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Epigallocatechin-3-gallate, constituent of green tea, suppresses the LPS-induced phenotypic and functional maturation of murine dendritic cells through inhibition of mitogen-activated protein kinases and NF-κB

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Abstract

The effects of epigallocatechin-3-gallate (EGCG) on dendritic cells (DC) maturation were investigated. EGCG, in a dose-dependent manner, profoundly inhibited CD80, CD86, and MHC class I and II expression on bone marrow-derived murine myeloid DC. EGCG restored the decreased dextran-FITC uptake and inhibited enhanced IL-12 production by LPS-treated DC. EGCG-treated DC were poor stimulators of naïve allogeneic T-cell proliferation and reduced levels of IL-2 production in responding T cells. EGCG-pretreated DC inhibited LPS-induced MAPKs, such as ERK1/2, p38, JNK, and NF-κB p65 translocation. Therefore, the molecular mechanisms by which EGCG antagonized LPS-induced DC maturation appeared to involve the inhibition of MAPK and NF-κB activation. These novel findings provide new insight into the immunopharmacological role of EGCG and suggest a novel approach to the manipulation of DC for therapeutic application of autoimmune and allergic diseases.

Keywords: Epigallocatechin-3-gallate; Dendritic cell; Mitogen-activated protein kinases; NF-κB

Dendritic cells (DC) are the most potent antigen-presenting cells (APCs). Immature DC residue in peripheral tissues effectively capture and process exogenous protein, and then migrate to peripheral lymphoid tissues. After the uptake of antigens and exposure to inflammatory agents, DC undergo a process of maturation such that they have a greatly diminished capacity for antigen uptake and processing [1,2]. The maturing DC migrate to lymphoid organs, where they stimulate naïve T cells through the signals of costimulatory molecules and both major histocompatibility complex (MHC) molecules presenting antigen-peptides [3,4]. DC are also highly responsive to inflammatory cytokines and bacterial products, such as TNF-α and lipopolysaccharide (LPS), respectively. When encountered in peripheral organs, these products induce a series of phenotypic and functional changes in DC [5,6]. Similar changes indicative of maturation have also been reported following infection with mycoplasma, viruses, intracellular bacteria, and parasites [7–9].

Epigallocatechin-3-gallate (EGCG), a major ingredient of green tea, has been known to have a variety of physiological activities, such as anti-carcinogenic, anti-oxidant, anti-angiogenic, and anti-viral activities

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[10–12]. EGCG was also reported to function as an anti-diabetic [13,14]. A previous study demonstrated that EGCG protected cytokine-induced β-cell damage, which is partly mediated by suppression of NF-κB activity [15]. Recently, EGCG was shown to suppress angiogenesis [16] and the inhibition of urokinase activity [17], mitogen-activated protein kinase (MAPK) activation [17,18], lipooxygenase and cyclooxygenase (COX) activities [19], and arrest of the cell cycle [20,21] in tumor cells. Effects of EGCG on the activation of MAPK members in selected cell lines have also been reported. It was shown that treatment with EGCG inhibited the ultraviolet-B-induced activation of p38-MAPK in a human keratinocyte cell line [22], while others have shown that EGCG activated all three MAPK families including the extracellular signal-regulated kinase (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 in HeLa cells [23]. In vascular smooth cells, EGCG inhibited the platelet-derived growth factor-β-induced activation of ERK1/2 in a dose-dependent manner [18]. In addition, EGCG selectively inhibited interleukin-1β-induced activation of JNK, but not ERK1/2 or p38 MAPK, in human osteoarthritis chondrocytes [24]. Thus, it appears that the MAPK activating or inhibitory effects of EGCG may be stimulus and/or cell type-dependent. Until now, the mechanisms behind the effects of EGCG on LPS-induced mature DC were little known.

In this study, we investigated whether EGCG affects the phenotypic and functional maturation of murine DC via MAPKs. We have shown for the first time EGCG inhibiting the phenotypic and functional maturation of DC and suppressing the LPS-induced activation of ERK1/2, JNK, and p38 MAPK in DC. Also, EGCG was observed decreasing the nuclear translocation of NF-κB p65.

Materials and methods

Animals and chemicals. Male 8- to 12-week-old C57BL/6 (H-2K^b and I-A^b) and BALB/c (H-2K^d and I-A^d) mice were purchased from the Korean Institute of Chemistry Technology (Daejeon, Korea). They were housed in specific pathogen-free enclosures in our animal facility for at least 1 week before use. EGCG was purchased from Sigma.

