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SK-126, a synthetic compound, regulates the production of inflammatory cytokines induced by LPS in antigen-presenting cells

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ABSTRACT

A variety of mediators released by immune cells triggers or enhances specific aspects of the inflammatory response. Dendritic cells (DCs) play an essential role in the innate immune system by shaping the adaptive immune responses and by controlling the production of cytokines in response to inflammatory stimuli. In the present study, we investigated whether SK-126, a pyridine derivative based on gentianine originated from a natural product, can affect the LPS-induced inflammatory cytokine production in DC. Interestingly, treatment of mouse bone marrow-derived dendritic cells (BMDCs) and the murine dendritic cell line, DC 2.4, with SK-126 completely suppressed LPS-induced TNF-α expression at both transcriptional and protein levels. In contrast to TNF-α, SK-126 enhanced IL-10 expression at both transcriptional and protein levels. To determine signaling pathways involved in the regulation of inflammatory cytokines, we examined the involvement of MAPK and the transcription factor, NF-κB. SK-126 enhanced ERK1/2 and p38 activation following LPS stimulation, but it did not induce phosphorylation of SAPK/JNK and NF-κB. Also, STAT3 phosphorylation after LPS stimulation was increased by SK-126 to a large extent. Using specific inhibitors, we confirmed that SK-126 has dual effects in which it suppresses TNF-α production and enhances IL-10 production via the up-regulation of ERK1/2 and p38. Finally, LPS-induced inflammatory responses such as TNF-α production *in vivo* were significantly reduced by treatment with SK-126. Therefore, our findings suggest that SK-126 may be a useful drug candidate to treat inflammatory diseases in which pro- or anti-inflammatory cytokines play a significant role in their pathogenesis.

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Abbreviations: DCs, dendritic cells; LPS, lipopolysaccharide; TNF, tumor necrosis factor; IL-10, interleukin-10; NF-κB, nuclear factor-κB; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase.

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1. Introduction

Dendritic cells (DCs), potent antigen-presenting cells (APC), function as sentinels *in vivo*, initiators of immune responses, potent stimulators of T cells, and inducers of tolerance [1,2]. DCs originate from bone marrow precursors and migrate to almost every tissue, where they reside in an immature state. Immature DCs capture antigen and process antigen in inflammatory tissues. Thereafter, mature DCs that have captured antigen migrate to secondary lymphoid tissues and present antigen to naive T cells and initiate T cell responses [3,4].

Toll-like receptors (TLR), which are expressed on the surface of DCs at high levels, mediate innate and adaptive immune responses upon exposure to pathogen-associated molecules including lipopeptides, lipopolysaccharides, and nucleic acids [5]. The engagement of TLRs by these ligands results in a potent inflammatory response characterized by the release of pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6, IL-12, and IL-18. Among several TLR ligands, LPS capable of initiating TLR4 signaling activates immune cells and triggers production of pro-inflammatory cytokines. Through recruitment of adaptor proteins such as MyD88 and TRIF, TLR4 activates MAPK and NF-κB [5–7]. In particular, MAPKs play an essential role in the regulation of survival, maturation, and cytokine secretion in DCs. ERK and p38 differentially regulate the production of pro- and anti-inflammatory cytokines in DCs. ERK plays a critical role in mediating TNF-α synthesis, whereas p38 regulates IL-10 synthesis by TLR activation [8–10]. It has been shown that inappropriate regulation of MAPK activation induces autoimmune diseases [11,12]. Thus, control of the MAPK pathways offers several potential therapeutic opportunities for autoimmune diseases.

In this study, we investigated the effect of SK-126 on the production of inflammatory cytokines in DCs. SK-126 is a synthetic compound based on gentianine, one of the major components of *Gentiana Macrophylla Radix*. It significantly suppressed TNF-α production and enhanced IL-10 production via the regulation of ERK1/2 and p38 activation. Therefore, SK-126 may be a useful drug candidate to treat inflammatory diseases in which pro- and anti-inflammatory cytokines play a significant role in their pathogenesis.

2. Materials and methods

2.1. Mice and cell lines

C57BL/6 and Balb/c mice were purchased from Daehan Biolink (Umsong, Republic of Korea). Mice were maintained in specific pathogen-free conditions and used at 5–7 weeks. The experiments employing the mice were performed in accordance with institutional guidelines. The DC 2.4 cell line, which was established as a murine dendritic cell line, was kindly provided by Dr. K. Rock of Harvard Medical School [13].

2.2. Preparation of SK-126 and cell culture

SK-126, a pyridine derivative based on a lead compound derived from a natural product, was provided by SK Chemicals (Suwon, Republic of Korea). Briefly, SK-126, 2-ethyl-8-(4-fluorophenyl)-6-methyl-3,4-dihydro-2H-[2,7]naphthyridin-1-one, was synthesized by using a five-step procedure. 2-Chloro-4,6-dimethyl-nicotinonitrile readily underwent Suzuki-coupling reaction with 4-fluorophenylboronic acid to afford 2-(4-fluorophenyl)-4,6-dimethyl-nicotinonitrile. Treatment of this coupled product with *N*,*N*-dimethylformamide dimethyl acetal followed by sulfuric acid yielded the cyclized compound, 8-(4-fluorophenyl)-6-methyl-2H-[2,7]naphthyridin-1-one. Finally, ethylation and hydrogenation of the lactam provided SK-126 which was purified by silica gel column chromatography and subsequent recrystallization. NMR data of the final compound showed a purity of over 95% (data not shown). Dendritic cells and DC 2.4 cells were cultured in RPMI 1640 (Gibco/Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FBS (Gibco/Invitrogen). Growth factors used in the primary culture of DCs were recombinant mouse GM-CSF and IL-4 (Endogen, Woburn, MA). FITC-conjugated antibodies to CD11c, CD54, CD80, and PE-conjugated antibodies to CD86, and MHC class II were purchased from BD Pharmingen (Palo Alto, CA). LPS and poly I:C were purchased from Sigma (St. Louis, MO) and CpG ODN from InvivoGen (San Diego, CA). PD98059, SB203580, and wortmannin were purchased from Stressgen (Victoria, BC, Canada). Cucurbitacin I (JSI-124), a STAT3 inhibitor, was purchased from Calbiochem (La Jolla, CA). Poly-L-lysine was purchased from Sigma. Alexa Fluor® 594-conjugated donkey anti-rabbit IgG antibody was purchased from Invitrogen. Mounting medium with DAPI was purchased from Vector (Burlingame, CA).

