be convincingly demonstrated. Thus, it is quite important to regulate DC differentiation via the manipulation of exogenous or endogenous factors.

In previous studies, many researchers have reported that apigenin exerts a variety of biological effects, including anti-inflammatory, anticarcinogenic, and free radical-scavenging effects in many in vitro systems (Kim et al., 1998). Apigenin also inhibits the phorbol 12-myristate 13-acetate-induced activity of Elk-1 and c-Jun as well as MAPK signaling (Yin et al., 1999). In a recent study, the investigators showed that apigenin is a potent inhibitor of the nuclear transcription factor NF-κB, which is thought to perform a pivotal role in the regulation of cell growth, apoptosis, and cell cycle regulation. However, the cellular targets of apigenin in the immune system remain poorly understood, and its effects on DC have yet to be thoroughly elucidated.

In this study, we attempted to characterize the effects of apigenin on the maturation and function of LPS-exposed DC, including the expression of MHC molecules and costimula-

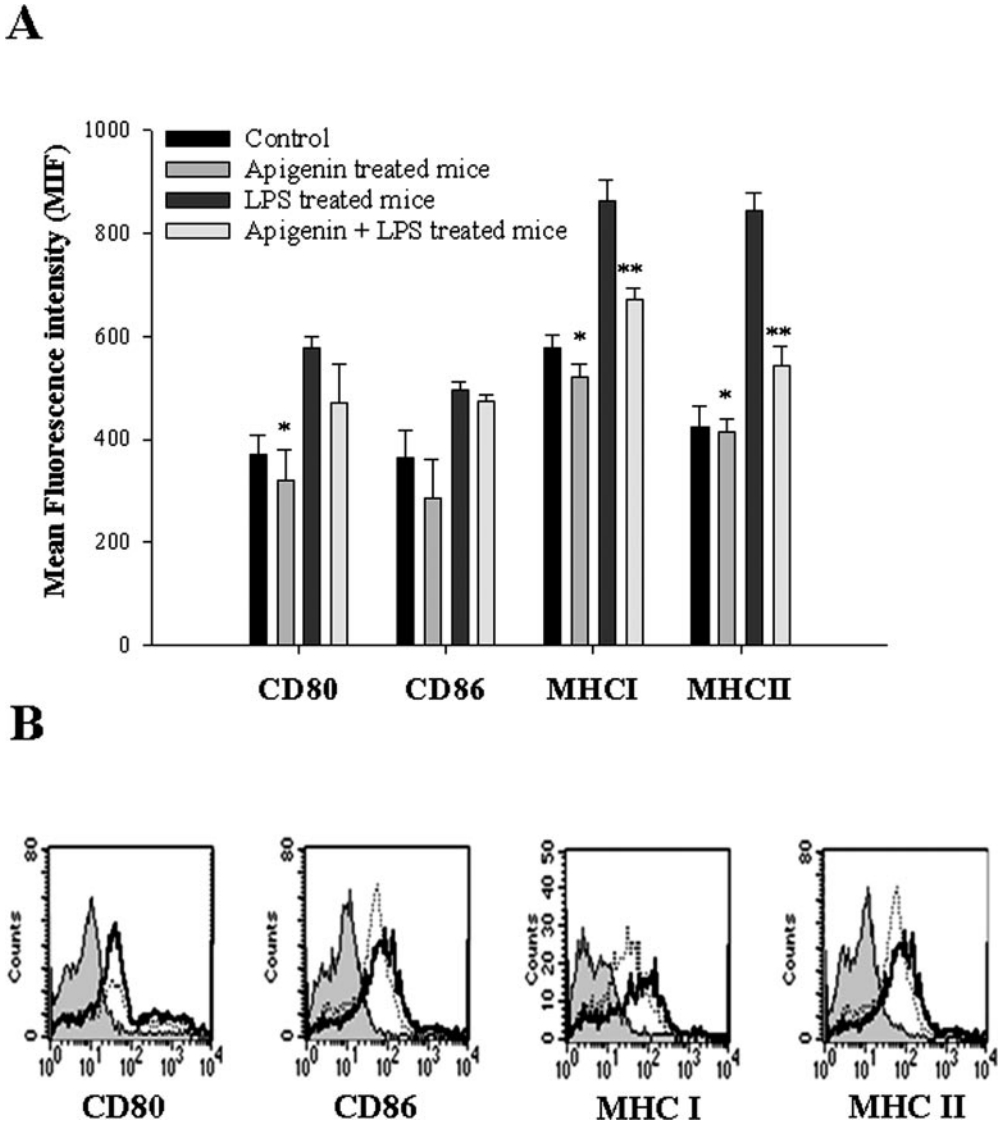


Fig. 6. In vivo administration of apigenin suppressed the phenotypic maturation of LPS-challenged splenic DC. Mice were injected intraperitoneally with 5 mg/kg apigenin every 3 days. One hour after the last injection, the mice were injected in a lateral tail vein with 1 mg/kg LPS. At 24 h, the mice were sacrificed and the splenic DC were generated, as was described under *Materials and Methods*. The cells were harvested and analyzed via two-color flow cytometry. The cells were gated on $CD11c^+$ for mean fluorescence intensity (MFI) (A) and positive populations (B). The histogram is from a single experiment that is representative of three.

tory molecules, IL-12 production, endocytosis, and the stimulation of allogeneic T-cell proliferation. Our results revealed that apigenin is a potent inhibitor of DC maturation, under both in vitro and in vivo conditions. These data provide us with new insight into the immunopharmacological aspects of apigenin. Moreover, this readily available drug may provide a simple, inexpensive, and highly effective means for the manipulation of the immunostimulatory capacity of DC. It remains to be determined, however, whether the profound suppressive effects on DC maturation evinced by apigenin are actually attributable to a nonspecific inhibitory effect. Thus, we also evaluated the ability of apigenin-treated DC to internalize FITC-dextran by means of mannose receptor-mediated endocytosis. This mechanism is a distinctive characteristic of mature DC (as opposed to immature DC) (Sallusto et al., 1995). The endocytotic capacity of apigenin-treated DC was profoundly increased, indicating that apigenin inhibits the phenotypical and functional maturation of DC. Our data also revealed that apigenin causes $CD11c^+$ DC to generate IL-12 in the presence of LPS and confirmed its inhibitory effects on the expression of intracellular IL-12 p40/p70 and IL-12 p70. We also conducted an evaluation of MAPK to characterize the mechanism underlying the ability of apigenin to inhibit LPS-induced DC maturation. MAPK,