Generation of bone marrow-derived murine myeloid DC. Bone marrow (BM)-derived murine myeloid DC were generated as described previously in detail [25], with minor modifications. Briefly, bone marrow cells from the femora and tibiae of C57BL/6 mice were flushed and depleted of RBC by hypotonic lysis using RBC lysing buffer (Sigma). Cells were grown from precursors at a starting concentration of 1×10^6 cells/ml in RPMI 1640 supplemented with 10% v/v heat-inactivated FBS, L-glutamine, non-essential amino acids, sodium pyruvate, penicillin–streptomycin, Hepes, and 2-ME (all from Sigma) for 3 h. Then, non-adherent cells were washed out. Twenty ng/ml rmGM-CSF and 20 ng/ml rmIL-4 were given to the culture medium referred to subsequently as complete medium. In parallel experiments, either 10, 50 or 100 μM EGCG was added to the culture medium on day 5. On the 6th day of culturing, non-adherent and loosely adherent cells were collected as BM-DC.

Flow cytometric analysis. On day 7, BM-DC were harvested, washed with PBS, and resuspended in ice-cold FACS washing buffer (2% FBS 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-H-2K^b (MHC class I), anti-I-A^b (MHC class II), anti-CD80, and anti-CD86 with FITC-conjugated anti-CD11c antibody (PharMingen, San Diego, CA) for 30 min at 4 °C. Stained cells were analyzed using a FACSCalibur flow cytometer (Becton–Dickinson, San Jose, CA).

Cytokine 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 (PharMingen) according to manufacturer's instructions. Intracellular IL-12p40/p70 was 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 from DC was measured using an ELISA kit (PharMingen), according to manufacturer's instructions. The detection limits for IL-12p70 were 7.8 pg/ml.

Endocytosis assay. To analyze endocytosis of DC, 1×10^5 cells were incubated at 37 °C for 1 h with 1 mg/ml FITC-dextran (42,000 Da; Sigma). After incubation, cells were washed twice with cold HBSS and stained using a PE-conjugated anti-CD11c antibody (PharMingen). Double stained DC were analyzed by a FACSCalibur flow cytometer. In addition, parallel experiments were performed at 4 °C to show that the uptake of dextran by BM-derived murine DC is inhibited at low temperatures.

Mixed lymphocyte reaction induced by DC. Responder T cells, used for the allogeneic T-cell reaction, were isolated by being passed through mononuclear cells from BALB/c mice in a MACS column (Miltenyi Biotec, Germany). They were mainly composed of CD3$^+$ cells (>93%) when determined by staining with FITC-conjugated anti-CD3 antibody (PharMingen). BM-derived murine DC were treated with 50 μg/ml mitomycin C (Sigma) for 1 h and added in graded doses to 1×10^5 allogeneic T cells in U-bottomed 96-well microtiter culture plates. During the last 18 of the 72 h culturing, cell proliferation was quantified by [^{3}H]thymidine (NEN-DuPont, Boston, MA) uptake of cells incubated with 0.5 μCi of [methyl-^{3}H]thymidine (NEN-DuPont). The cells were harvested onto glass fiber filters (Inotech Biosystems, Zurich, Switzerland) and the radioactivity was measured in a scintillation counter. Results are presented as mean c.p.m. of triplicated cultures.

Assessment of ERK, p38 kinase, and JNK activation by Western blotting. The cells were exposed to LPS (100 ng/ml) in the absence or presence of 100 μM EGCG-pretreatment. Then following 15 or 30 min of incubation at 37 °C, cells were washed twice with cold PBS and lysed with modified RIPA buffer (1.0% NP-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, 1.0 μl/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, Rockford, IL). Equivalent amounts of proteins were separated by SDS–10% PAGE and analyzed by Western blotting using an anti-phospho-ERK (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 1 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 the ECL system (Amersham), by following manufacturer's instructions.

Preparation of nuclear extracts and assessment of NF-κB activation by Western blotting. 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).

Statistics. The results were expressed as means ± SD of the indicated number of experiments. The statistical significance was estimated using a Student's t test for unpaired observations. A p value of <0.05 was considered to be significant.

