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

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Running Title : Apigenin inhibits Immunostimulatory Function of DC

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Abbreviations: DC, dendritic cells; MAPK, mitogen-activated protein kinase; BM, bone marrow; APC, antigen-presenting cells; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; TNCB, 2,4,6-trinitrochlorobenzene; CHS, contact hypersensitivity.

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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 MHC class I and II molecules expressions on DC were significantly suppressed, and LPS-induced 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 MAPK, the nuclear translocation of its NF-κB p65 subunit, and the induction of the Th1 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 may also prove useful in the development of adjuvant therapies for individuals suffering from acute or chronic DC-associated diseases.

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Introduction

Dendritic cells (DC) are antigen-presenting cells (APC) 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 (Banchereau J et al., 1998; Steinman RM et al., 1991). 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 naïve T cells via the signaling of both major antigen-presenting histocompatibility complex (MHC) molecules and costimulatory molecules (Austyn JM, 1998). DC have also been shown to be highly responsive to inflammatory cytokines and bacterial products, including tumor necrosis factor-alpha (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 T et al., 1996; Cella M, 1997). Similar maturation-indicative changes have also been reported after infections with *Mycoplasma,* viruses, intracellular bacteria, and parasites (Salio M et al., 2000; Kolb-Maurer A et al., 2000). DC located in the peripheral tissues tend to be both phenotypically and functionally immature; (Banchereau, J and Steinman R. M, 1998) consequently, they are unable to induce primary immune responses, as they do not express the requisite costimulatory molecules, nor do they express antigenic

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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 IL-12 upon stimulation (Banchereau J et al., 2000).

The flavonoids comprise a family of common phenolic plant pigments that have been identified as dietary anticarcinogens and antioxidants (Chen JW et al., 2002). We reported in a previous study that a variety of phytochemicals exhibit profound immunoregulatory activity, particularly in the DC (Kim GY et al., 2004, 2005; Ahn SC et al., 2004). 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 G et al., 2000). Apigenin has demonstrated antiinflammatory, anticarcinogenic, and free-radical scavenging activities in a variety of *in vitro* systems (Kim, HP et al., 1998). In a recent study, investigators identified apigenin as a potent inhibitor of the nuclear transcription factor NF-κB, which may perform a pivotal function in the regulation of cell growth, apoptosis, and the regulation of the cell cycle (Hastak K et al., 2003). Studies using human leukemia cells, as well as carcinoma cells in the breast, colon, and elsewhere, have revealed that

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apigenin inhibits cell growth via the induction of cell-cycle arrest and apoptosis (Wang W et al., 2000). It also attenuates pro-inflammatory cytokine production in LPS-stimulated peripheral blood mononuclear cells (PBMC) via the selective elimination of monocytes and macrophages, inhibits TNF-α–induced intercellular adhesion molecule-1 up-regulation *in vivo*, and inhibits interleukin (IL)-1 α-induced prostaglandin synthesis and TNF-α-induced IL-6 and IL-8 production (Sander Hougee *et al*., 2005). Moreover, it actively inhibits IκB kinase (IKK) activity, IκBα degradation, nuclear factor-κB (NF-κB) DNA-protein binding activity, NF-κB luciferase activity, and mitogen-activated protein kinase (MAPK) activity (Chen CC 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

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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 APC 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.

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Materials and Methods

Animals and chemicals: Male 8–12-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.

Reagents and Abs: Recombinant mouse (rm)GM-CSF and rmIL-4 were purchased from R&D Systems. Apigenin, dextran- FITC (molecular mass, 40,000), and LPS (from *Escherichia coli* 055:B5) were obtained from Sigma-Aldrich. An endotoxin fil edter (END-X) and an endotoxin removal resin (END-X B15) were acquired from Associates of Cape Cod. Cytokine ELISA kits for murine IL-12 p70, IL-4, and IFN-γ were purchased from BD Pharmingen. FITC- or 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), 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, biotinylated anti- CD11c (N418) mAb, were purchased from BD Pharmingen. To detect protein levels, anti-phospho-ERK, anti-ERK, anti-phospho-

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p38, anti-p38,anti-p-IκB, anti-IκB, anti-phospho-JNK, and anti-JNK from Cell Signaling, and anti-p65 Ab from Abcam were purchased.

Isolation and culture of DC: DC was generated from murine BM cells, as described by Inaba et al. (1992) and Porgador A et al. (1995) with modifications. Briefly, BM was 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 OptiMEM (Invitrogen Life Technologies) supplemented with 10% heat-inactivated 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 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) followed by positive selection through paramagnetic columns (LS columns; Miltenyi Biotec) according to the manufacturer's instructions.

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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 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 4ºC and stained with phycoerythrin (PE)-conjugated anti- H-2Kb [major histocompatibility complex (MHC) class I], anti-I-Ab (MHC class II), anti-CD80, and anti-CD86 with fluorescein isothiocyanate (FITC)-conjugated anti-CD11c (PharMingen, San Diego, CA) for 30 min at 4ºC. The stained cells were analysed using a FACSCalibur flow cytometer

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(Becton Dickinson, San Jose, CA).

Quantitation of Ag 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 (40,000 molecular mass; Sigma-Aldrich) at a concentration of 1 mg/ml. 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 Ag is lost if DC are cultured without GM-CSF (Rescigno M et al., 1998)

Cytokines assay: Cells were first blocked with 10% v/v normal goat serum for 15 min at 4 ºC and then stained with FITC-conjugated $CD11c^+$ antibody for 30 min at 4 ºC. Cells stained with the appropriate isotype-matched Ig were used as negative controls. The cells were fixed and permeated with the Cytofix/Cytoperm kit (PharMingen) according to manufacturer's instructions. Intracellular IL-12p40/p70, IL-10, and IFN-γ

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were stained with fluorescein R-phycoerythrin (PE)-conjugated antibodies (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 (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, Gladbach, Germany). Staining with fluorescein isothiocyanate (FITC)-conjugated anti−CD3 antibodies (BD PharMingen, San Diego, Calif, USA) 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 previously described (Lyons AB 2000). The cells were resuspended in 1 μM CFSE in phosphate-buffered saline (PBS). After being shaken for 8 minutes at room temperature, the cells were washed once in pure fetal bovine serum (FBS) and twice in PBS with 10% FBS. DC (1×10^4) or DC exposed to apigenin (20 μM/mL) or LPS (100 ng/mL) for 24 hours were cocultured with 1×10^5 allogenic CFSE-labeled T lymphocytes in 96-well, U-bottom plates (Nunc). A negative control ($CD3^+$ lymphocytes alone) and a

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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 LPS (200 ng/ml) in the absence or presence of 20 μM apigenin pretreatment. Then following 15 or 30 min of incubation at 37 ºC, cells were washed twice with cold PBS and lased with modified RIPA buffer (1.0% NP-40, 1.0% sodium deoxycholate, 150nM NaCl, 10mM Tris–HCl [pH 7.5], 5.0mM sodium pyrophosphate, 1.0mM $NaVO_4$, 5.0mM NaF, 10μM/ml leupeptin, and 0.1mM phenylmethylsulfonyl fluoride) for 15 min at 4 ºC. Lysates were cleared by centrifuging at 14,000g for 20 min at 4 ºC. The protein content of cell lysates was determined using the Micro BCA assay kit (Pierce, Rockford, IL). Equivalent amounts of proteins were separated by SDS–10% PAGE and analyzed by Western blotting using an antiphospho- ERK1/2 (p-ERK, Santa Cruz, CA), anti-phospho-JNK (p-JNK, Santa Cruz, CA), or anti-phospho-p38 (p-p38, Santa Cruz, CA) MAP kinase mAb for 2 h, as described by the manufacturer of the antibodies. Following washing three times with TBST, membranes were incubated with secondary HRP-conjugated anti-mouse IgG for 1 h. After washing, the blots were developed using

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the ECL system (Amersham), by following manufacturer's instructions. DC nuclear extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL), 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, CA).