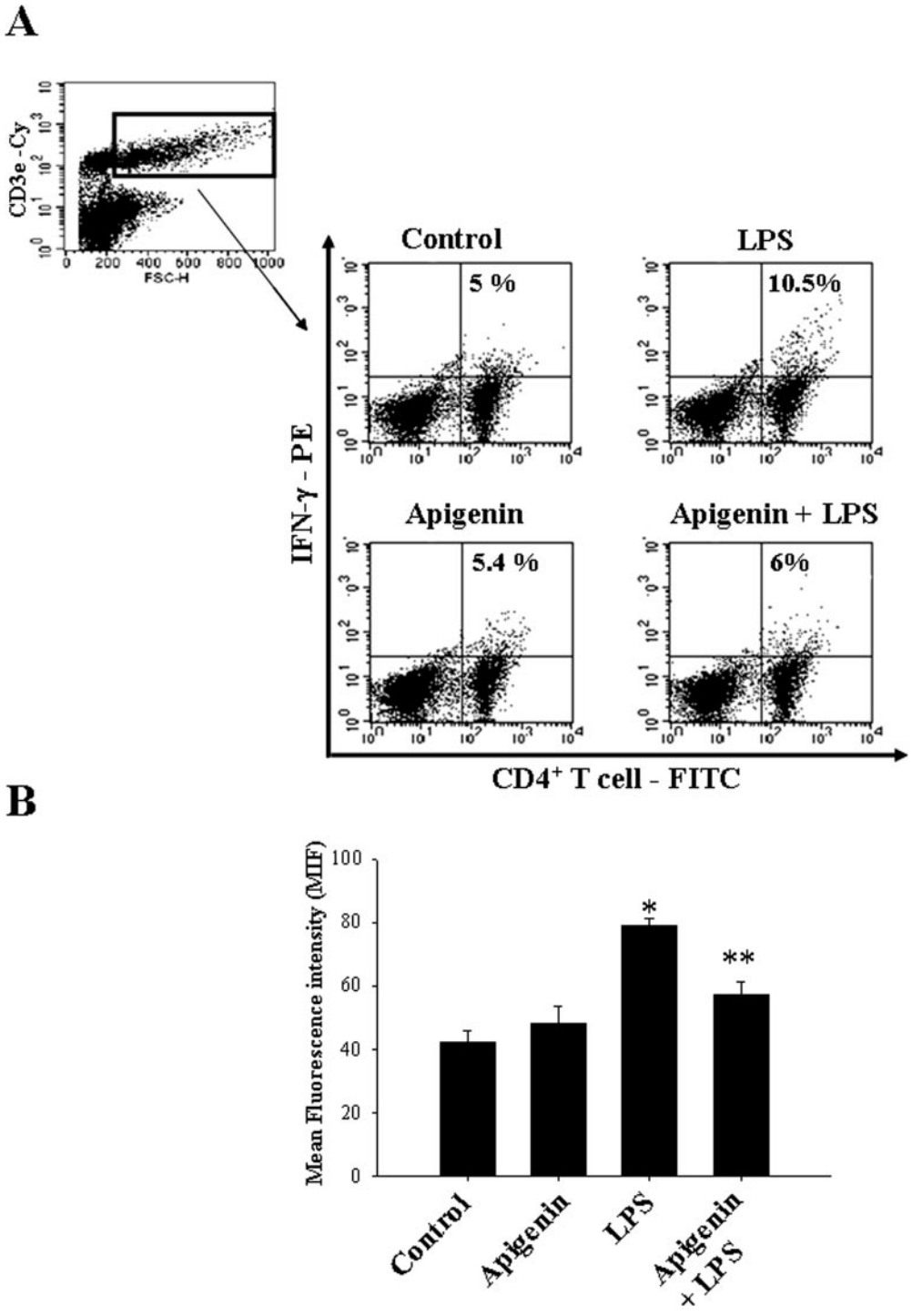


Fig. 7. Apigenin impaired INF-γ production in the $CD4^+$ T cells of LPS-treated mice. Mice were injected i.p. with 5 mg/kg apigenin every 3 days. One hour after the last injection, the mice were injected with 1 mg/kg LPS in a lateral tail vein. They were sacrificed at 24 h, and the T cells were generated as was described under *Materials and Methods*. A, $CD4^+$ T cells were subsequently analyzed using an intracellular cytokine staining technique. The cells were gated on $CD4^+$. B, MIF. The histogram is from a single experiment that is representative of three (**, $p < 0.01$ versus LPS-treated mice).

including its p38, ERK1/2, and JNK subfamilies, are activated by a variety of stimuli, including DNA-damaging agents, cytokines, and growth factors. MAPK regulates gene expression via the phosphorylation of downstream transcription factors (Kyriakis and Avruch, 1996). Both JNK and p38 kinase activation have been associated with arrested development, responses to stress, and apoptosis (Yang et al., 1997). In addition to these physiological responses and activation patterns, MAPK activation also seems to vary with the type of MAPK involved as well as the cell type. In this study, apigenin was determined to exert a suppressive effect on the phenotypic and functional maturation of murine BM-DC, via the inhibition of p38 kinase, ERK1/2, and JNK. This mechanism may be relevant to the protective effects of apigenin that have been observed in autoimmune diseases, including arthritis, allergy, and diabetes. We also determined that the NF-κB signaling pathway may be inhibited by apigenin upon DC maturation.