Results

EGCG inhibited the phenotypic maturation of murine BM-DC in a dose-dependent manner

BM cells were cultured for 6 days in RPMI supplemented with GM-CSF (20 ng/ml) and IL-4 (20 ng/ml). Different concentrations of EGCG were added to a culture on day 6 in the presence or absence of LPS (100 ng/ml). Because EGCG > 200 μM was found to be cytotoxic to BM cells, EGCG was used at concentration of ⩽ 100 μM.

In the first set of experiments, we investigated the effects of different physiological concentrations of EGCG on DC maturation. BM-derived cells were cultured for 24 h in the presence of 0–100 μM EGCG, as described in Materials and methods. As shown in Fig. 1A, 100 μM EGCG was sufficient to reduce the expression of CD80, CD86, and MHC class I and II on $CD11c^+$ cells. The inhibitory effect of EGCG was dose-dependent and targeted primarily the expression of CD86 and MHC class II, which were down-regulated markedly at 100 μM (Fig. 1A). Stimulation of cells with LPS resulted in up-regulation of CD80, CD86, and MHC class I and II expression within 24 h (thick lines in Fig. 1B). A treatment of 100 μM EGCG in the presence of LPS impaired the expression of costimulatory molecules, CD80 and CD86. Interestingly, a slight down-regulation of MHC class I and II molecules was also observed (thin lines in Fig. 1B).

EGCG inhibited IL-12 production of DC

We examined the ability of BM-DC to produce proinflammatory cytokines, because DC, like mono-

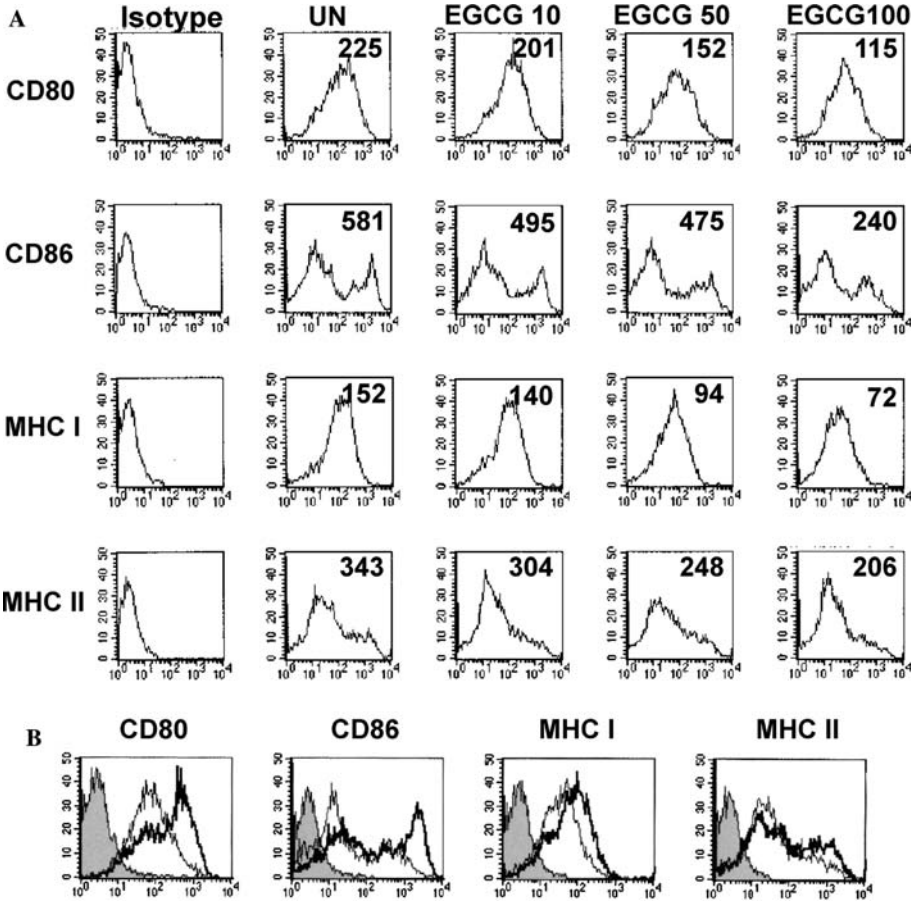

Fig. 1. EGCG suppresses the expression of the costimulatory molecules (CD80 and CD86), and MHC class I and II in a dose-dependent manner during DC maturation. DC were generated as described in Materials and methods. At day 6, cells were harvested and analyzed by two-color flow cytometry. Cells were gated on $CD11c^+$. EGCG was added at concentrations of 10, 50, and 100 μM to DC for 24 h. The expression of surface molecules was then analyzed (A). DC were left untreated (control) or were stimulated for 24 h with 100 ng/ml LPS in the absence (thick lines) or presence (thin lines) of 100 μM EGCG on day 6 (B). UN represents the chemically untreated control group. The histogram is from one representative experiment out of three performed.