Generation of DC from spleen and culture: Mice were injected intraperitoneally with apigenin (5 mg/kg) every 3 days before the administration of 1mg/kg of LPS in a lateral vein of the tail. 24 h after LPS challenge, they were scarified, their spleens were disrupted and the cells were centrifuged at 400 g for 5 min, resuspended in RPMI-1640 supplemented with 10% heat-inactivated FBS, l-glutamine, non-essential amino acids, sodium pyruvate, penicillin-streptomycin, HEPES and 2-ME (all from Sigma) for 2 hr, and then non-adherent cells were washed out. The residual adherent cells were maintained in the culture medium and incubated overnight at 37 ºC in a 5% CO_2 atmosphere. After incubation, DC (which exhibit adherence capacity in the first hours of culture) become non-adherent and floats in the medium. The cells were gated on $CD11c^+$ for DC.

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Generation of $CD4^+$ T cell from spleen and culture: Mice were injected intraperitoneally with apigenin (5 mg/kg) every 3 days before the administration of 1mg/kg of LPS in a lateral vein of the tail. 24 h after LPS challenge, they were scarified, their spleens were disrupted and the cells were centrifuged at 400 g for 5 min, resuspended in RPMI-1640 supplemented with 10% heat-inactivated FBS, l-glutamine, non-essential amino acids, sodium pyruvate, penicillin-streptomycin, HEPES and 2-ME (all from Sigma) for 4 hr. The residual adherent cells were maintained in the culture medium and incubated overnight at 37 ºC in a 5% CO_2 atmosphere. The cells were fixed and permeated with the Cytofix/Cytoperm kit (PharMingen) according to manufacturer's instructions. Intracellular IFN-γ was stained with fluorescein R-phycoerythrin (PE)-conjugated antibodies (PharMingen) in a permeation buffer. The cells were gated on $CD4^+$ T cell for T cell.

2,4,6-Trinitrobenzenesulfonic acid (TNBS)-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

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(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). The percentage increase in ear thickness was calculated using the following formula: 100 × ((thickness of challenged ear-thickness of unchallenged ear)/thickness of unchallenged ear).

Statistics: All results were expressed as the means ± SD 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 ANOVA, 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.

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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 OptiMEM supplemented with granulocyte-macrophage-colony stimulating factor (GM-CSF) at concentrations of 20 ng/mL and IL-4 20 ng/mL. Different apigenin concentrations were added to the cultures on day 6, with or without 200 ng/mL of 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 hours in the presence of 0 to 20 μM apigenin, as was described in the *Materials and Methods* section of this article. As is shown in Figure 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. These molecules were also upregulated within 24 hours of exposure to LPS (Fig. 1C, thick

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lines). Exposure to 20 μM apigenin in the presence of LPS was accompanied by an impaired expression of the costimulatory molecules, CD80 and CD86. Interestingly, a significant downregulation of these 2 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 pro-inflammatory molecules (Mosca PJ et al., 2000). Thus, we assessed the ability of BM-DC to generate pro-inflammatory cytokines. IL-12 expression has previously been identified as a specific marker for DC activity (An H et al., 2002). It is also an important marker for DC maturation, and can be employed in the selection of Th1-dominant adjuvants. The secretion of bioactive IL-12 p70 requires the coordinated expression of 2 of its subunits, p35 and p40, which are encoded for by 2 separate genes and are independently regulated (Lutz M 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 Figure 2*A*, 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

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expressed small amounts of IL-12 p40/p70, as compared with unstimulated DC, whereas IL-10 was not detectable. When the supernatants were analyzed using ELISA, IL-10 was also undetectable 24 hours after stimulation with 200 ng/mL of LPS. As is shown in Figure 2B, ELISA analyses revealed high levels of IL-12 p70 when the DC were stimulated for 24 hours with LPS (92.3 ± 4.2 ng/mL). Apigenin (64.1 ± 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 pro-inflammatory 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 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-

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positive cells ($CD11c^+$ + dextran-FITC) was identical for the apigenin-treated and non-treated 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 were also conducted at 4ºC, 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 which render it particularly appropriate for this application. 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 twofold 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 following transfer *in vivo* (Lyons AB, 2000). In order to determine whether apigenin exerts a detectable effect on allogeneic T-cell stimulation, we treated DC with apigenin for 24 hours. As shown in Figure 4B, the LPS-treated DC exhibited

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a more profound proliferation rate than did the controls, whereas apigenin appeared to impair the proliferation response in allogeneic T cells elicited by LPS-activated DC. Importantly, 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, as 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. Naïve allogeneic T cells primed with mature DC differentiated into Th1 lymphocytes when they generated high levels of INF-γ and low levels of IL-4 (Fig 4C). By way of 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 apigenin's effects on the T-cell–differentiating properties of DC are a consequence of the inhibition of IL-12 production.

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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 M et al., 1998; Rana P et al., 2004). The activation of MAPK and NF-κB are important events in DC maturation (Rescigno M et al., 1998). LPS activates p-p38 kinase, p-ERK1/2, and p-JNK (Fig. 5A). In order 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 prior to the application of LPS stimulation. Pretreatment with 20 μM apigenin resulted in a marked inhibition of the LPS-induced upregulation 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 M 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

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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 minutes of exposure. Conversely, pretreatment with 20 μM apigenin resulted in the suppression of the LPS-enhanced nuclear translocation of NF-κB p65 (Fig. 5B).

Intraperitoneal administration of apigenin inhibits LPS-induced DC maturation: In order 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 downregulated 24 hours after LPS challenge. These *in vivo* results indicate that apigenin pretreatment inhibits the phenotypic maturation of LPS-exposed DC.

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Apigenin impairs INF-γ production by $CD4^+$ T cells in LPS-treated mice: Naïve 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 downregulated 24 hours 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

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challenged with a hapten. By way of contrast, the apigenin-treated DC failed to elicit a significant immune response under identical conditions (Fig 8). Additionally, 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.

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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 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 (Starzl TE et al., 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 TE et al., 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 antiinflammatory, anticarcinogenic, and free-radical scavenging effects--in many *in vitro* systems (Kim, HP et al., 1998). Apigenin also inhibits the PMA-induced activity of Elk-1 and c-Jun, as well as MAPK signaling (Yin F et al., 1999). In a recent study, the investigators showed that apigenin is a potent

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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 costimulatory 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 insights 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 F et al., 1995). The endocytotic capacity of apigenin-treated DC was profoundly increased, thereby

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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 in order to characterize the mechanism underlying the ability of apigenin to inhibit LPS-induced DC maturation. MAPK, including its p38, ERK1/2, and JNK subfamilies, are activated by a variety of stimuli, including DNA-damaging agents, cytokines, and growth factors. MAPK regulate gene expression via the phosphorylation of downstream transcription factors (Kyriakis JM et al., 1996). Both JNK and p38 kinase activation have been associated with arrested development, responses to stress, and apoptosis (Yang X et al., 1997). In addition to these physiological responses and activation patterns, MAPK activation also appears 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.

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Recent evidence suggests that cytokine production in DC varies with the particular DC subset or its stimuli (Kadowaki N 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 K et al., 199). A growing body of evidence suggests that the differentiation of Th1 cells is regulated primarily by DC-derived cytokines, including IL-12 (Rissoan M et al., 1999) and IFN-γ (Brinkmann V et al., 1993). Additionally, 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 exhibited no significant changes in the quantity of IL-4 they produced. These results indicate that apigenin is a potent inhibitor of DC maturation. As 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 wide 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

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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 appears 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.

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 MB et al., 1999). The ability of the DC to induce T-cell–mediated immune responses was assessed using TNBS-DC to sensitize for CHS in naive syngeneic recipients. CHS sensitization was confirmed in the recipients of SC injections containing 10^5 TNBS-DC, but 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

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MAPK and NF-κB p65 subunit activation. In order to ensure that these effects are attributable to DC and not to contaminating cells in BM-derived cell cultures, the DC were purified (>90%) prior to 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.

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Footnotes:

[1]These authors contributed equally to this work.