Recent evidence suggests that cytokine production in DC varies with the particular DC subset or its stimuli (Kadowaki et al., 2001). IL-12, in particular, exerts multiple immunoregulatory functions that activate the Th1 subset, which plays a pivotal role in the induction of inflammation (Triantaphyllopoulos et al., 1999). A growing body of evidence suggests that the differentiation of Th1 cells is regulated primarily by DC-derived cytokines, including IL-12 (Rissoan et al., 1999) and IFN-γ (Brinkmann et al., 1993). In addition, our results indicate that LPS-stimulated CD11c$^+$ DC skewed naive T cells toward differentiation into IFN-γ-producing T cells. Apigenin was shown to significantly impair the ability of these cells to proliferate and initiate Th1 responses. Naive T cells stimulated with apigenin-treated DC generated lower levels of IFN-γ, but they exhibited no significant changes in the quantity of IL-4 that they produced. These results indicate that apigenin is a potent inhibitor of DC maturation. Because Th1 cells are either functionally immunogenic or provide protection against invading pathogens, the inhibition of DC-mediated Th1 polarization may constitute an apigenin-associated immunosuppressive mechanism. However, the inhibition of Th1 development exerts negative effects on the regulation of a variety of immune cells. Indeed, Th1 development was abolished when the above-mentioned molecules were inhibited during antigen presentation. Thus, the present finding that apigenin inhibits the expression of LPS-induced costimulatory molecules on the surfaces of the DC may point to a new strategy by which T-cell responses can be driven toward the Th2 type of response. It seems that the inhibition of the expression of costimulatory molecules on the surfaces of DC treated with apigenin results in low IL-12 production levels and a diminished ability to induce Th1 polarization. Indeed, we observed that the production of IL-12 in the presence of apigenin is inhibited in DC treated with LPS plus apigenin, compared with DC treated with LPS alone.