cytes and macrophages, are thought to be a source of proinflammatory sites [26,27]. It has been reported that the expression of IL-12 is a more specific marker of functionally activated DC [28,29]. Therefore, we analyzed both intracellular IL-12p40/p70 and bioactive IL-12p70 production in EGCG-treated DC. As shown in Fig. 2A, intracellular staining of FITC-labeled $CD11c^+$ with PE-labeled IL-12p40/p70 mAbs revealed that EGCG-treated DC (100 μM) expressed lower amounts of IL-12p40/p70 compared with untreated DC (Fig. 2A). The inhibitory effect of EGCG on IL-12p70 production was also analyzed using ELISA. The BM-DC control group secreted a low concentration of IL-12p70 (27.5 ± 4.1 pg/ml). LPS-treated DC secreted higher concentrations of IL-12 than untreated-DC (98.2 ± 10.35 pg/ml). As shown in Fig. 2B, EGCG inhibited the production of bioactive IL-12p70 in LPS-stimulated DC (35.2 ± 8.3 pg/ml), indicating that exposure to EGCG impaired the capability of DC to produce bioactive IL-12p70 (Fig. 2B).

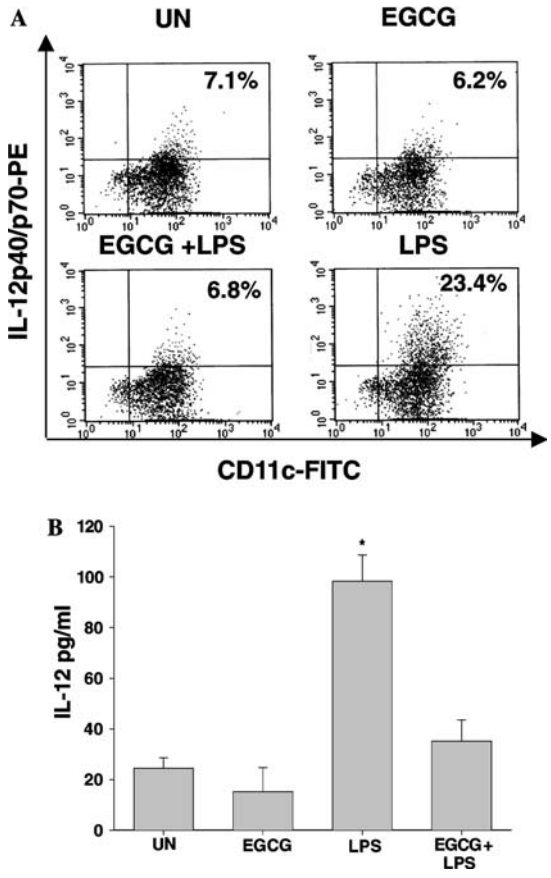

Fig. 2. EGCG impaired IL-12 production by murine DC. Murine DC were stimulated by EGCG (100 μM) for 24 h in the absence or presence of LPS. $CD11c^+$ DC was subsequently analyzed by intracellular cytokine staining (A). DC (5×10^5 cells/ml) were cultured for 24 h and bioactive IL-12 p70 production was analyzed using ELISA from culture supernatants (B). UN represents the chemically untreated control group. The data represent means (±SD) of three separate experiments.

EGCG enhanced the endocytosis of Dex-FITC in LPS-treated DC

The expression of surface molecules and IL-12 production indicated that exposure to EGCG profoundly inhibited the phenotypic and functional maturation of in vitro-generated murine DC. However, these results did not exclude the possibility that EGCG caused a general inhibition to DC physiological functions. Subsequently, we investigated the ability of EGCG-treated DC to endocytose dextran-FITC. After incubating murine DC with EGCG in the absence or presence of LPS, dextran-FITC was added to the culture medium. The percentage of double positive cells ($CD11c^+ \times$ dextran-FITC) did not differ from EGCG-treated DC and untreated DC. The percentage of LPS-stimulated DC was less than that of untreated DC. Also, the EGCG-treated DC showed a