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Legends of Figures:

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 in the *Materials and Methods* section of this article. On day 6, the cells were harvested and analyzed using 2-color flow cytometry. Apigenin exerts no influence on growth and no cytotoxicity in $CD11c^+$ DC (A). The cells were gated on $CD11c^+$. Apigenin was added to the DC for 24 hours at concentrations of 5, 10, and 20 μM. The expression of surface molecules was then analyzed (B). DC were left untreated (control) or were stimulated for 24 hours with 200 ng/mL LPS in the absence or presence (gray line, control; thin line, apigenin plus LPS; thick line, LPS) of 20 μM apigenin on day 6 (C). UN represents the chemically untreated control group. The histogram is from one representative experiment out of 3.

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 apigenin (20 μM) for 24 hours with or without LPS. $CD11c^+$ DC were subsequently analyzed via intracellular cytokine staining (A). The cells were gated on $CD11c^+$. The DC (5×10^5 cells/mL) were cultured for 24 hours, and the production of bioactive IL-12 p70 in the culture supernatants (B)

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was analyzed using ELISA. The results shown are from one representative experiment out of 3 (*,$p < 0.01$ vs unstimulated DC (control); **,$p < 0.01$ vs LPS-stimulated DC).

Fig 3. Apigenin-stimulated DC exhibited increased antigen uptake. DC (1×10^5 cells) were treated with 20 μM of apigenin with or without LPS (200 ng/mL) for 24 hours. 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 cold HBSS and stained with PE-conjugated anti-CD11c antibody. The endocytic activity of the controls was determined after exposure to FITC-dextran at 4ºC. The numbers represent the percentages of cells. Medium designates the chemically untreated control group. In order to confirm these results, we repeated these experiments 3 times. The cells were gated on $CD11c^+$. The numbers indicate the percentage of $CD11c^+$ cells. The results represent 2 separate experiments, which 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 hours in medium alone, apigenin 20 μM, LPS 200 ng/mL, or apigenin with LPS. The DC were washed and then cocultured with T cells. (*A)*, Clustering was assessed after 64

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hours. B, the DC were cultured in medium with or without apigenin for 24 h. The treated DC were harvested and washed thoroughly to remove the apigenin. (B) A mixed lymphocyte reaction (MLR) was allowed to proceed for 4 days, as described in the *Materials and Methods* section of this article. (C) The cells were then examined after 48 hours for cytokine release using ELISA. The data are expressed as ng/mL/10^6 cells ± SD of triplicate cultures (**, P <.005 vs T cells primed with immature DC). Medium represents the chemically untreated control group. Similar results were obtained in 3 separate experiments.

Fig 5. Apigenin decreased MAPK and NF-κB translocation in LPS-stimulated DC. The DC were pretreated with 20 μM for 30 minutes prior to LPS stimulation (200 ng/mL). The cell lysates were prepared and blotted with antiphospho-ERK1/2 (p-p44/42), anti-ERK1/2 (p44/42), anti-phospho-p38 (p-p38), anti-p38 (p38), and anti-phospho-IκBα Abs (A). LPS-induced nuclear translocation of the NF-κB p65 subunit was inhibited by apigenin (B). The DC were pretreated with apigenin for 30 minutes and stimulated with LPS 200 ng/mL for the indicated lengths of time. Nuclear extracts were blotted with anti-p65 Ab. The bound antibodies were visualized using biotinylated goat anti-rabbit IgG. The results shown represent 3 independent experiments. UN represents the

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chemically untreated control group.

Fig 6. *In vivo* administration of apigenin suppressed the phenotypic maturation of LPS-challenged splenic DC. Mice were injected intraperitoneally with apigenin 5 mg/kg every 3 days. One hour after the last injection, the mice were injected in a lateral tail vein with 1 mg/kg of LPS. At 24 hours, the mice were sacrificed and the splenic DC were generated, as was described in the *Materials and Methods* section of this article. The cells were harvested and analyzed via 2-color flow cytometry. The cells were gated on $CD11c^+$ for mean fluorescence intensity (MFI) (A) and positive populations (B). The histogram is from one representative experiment out of three.

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

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out of 3 (**, *P* <.01 vs LPS-treated mice).

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 were pulsed with 0.1% w/v TNBS and injected SC on day 0, as was described in the *Materials and Methods* section of this article. In the control groups, DC were either not TNBS-pulsed ([-] control) and injected s.c, or the animals were shaved and the skin of their abdomen painted with 7% w/v TNCB ([+] control). After 7 days, the ear thickness of the experimental mice was measured. The results represent the mean ± SD percentage increase in ear swelling for 6 treatment group animals and 6 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* <.01 vs apigenin-treated DC or [-] control).

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Table 1. Apigenin markedly inhibits the expression of costimulatory molecules (CD80, CD86 and CD40), MHC class I and II on LPS-stimulated CD11c+ DC

	% of positive cell [a] (MFI) [b]			
Surface Ag	**Medium**	**Apigenin**	**LPS**	**Apigein + 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)**

[a]BM-derived DC were cultured in the absence or presence of apigenin (20 μM) following the LPS (200 ng/ml) stimulation for 24 h. The expression of surface molecules was analyzed by FACSCalibur. Two-color flow cytometry was used to determine the level of Ag expression on CD11c+ DC.

[b]MFI, Mean fluorescence intensity. The results are from one experiment of three performed.

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

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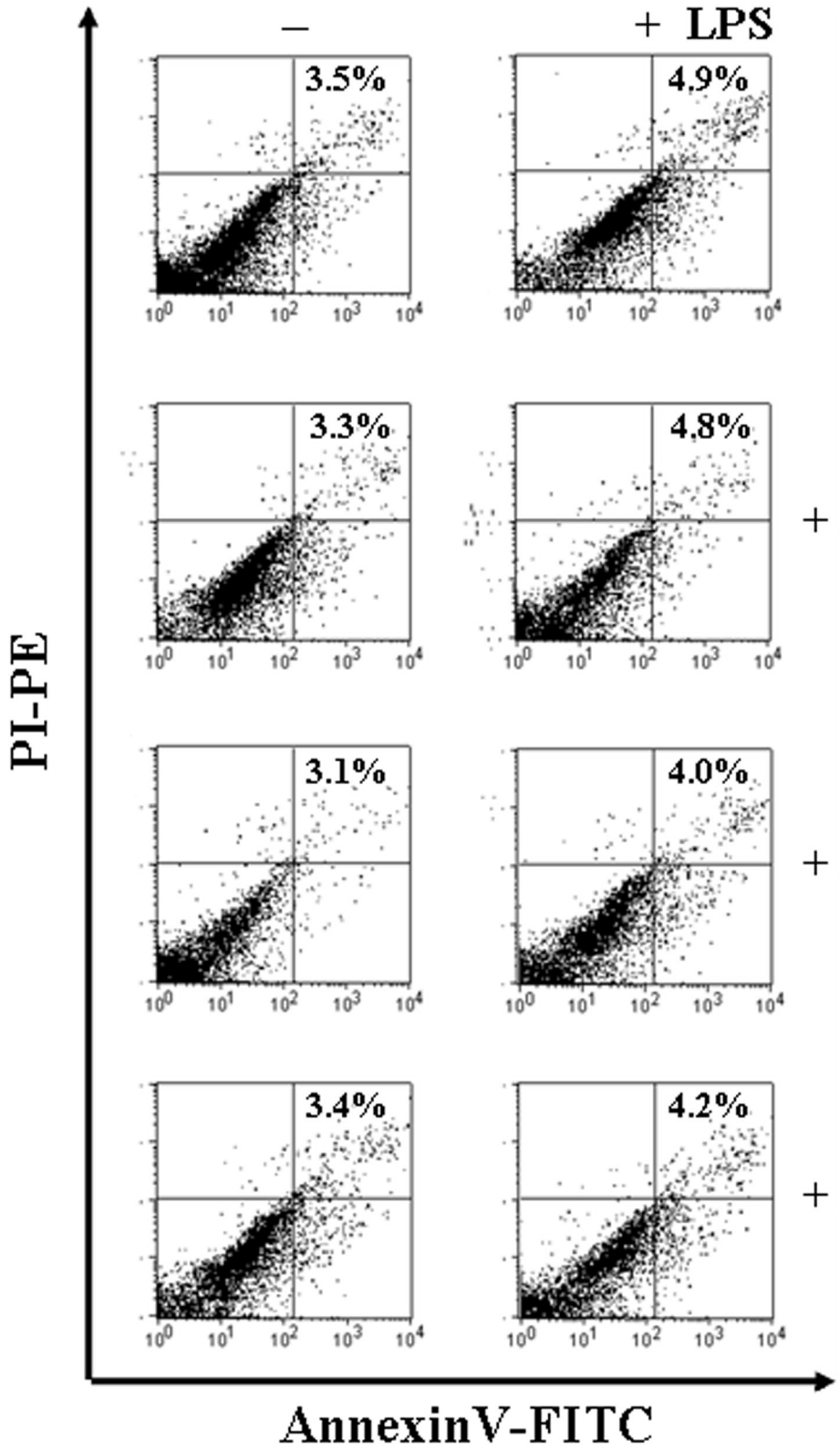