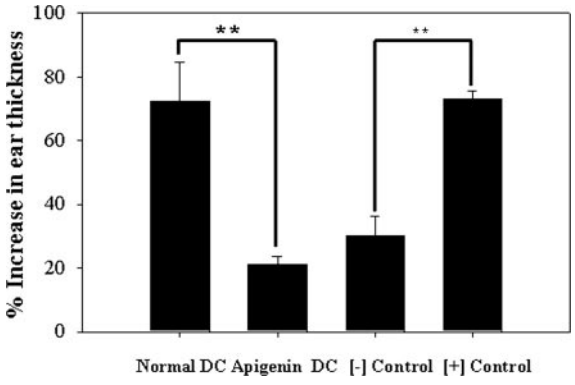


Fig. 8. Apigenin-treated DC fail to induce a normal cell-mediated immune response. A culture consisting of approximately 10^6 purified DC in the presence or absence of 20 μM apigenin was pulsed with 0.1% (w/v) TNBS and injected s.c. on day 0, as described under *Materials and Methods*. In the control groups, DC were either not TNBS-pulsed ([−] control) and injected s.c., or the animals were shaved and the abdominal skin was painted with 7% (w/v) TNCB ([+] control). After 7 days, the ear thickness of the experimental mice was measured. The results represent the mean ± S.D. percentage of increase in ear swelling for six treatment group animals and six control group animals. Treatment with the vehicle alone did not induce swelling. The p values were calculated using Student's t test for independent samples (**, $p < 0.01$ versus apigenin-treated DC or [−] control).

Based on our observations, we were able to hypothesize that the ability of apigenin-treated DC to stimulate naive T cells in vivo and to initiate cell-mediated immune responses should be impaired. It was recently demonstrated that as few as 10^5 TNBS-pulsed murine BM-derived DC (TNBS-DC) induced a profound CHS (Lappin et al., 1999). The ability of the DC to induce immune responses mediated by T cells was assessed using TNBS-DC to sensitize for CHS in naive syngeneic recipients. CHS sensitization was confirmed in the recipients of s.c. injections containing 10^5 TNBS-DC, but it was not observed in the recipients of apigenin-pretreated TNBS-DC. These results indicate that the decreased T-cell stimulatory capacity of apigenin-treated, BM-derived DC cannot be readily reversed.

These results indicate that the primary effects of apigenin involve the suppression of MAPK and NF-κB p65 subunit activation. To ensure that these effects are attributable to DC and not to contaminating cells in BM-derived cell cultures, the DC were purified (>90%) before the analysis phase of each assay. All of our results show that apigenin is a potent inhibitor of LPS-induced DC maturation.

Th1/Th2 polarization has reportedly been regulated by microenvironmental conditions, including the concentration of antigen and/or other extracellular stimuli. Our findings show that apigenin affects the ability of DC to induce Th1/Th2 polarization via the modulation of costimulatory molecule expression and IL-12 production in DC. These findings also provide new insight into the immunopharmacology of apigenin.

References

Ahn SC, Kim GY, Kim JH, Baik SW, Han MK, Lee HJ, Moon DO, Lee CM, Kang JH, Kim BH, et al. (2004) Epigallocatechin-3-gallate, constituent of green tea, suppresses the LPS-induced phenotypic and maturation of murine dendritic cells through inhibition of mitogen-activated protein kinase and NF-κB. *Biochem Biophys Res Commun* **313:**148–155.

An H, Yu Y, Zhang M, Xu H, Qi R, Yan X, Liu S, Wang W, Guo Z, Guo J, et al. (2002) Involvement of ERK, p38 and NF-κB signal transduction in regulation of TLR2, TLR4 and TLR9 gene expression induced by lipopolysaccharide in mouse dendritic cells. *Immunology* **106:**38–45.