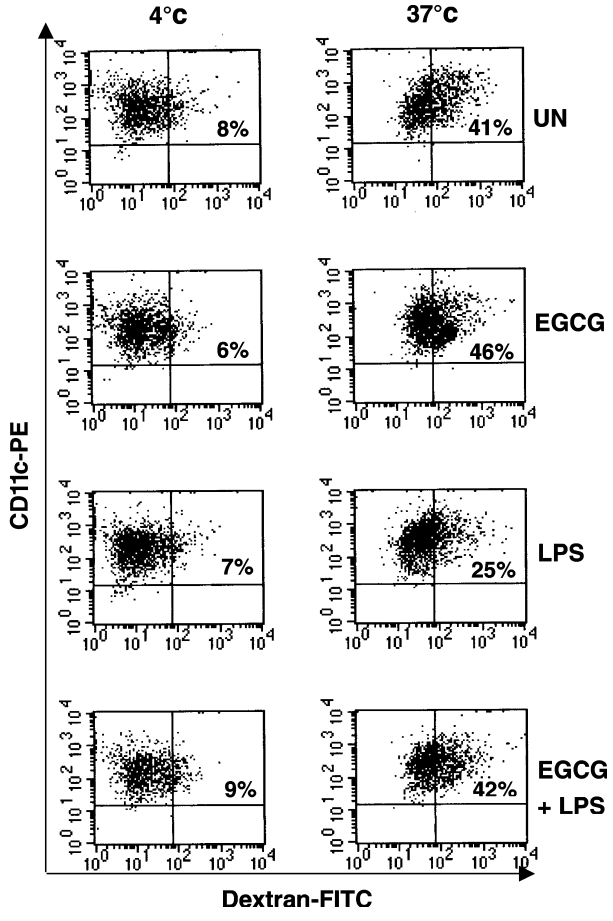

Fig. 3. DC stimulated with EGCG increased Ag uptake. DC (1×10^5 cells) were treated with 100 μM EGCG either in the absence or presence of LPS (100 ng/ml) for 24 h. The endocytic activity of the DC was determined by flow cytometer after treatment with FITC-dextran. Thereafter the cells were washed twice with cold HBSS and stained using the PE-conjugated anti-CD11c antibody. The control endocytic activity was determined after FITC-dextran treatment at 4 °C. The numbers represent the percentages of cells. UN represents the chemically untreated control group. To confirm the results, we repeated these experiment three times.

higher endocytic capacity for dextran-FITC than LPS-stimulated DC (Fig. 3), again indicating that EGCG-treated DC were phenotypically and functionally immature DC. The same experiments were also performed at 4 °C to show that the uptake of dextran-FITC by DC is inhibited at low temperatures. These results indicate that endocytosis by EGCG increases when immature DC are augmented.

EGCG impaired the allostimulatory capactity of DC

To elucidate whether EGCG had any effect on allogeneic T-cell stimulation, DC were treated for 24 h with EGCG. As shown in Fig. 4, LPS-treated DC stimulated proliferative responses more effectively than those of the control group, while EGCG impaired proliferative responses of allogeneic T cells by LPS-activated DC. Also, untreated DC were approximately 2-fold more efficient in T-cell stimulation. Additionally T-cell activation was assessed by IL-2 release. Because DC did not produce IL-2, activated T cells in the cocultures can be the only source of IL-2. DC stimulated with EGCG showed a decrease in IL-2 secretion from T cells. In addition, DC, in the presence of LPS and treated with EGCG, had IL-2 secretions of T cells strongly inhibited as compared with those of LPS-stimulated DC (Fig. 4B). Importantly, maturation induced by LPS stimulation (24 h, 100 ng/ml) strongly promoted the allostimulatory capacity of the untreated DC, whereas exposure to EGCG only marginally impaired their allostimulatory capacity. This observation indicates that the EGCG-treated DC were at least partially maturation-resistant.