Fig. 1

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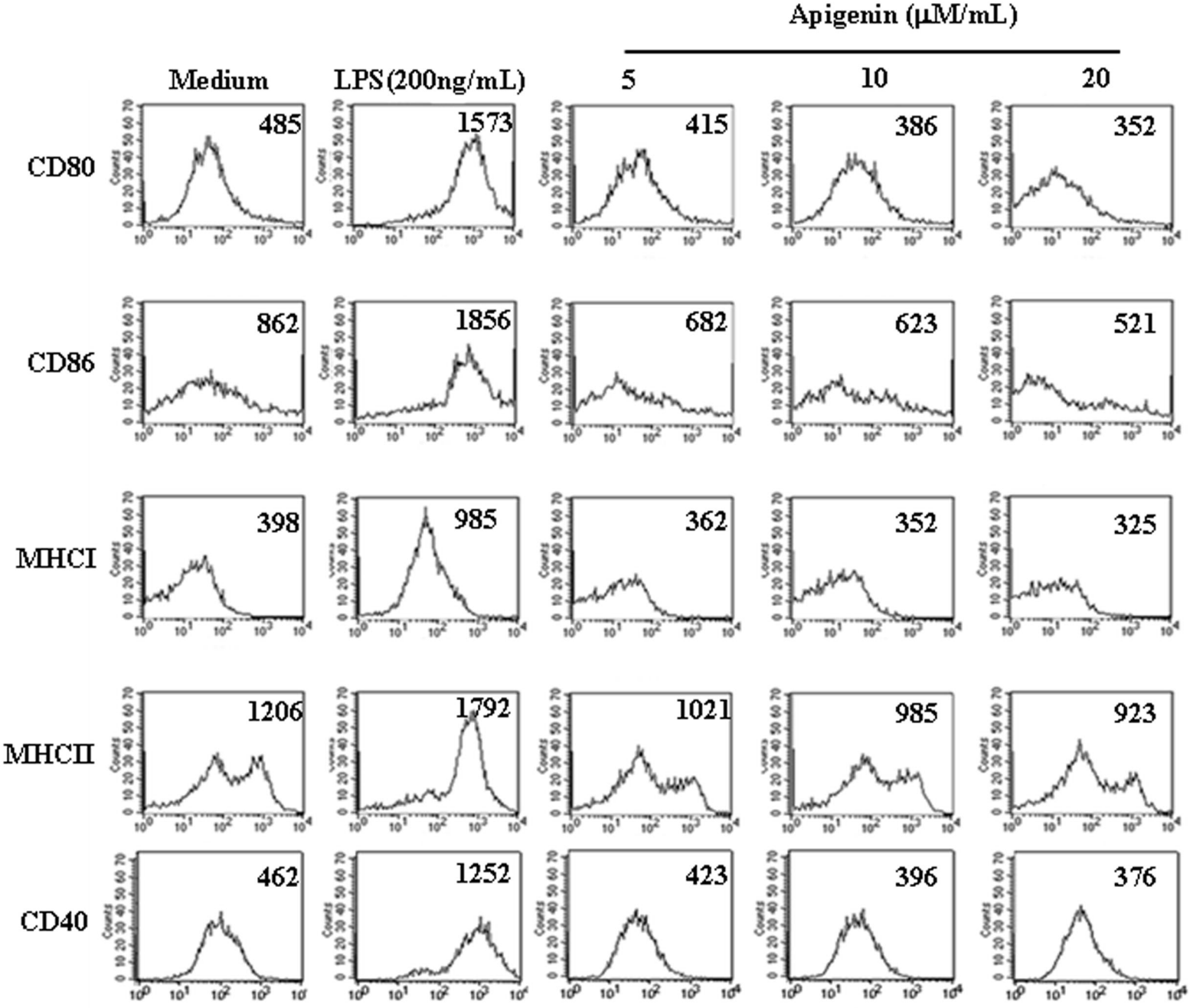
B



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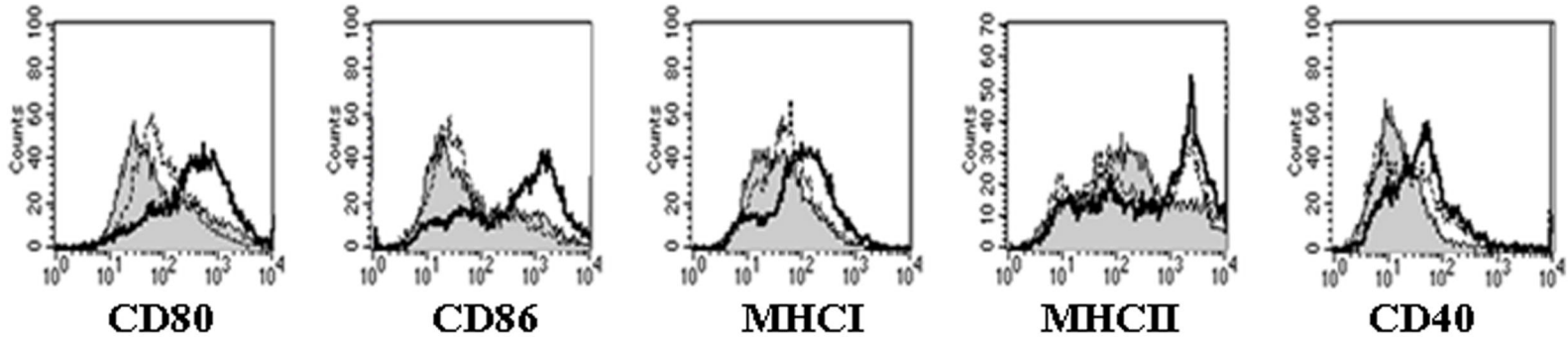