Austyn JM (1998) Dendritic cells. *Curr Opin Hematol* **5:**3–15.

Banchereau J, Briere F, Caux C, Davoust Caux, Lebecque S, Liu YJ, Pulendran B, and Palucka K (2000) Immunobiology of dendritic cells. *Annu Rev Immunol* **18:** 767–811.

Banchereau J and Steinman RM (1998) Dendritic cells and the control of immunity. *Nature (Lond)* **392:**245–252.

Brinkmann V, Geiger T, Alkan S, and Heusser CH (1993) Interferon-γ increases the frequency of interferon-g-producing human CD4+ T cells. *J Exp Med* **178:**1655–1663.

Cella M, Sallusto F, and Lanzavecchia A (1997) Origin, maturation and antigen presenting function of dendritic cells. *Curr Opin Immunol* **9:**10–16.

Chen CC, Chow MP, Huang WC, Lin YC, and Chang YJ (2004) Flavonoids inhibit tumor necrosis factor-α-induced up-regulation of intercellular adhesion molecule-1 (ICAM-1) in respiratory epithelial cells through activator protein-1 and nuclear factor-κB: structure–activity relationships. *Mol Pharmacol* **66:**683–693.

Chen JW, Zhu ZQ, Hu TX, and Zhu DY (2002) Structure-activity relationship of naturalflavonoids in hydroxyl radical-scavenging effects. *Acta Pharmacol Sin* **23:** 667–672.

De Smedt T, Pajak B, and Muraille E (1996) Regulation of dendritic cell numbers and maturation by lipopolysaccharide in vivo. *J Exp Med* **184:**1413–1424.

Duthie G and Crozier A (2000) Plant-derived phenolic antioxidants. *Curr Opin Clin Nutr Metab Care* **3:**447–451.

Hastak K, Gupta S, Ahmad N, Agarwal MK, Agarwal ML, and Mukhtar H (2003) Role of p53 and NF-kappaB in epigallocatechin-3-gallate-induced apoptosis of LNCaP cells. *Oncogene* **22:**4851–4859.

Inaba K, Inaba M, Romani N, Aya H, Deguchi M, S Ikehara, S Muramatsu, and Steinman RM (1992) Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony stimulating factor. *J Exp Med* **176:**1693–1702.

Kadowaki N, Ho S, Antonenko S, Malefyt RW, Kastelein RA, Bazan F, and Liu YJ (2001) Subsets of human dendritic cell precursors express different Toll-like receptors and respond to different microbial antigens. *J Exp Med* **194:**863–869.

Kim GY, Lee MY, Lee HJ, Moon DO, Lee CM, Jin CY, Jeong YK, Chun KT, Lee JY, Choi IH, et al. (2005) Effect of water-soluble preteoglycan isolated from *Agaricus blazei* on the maturation of murine bone marrow-derived dendritic cells. *Int Immunopharmacol* **5:**1523–1532.

Kim GY, Oh WK, Shin BC, Shin YI, Park YC, Ahn SC, Lee JD, Bae YS, Kwak JY, and Park YM (2004) Proteoglycan Isolated from *Phellinus linteus* inhibits tumor growth through mechanisms leading to an activation of CD11c+ CD8+ DC and type I helper T cell-dominant immune state. *FEBS Lett* **576:**391–400.

Kim HP, Mani I, Iversen L, and Ziboh VA (1998) Effects of naturally-occurring flavonoids and biflavonoids on epidermal cyclooxygenase and lipoxygenase from guinea-pigs. *Prostaglandins Leukot Essent Fatty Acids* **58:**17–24.

Kolb-Maurer A, Gentschev I, and Fries HW (2000) Listeria monocytogenes-infected human dendritic cells: uptake and host cell response. *Infect Immun* **68:**3680–3688.

Kyriakis JM and Avruch J (1996) Protein kinase cascades activated by stress and inflammatory cytokines. *Bioessays* **18:**567–577.

Lapointe R, Toso JF, Butts C, Young HA, and Hwu P (2000) Human dendritic cells require multiple activation signals for the efficient generation of tumor antigen-specific T lymphocytes. *Eur J Immunol* **30:**3291–3298.