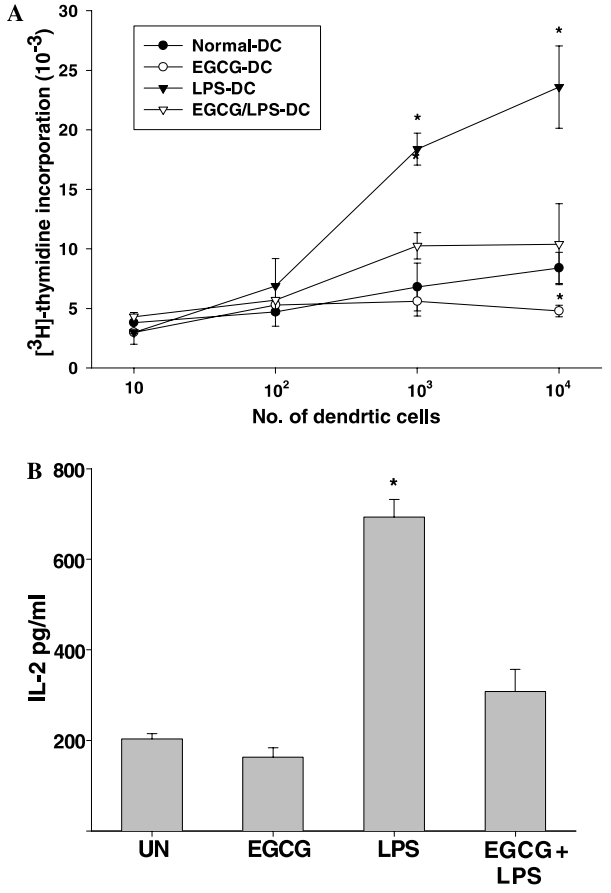

Fig. 4. EGCG decreased the proliferation of allogeneic T cells and IL-2 production through maturation of DC. DC were cultured in medium with or without 100 μM EGCG for 24 h. The treated DC were harvested and washed extensively to remove EGCG. An mixed lymphocyte reaction (MLR) was conducted for 5 days, as described in Materials and methods. A background level of [^{3}H]TdR uptake was determined by measuring reactions without stimulators (A). Analysis of IL-2 levels was measured in 48 h-MLR supernatants (stimulator–responder ratio, 0.1) by ELISA (B). UN represents the chemically untreated control group. Similar results were obtained in three separate experiments.

EGCG directly suppressed LPS-induced activation of the MAPKs and NF-κB in DC

LPS stimulation has been shown to activate MAPK and NF-κB signal pathways in DC [30,31]. LPS activated p-ERK, p-p38 kinase, and p-JNK (Fig. 5A). To evaluate the effect of EGCG on p-ERK, p-p38 kinase, and p-JNK in DC, immature DC were treated with EGCG before LPS stimulation. A pretreatment with

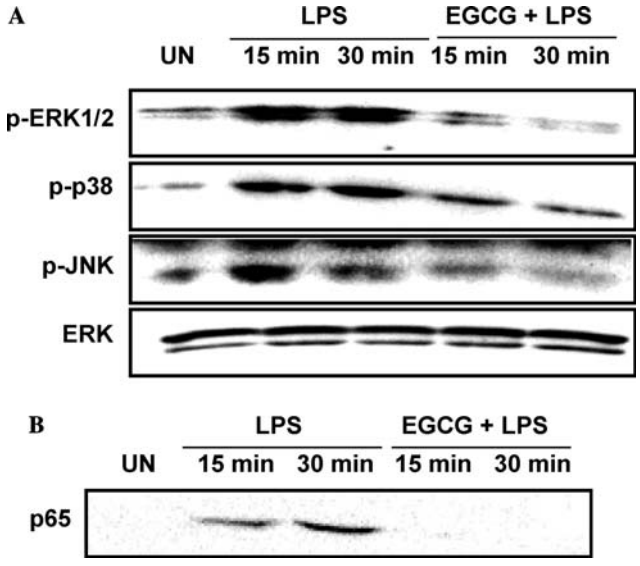

Fig. 5. EGCG decreased the activation of mitogen-activated protein kinases (MAPK) in LPS-stimulated DC. DC were pretreated with 100 μM EGCG for 1 h before LPS stimulation (100 ng/ml). Cell lysates were prepared and blotted with anti-phospho-ERK1/2 (p-p44/42), anti-ERK1/2 (p44/42), anti-phospho-p38 (p-p38), and anti-p38 (p38) Abs (A). LPS-induced nuclear translocation of NF-κB p65 subunit was inhibited by EGCG (B). DC were pretreated with EGCG for 1 h and stimulated with 100 ng/ml LPS for the indicated time. Nuclear extracts were blotted with anti-p65 Ab. The bound antibody was visualized with biotinylated goat anti-rabbit IgG. The results shown were representative of three independent experiments. UN represents the chemically untreated control group.