Fig. 1 Continued

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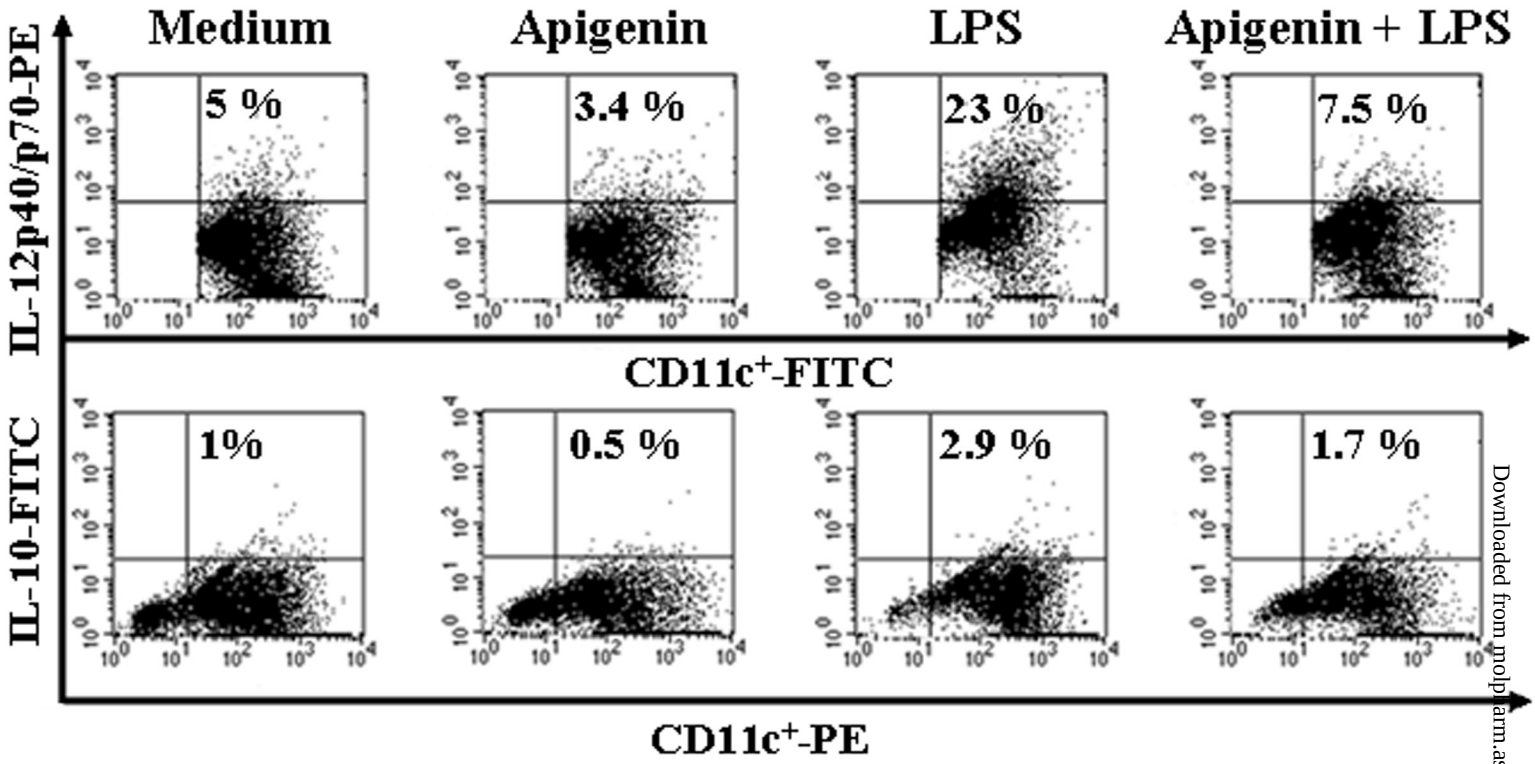

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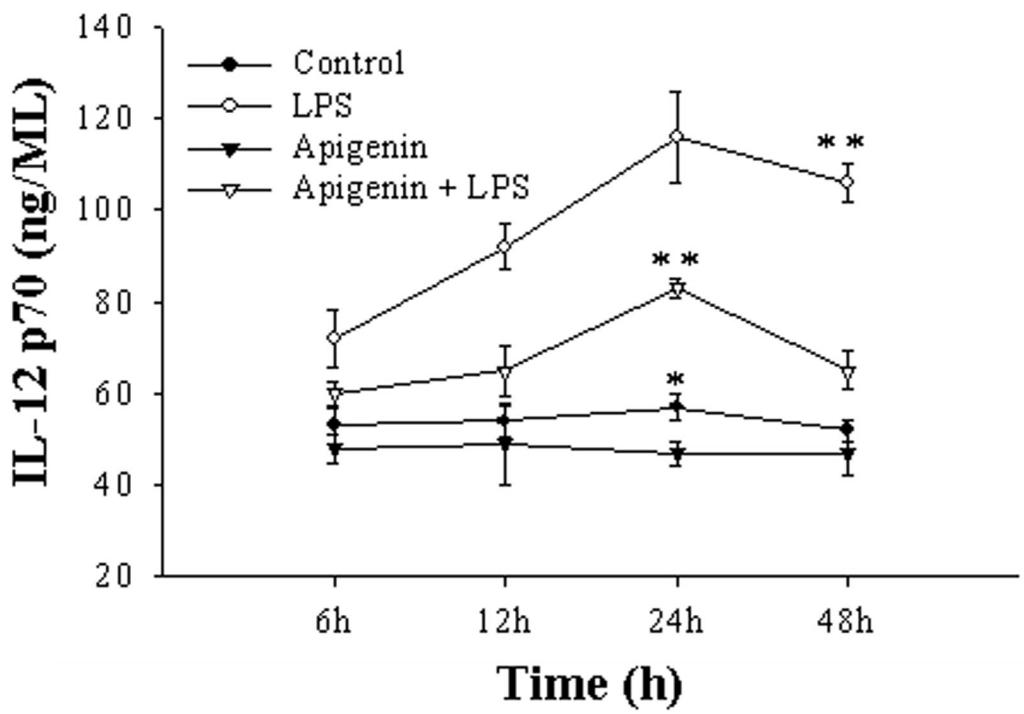

Fig. 2

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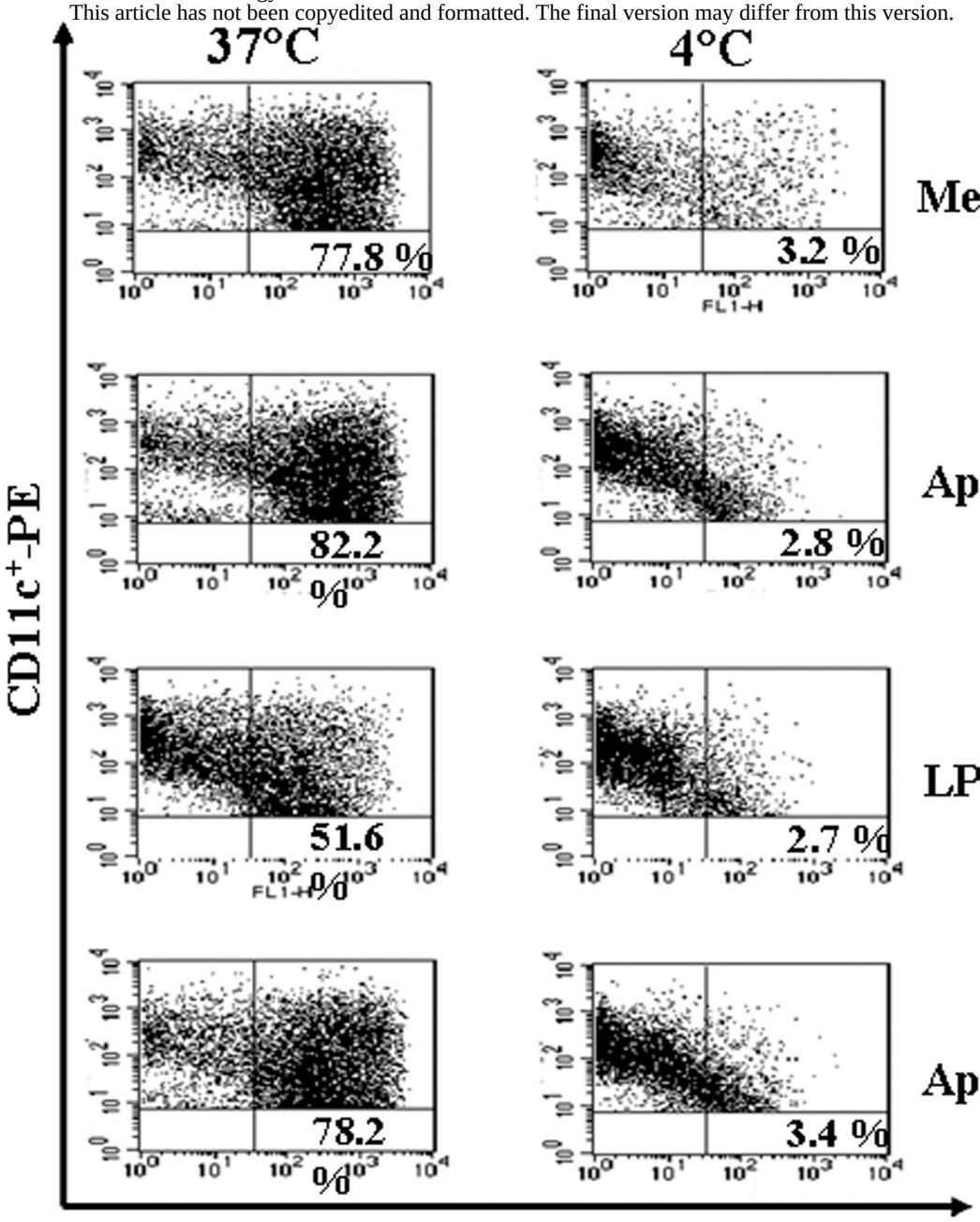