Lappin MB, Weiss JM, Delattre V, Mai B, Dittmar H, Maier C, Manke K, Grabbe S, Martin S, and Simon JC (1999) Analysis of mouse dendritic cell migration in vivo upon subcutaneous and intravenous injection. *Immunology* **98:**181–188.

Luft T, Jefford M, Luetjens P, Hochrein H, Masterman KA, Maliszewski C, Shortman K, Cebon J, and Maraskovsky E (2002) IL-1β enhances CD40 ligand-mediated cytokine secretion by human dendritic cells (DC): a mechanism for T cell-independent DC activation. *J Immunol* **168:**713–722.

Lutz MB, Assmann CU, Girolomoni G, and Ricciardi-Castagnoli P (1996) Different cytokines regulate antigen uptake and presentation of a precursor dendritic cells line. *Eur J Immunol* **26:**586–594.

Lyons AB (2000) Analysing cell division in vivo and in vitro using flow cytometric measurement of CFSE dye dilution. *J Immunol Methods* **243:**147–154.

Mosca PJ, Hobeika AC, Clay TM, Nair SK, Thomas EK, Morse MA, and Lyerly HK (2000) A subset of human monocyte-derived dendritic cells expresses high levels of interleukin-12 in response to combined CD40 ligand and interferon-gamma treatment. *Blood* **96:**3499–3504.

Porgador A and Gilboa E (1995) Bone marrow-generated dendritic cells pulsed with a class I-restricted peptide are potent inducers of cytotoxic T lymphocytes. *J Exp Med* **182:**255–260.

Rescigno M, Martino M, Sutherland CL, Gold MR, and Ricciardi-Castagnoli P (1998) Dendritic cell survival and maturation are regulated by different signaling pathways. *J Exp Med* **188:**2175–2180.

Rissoan MC, Soumelis V, Kadowaki N, Grouard G, Briere F, de Waal Malefyt R, and Liu YJ (1999) Reciprocal control of T helper cell and dendritic cell differentiation. *Science (Wash DC)* **283:**1183–1186.

Salio M, Cerundolo V, and Lanzavecchia A (2000) Dendritic cell maturation is induced by *Mycoplasma* infection but not necrotic cells. *Eur J Immunol* **30:**705–708.

Sallusto F, Cella M, Danieli C, and Lanzavecchia A (1995) Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: down-regulation by cytokines and bacterial products. *J Exp Med* **182:**389–400.

Hougee S, Sanders A, Faber J, Graus YM, van den Berg WB, Garssen J, Smit HF, and Hoijer MA (2005) Decreased pro-inflammatory cytokine production by LPS-stimulated PBMC upon in vitro incubation with the flavonoids apigenin, luteolin or chrysin, due to selective elimination of monocytes/macrophages. *Biochem Pharmacol* **69:**241–248.

Starzl TE and Zinkernagel RM (1998) Antigen localization and migration in immunity and tolerance. *N Engl J Med* **339:**1905–1913.

Steinman RM (1991) The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* **9:**271–296.

Thomson AW, Lu L, Murase N, Demetris AJ, Rao AS, and Starzl TE (1995) Microchimerism, dendritic cells progenitors and transplantation tolerance. *Stem Cells* **13:**622–639.

Triantaphyllopoulos KA, Williams RO, Tailor H, and Chernajovsky Y (1999) Amelioration of collagen-induced arthritis and suppression of interferon-α, interleukin-12, and tumor necrosis factor-α production by interferon-g gene therapy. *Arthritis Rheum* **42:**90–99.

Wang W, Heideman L, Chung CS, Pellin JC, Koehler KJ, and Birt DF (2000) Cell-cycle arrest at G_2/M and growth inhibition by apigenin in human colon carcinoma cell lines. *Mol Carcinog* **28:**102–110.

Yang X, Khosravi-Fa R, Chang HY, and Baltimore D (1997) Daxx, a novel Fas-binding protein that activate JNK and apoptosis. *Cell* **89:**1067–1076.

Yin F, Giuliano AE, and Van Herle AJ (1999) Signal pathways involved in apigenin inhibition of growth and induction of apoptosis of human anaplastic thyroid cancer cells (ARO). *Anticancer Res* **19:**4297–4303.

Address correspondence to: Dr. Yeong-Min Park, Department of Microbiology and Immunology and National Research Laboratory of Dendritic Cell Regulation, Pusan National University College of Medicine, Ami-dong 1-10, Seo-gu, Pusan 602-739, South Korea. E-mail: immunpym@pusan.ac.kr