100 μM EGCG remarkably inhibited LPS-induced up-regulation of p-ERK, p-p38, and p-JNK. Even though p-MAPK was inhibited, the ERK 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 [32], which plays a critical role in gene expression regulation. These results indicate that EGCG inhibits the MAPKs involved in the regulation of LPS-induced DC maturation. To study the role of NF-κB activation, immature DC were stimulated with LPS in the EGCG-pretreatment stage. To determine whether EGCG blocks LPS-induced activation of NF-κB, a nuclear extract was prepared from DC treated with LPS and EGCG; nuclear translocation of the NF-κB p65 subunit was detected by Western blot. LPS induced the nuclear translocation of the NF-κB p65 subunit within 30 min. Pretreatment with 100 μM EGCG suppressed NF-κB p65 nuclear translocation that was induced by LPS stimulation (Fig. 5B).

Discussion

EGCG has been reported to exert a variety of biological effects including anti-oxidant, anti-proliferative, and cancer chemopreventive activities [10–12]. Several studies also have reported that EGCG led to both growth inhibition and the induction of apoptosis [33–35]. Recently, a polyphenolic fraction from green tea was reported to prevent collagen-induced arthritis in mice [36]. Thus, these results suggest that polyphenolic compounds rich in anti-oxidants reduce the frequency of pathogenic Th1-type cells and associated pathogenic collagen-specific IgG2a antibody in the affected joint. These same joints also had significantly lower concentrations of inflammatory cytokines and other mediators of inflammation, such as TNF-α and cyclooxygenase-2, due to these polyphenolic compounds. Also, insulin-dependent diabetes mellitus develops as a consequence of the selective destruction of insulin-producing β-cells which is due to a variety of factors, including reactive oxygen species, nitric oxide (NO), and autoimmune responses [37]. EGCG induced a significant reduction in IL-1β and IFN-γ-induced NO production. Also it reduced levels of the inducible form of NO synthase through the inhibition of the NF-κB signal pathway on RINm5F β-cells [15].

In mice, the role of thymic DC in negative selection (but not positive selection) was confirmed by targeted expression of MHC class II molecules on DC [38]. In the periphery, the role of DC in establishing peripheral T-cell tolerance has not yet been completely demonstrated. Moreover, DC are also considered to play an important role in the establishment of hypersensitivity and transplantation tolerance [39–41]. Based on the above results, inhibition of DC maturation suppressed the inflammatory responses and hypersensitivity. However, the mechanisms involved in EGCG's effect on LPS-induced maturation of DC are not well known. In this study, we have analyzed the influence of EGCG on LPS-induced DC maturation and function, including the expression of MHC molecules and costimulatory molecules; IL-12 production; endocytosis; and stimulatory capacity for T-cell proliferation and IL-2 production in allogeneic T cells. Our results indicated that EGCG is a potent inhibitor of DC maturation. These findings provide new insight into the immunopharmacology of EGCG. Moreover, exposure to this readily available drug provides a simple, inexpensive, and highly effective means to manipulate the immunostimulatory capacity of DC.

We examined MAPKs in order to address EGCG's preventive mechanism on LPS-induced DC maturation. MAPKs, which include ERK, p38, and JNK subfamilies, are activated in response to stimuli, such as treatment with DNA damaging agents, growth factors, and cytokines [42–45]. MAPKs regulate gene expression through the phosphorylation of downstream transcription factors [42–45]. Activation of JN and p38 kinase is related to the stress response, growth arrest, and apoptosis [43–45], where ERK is important in mitogenesis and differentiation [46]. However, reports exist that JNK activation occurs independently of cell death [47], and furthermore, that JNK activation actually promotes proliferation and cellular transformation [48,49]. In addition to physiological response and activation pattern, MAPK activation is dependent upon types of MAPKs and the cell type. However, in the present study, we first provided evidence that activation of LPS-induced DC was related to MAPKs (ERK, p38, and JNK) and NF-κB. However, EGCG suppressed MAPKs and NK-κB in LPS-induced DC.

Taken together, this study showed that EGCG suppressed the phenotypic and functional maturation of murine DC through the inhibition of ERK, p38 kinase, and JNK. Moreover, this may be an important mechanism whereby EGCG protects against autoimmune diseases, including arthritis, allergy, and diabetes. In addition, we found that the NF-κB signaling pathway may be closely related to the EGCG function of inhibiting DC maturation. In summary, when taking all of the above information into account, EGCG has great potential as a health benefit to humans.

Acknowledgments

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