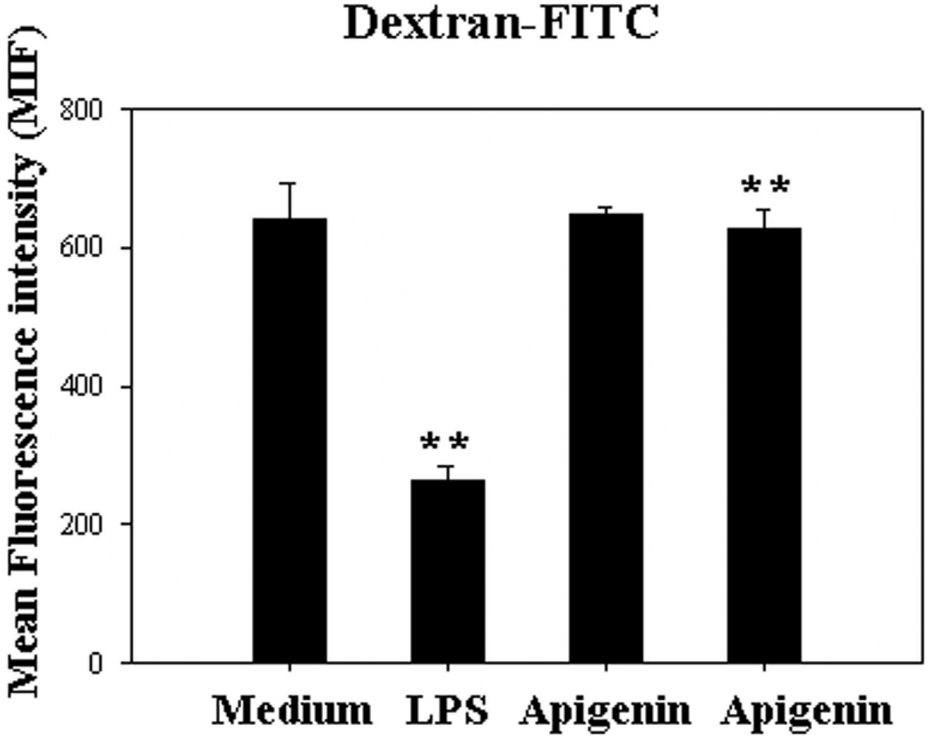

Fig. 3

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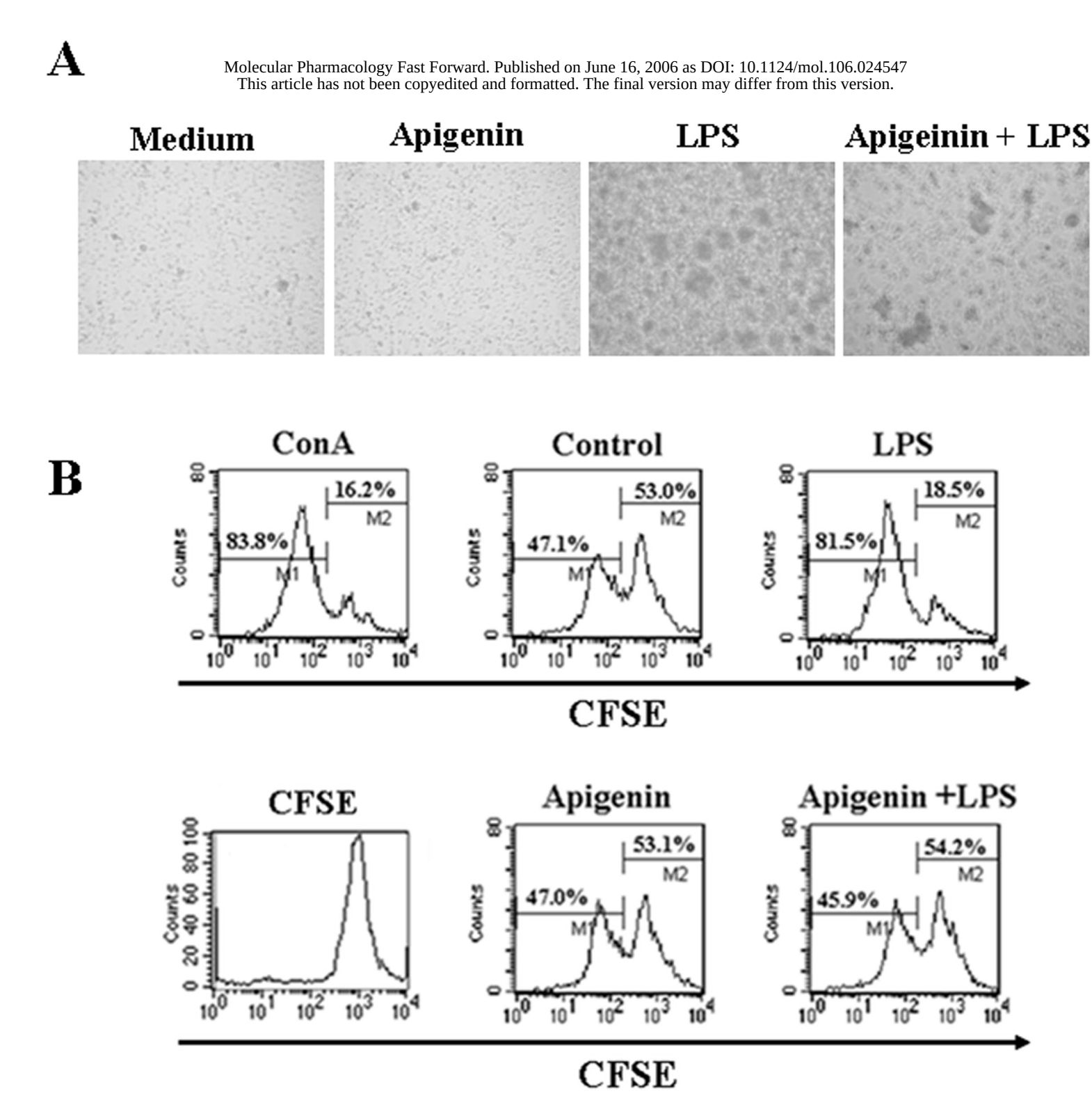

Fig. 4

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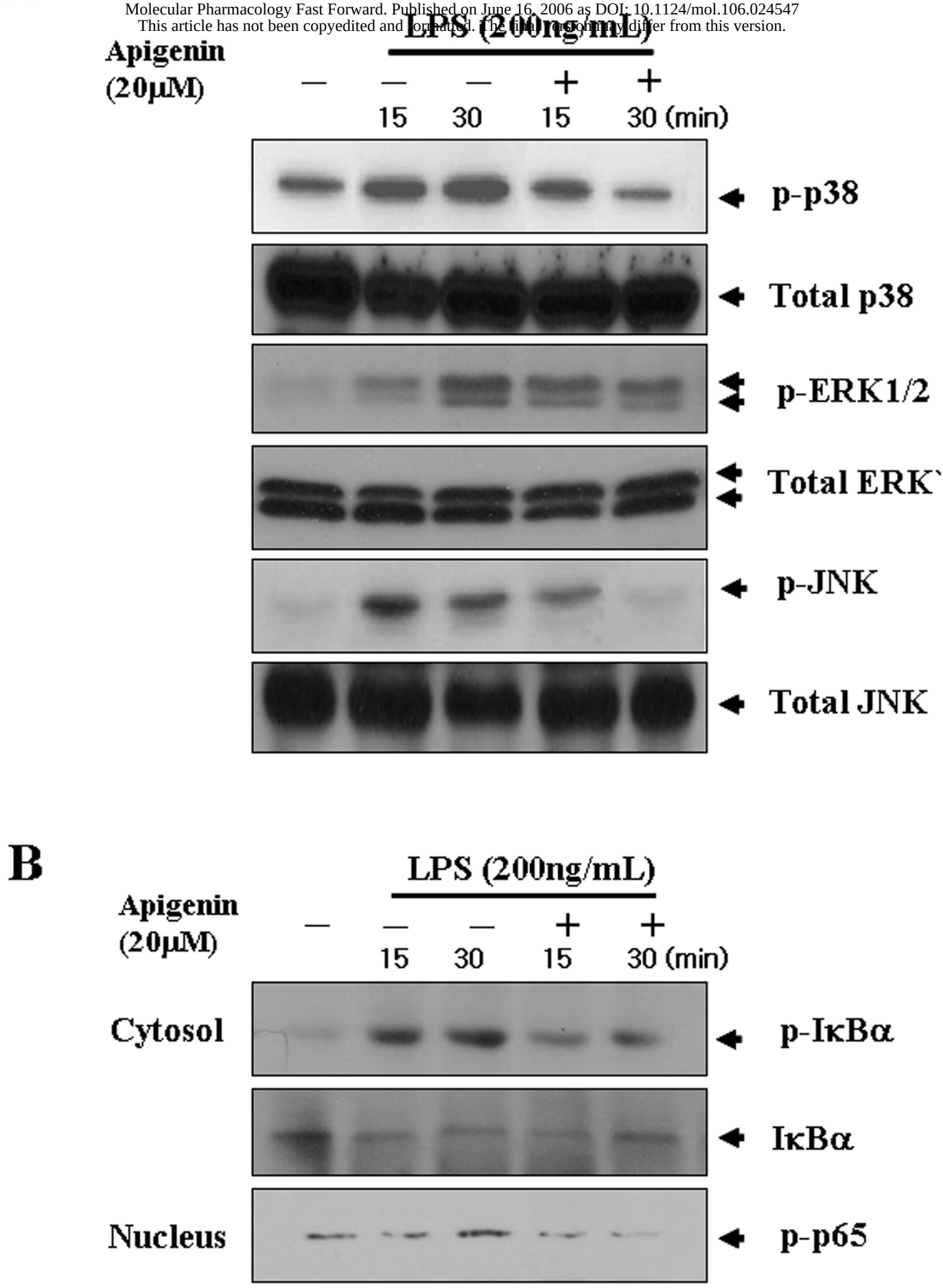

Fig. 5

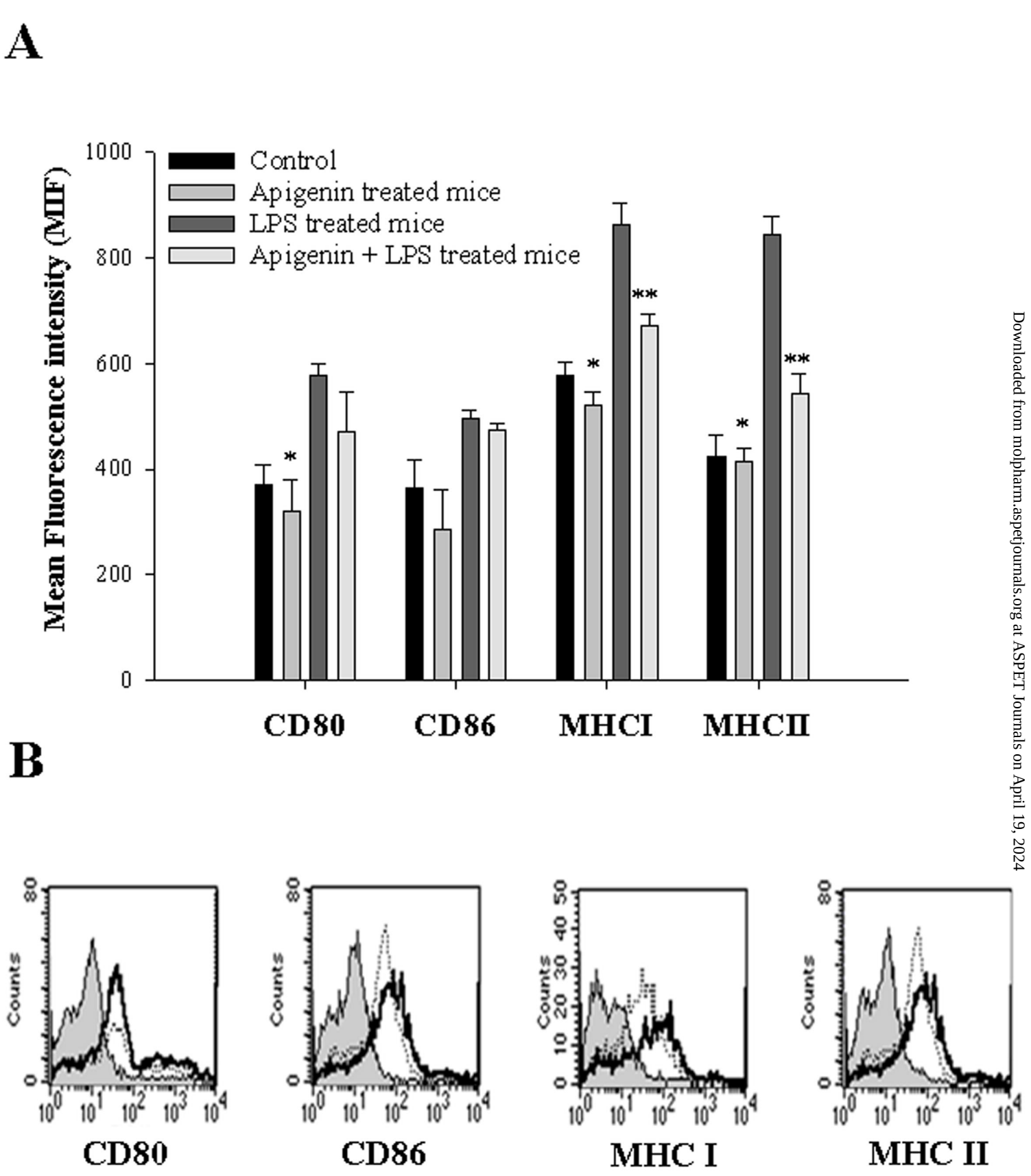

Fig. 6

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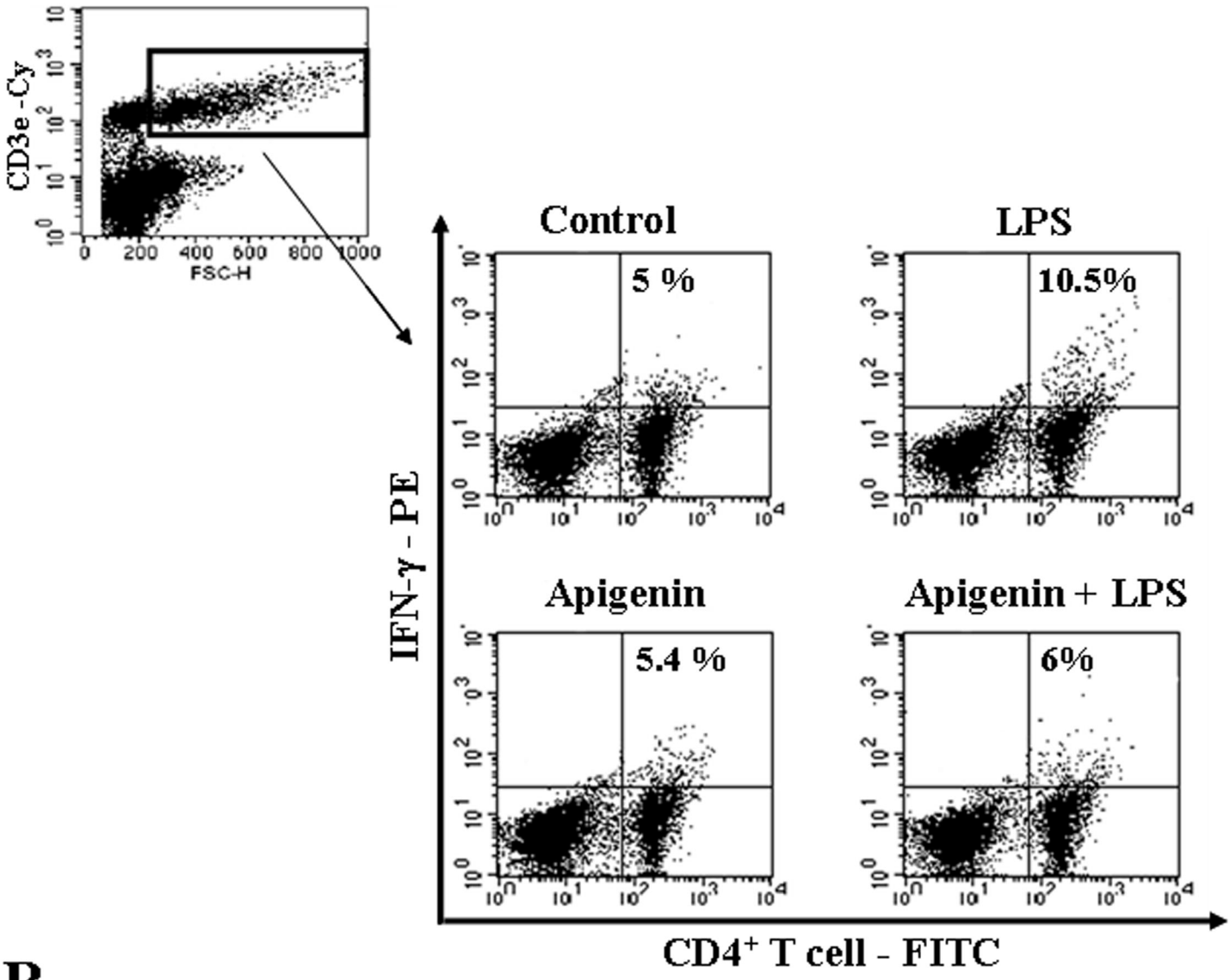

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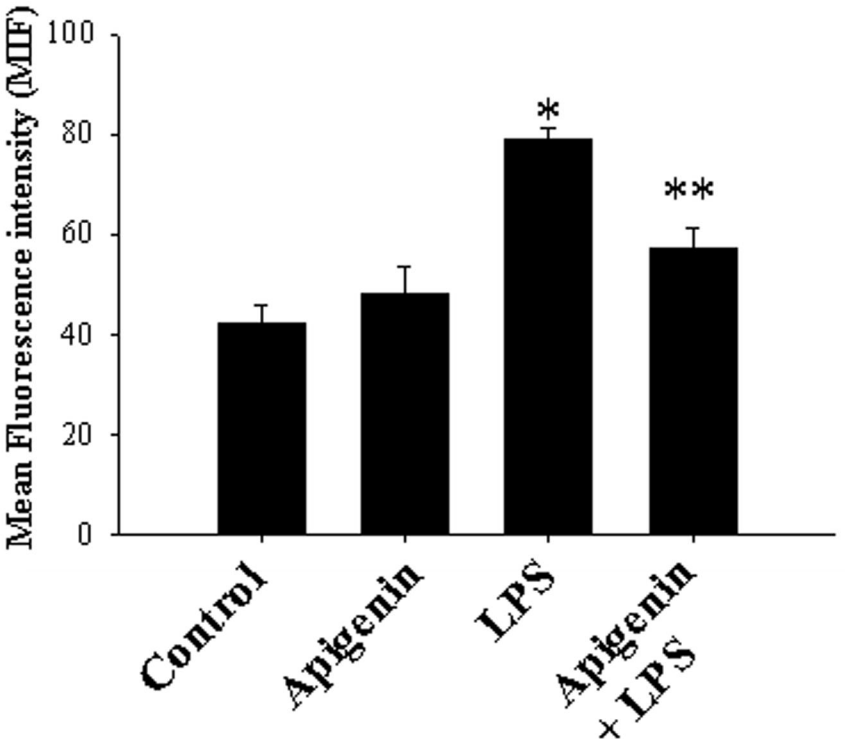

Fig. 7

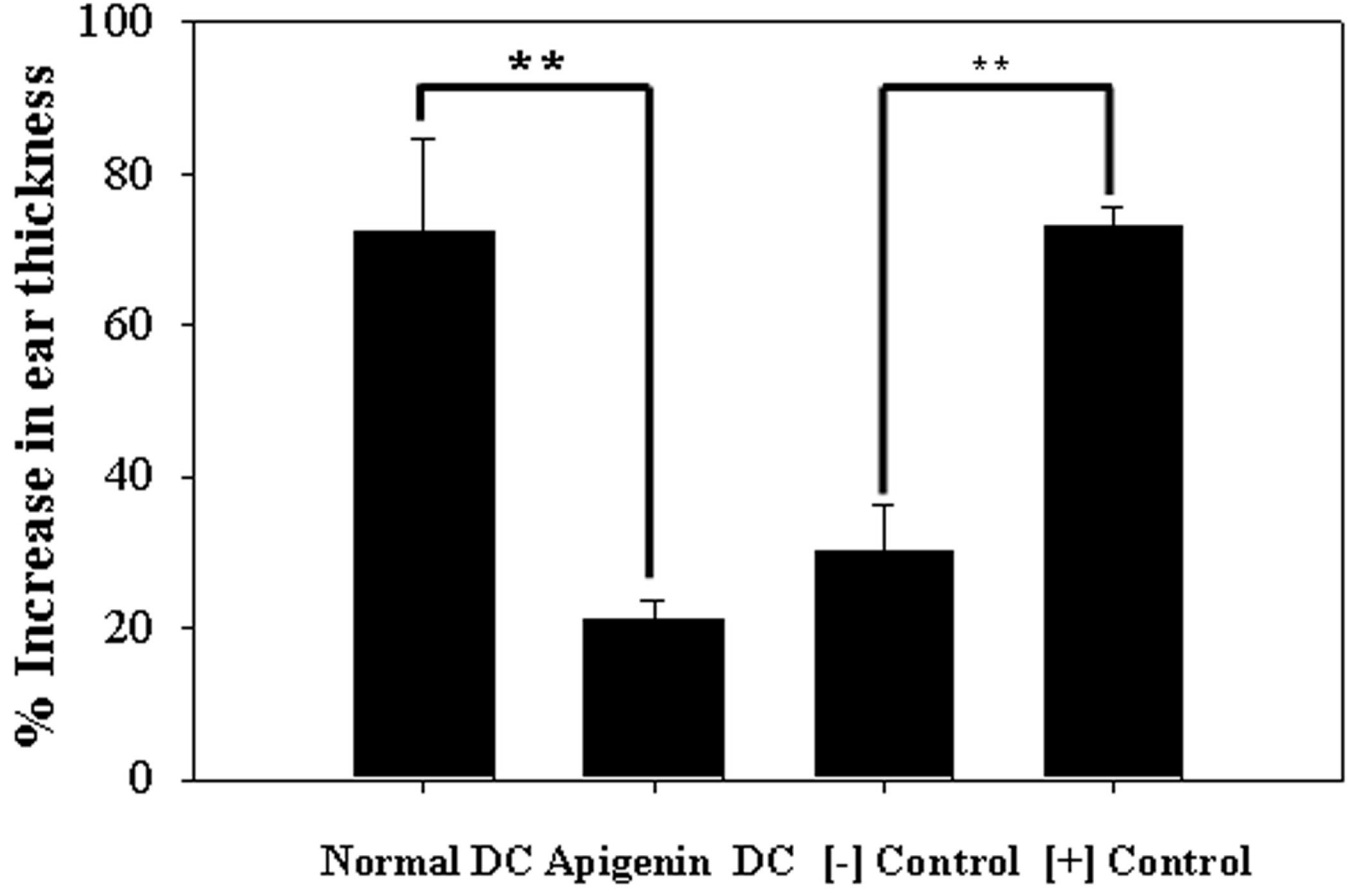

Fig. 8