2.3. Dendritic cell preparation from bone marrow

To obtain bone marrow-derived DCs, a method by Inaba et al. [14] was used. Briefly, bone marrow cells isolated from femurs of C57BL/6 mice were harvested and incubated for 30 min at 4 °C with an antibody cocktail containing seven monoclonal antibodies, designated RA3-3A1/6.1, J11d.2, J1J.10, GK1.5, M5/114.15.2, F4/80, and 3.168. The cells were washed with culture medium and treated with rabbit complement (Low-Tox^R–M, Cedarlane, Ontario, Canada) according to the manufacturer's instruction. Viable cells were then isolated by a density gradient centrifugation on Histopaque 1077 (Sigma–Aldrich) and washed twice with culture medium devoid of serum. The lymphocyte-depleted bone marrow cells were distributed in 24-well plates at 5–10 $\times$ 10^5 cells/ml. The cells were incubated in RPMI 1640 medium, supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), Hepes (10 mM), 5×10^{-5} M 2-ME (Sigma), and 10% heat-inactivated FBS (Gibco). The media were supplemented with mouse GM-CSF (10 ng/ml) and IL-4 (10 ng/ml). On days 2 and 4, non-adherent cells were discarded and culture media were replaced. On days 6 or 7, the non-adherent cells were harvested by a gentle swirling and used in subsequent experiments.

2.4. Flow cytometry

DCs produced by *in vitro* culturing were subjected to flow cytometric analysis using a FACSCalibur (Becton Dickinson, Mountain View, CA). Cells were allowed to react with appropriate antibodies against CD11c, CD54, CD80, CD86,

and MHC class II at 4 °C for 30 min and analyzed for their antigen expression.

2.5. RT-PCR and real-time PCR

RNA was isolated with Trizol (Gibco/Invitrogen). First strand cDNA was synthesized from 5 μg of total RNA using M-MLV reverse transcriptase (Promega, Madison, WI). The sequences of the primers used were as follows: TNF-α: forward, CCT CTC ATC AGT TCT ATG GC; reverse, AAC CTG GGA GTA GAC AAG GT. IL-1β: forward, GTG TGA CGT TCC CAT TAG AC; reverse, GTC CTG ACC ACT GTT GTT TC. TGF-β: forward, GCA ACA TGT GGA ACT CTA CC; reverse, AGT TCT TCT CTG TGG AGC TG. IL-10: forward, CTA TGC TGC CTG CTC TTA CT; reverse, GTA GAC ACC TTG GTC TTG GA. IL-12p35: forward, TCG ATC ATG AAG ACA TCA CA; reverse, GAT TCA GAG ACT GCA TCA GC. IL-12p40: forward, ATG TGT CCT CAG AAG CTA ACC; reverse, CCA AAT TCC ATT TTC CTT CT. β-actin was used as a loading control. Real-time PCR was performed at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s for 30–35 cycles (Palm-Cycler™, Corbett Life Science, Sydney, Australia). PCR products were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining.

2.6. Western blot analysis

Cells were pre-treated with the indicated concentration of SK-126 for 2 h and stimulated with 1 μg/ml of LPS for 30 min. After stimulation, cells were washed with RPMI 1640 medium and lysed in protein extraction solution (iNtRON Biotechnology, Seongnam, Republic of Korea). Proteins were separated on a 12% SDS-polyacrylamide gel and the proteins were blotted onto a PVDF membrane, which was then blocked by incubating with TBST (Tris-buffered saline, 0.05% Tween-20) containing 4% BSA or 5% skim milk. Membranes were incubated with specific antibodies and washed with TBST. Antibodies used were as follows: Akt, phospho-Akt (Ser473), p44/42 MAPK, phospho-p44/42 MAPK (Thr202/Tyr204), p38 MAPK, phospho-p38 MAPK (Thr180/Tyr182), SAPK/JNK, phospho-SAPK/JNK (Thr183/Tyr185), IκB-α, phospho-IκB-α (Ser32), STAT3, and phospho-STAT3 (Tyr705). All antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA). Antigen–antibody complexes were visualized after incubating the membrane with diluted goat anti-rabbit IgG (H + L) antibody coupled to horseradish peroxidase and detected by enhanced chemiluminescence.

2.7. Cytokine determination

Cells were pre-treated with indicated concentrations of SK-126 for 2 h and stimulated with 1 μg/ml of LPS for 18 h in order to measure TNF-α production or 24 h in order to measure IL-10 production. Blood sample was collected from the mouse. Cytokines in the supernatant of cells or serum were assayed using TNF-α ELISA kits (Pierce Biotechnology, Rockford, IL) and IL-10 ELISA kits (R&D Systems, Minneapolis, MN).

2.8. MTT assay

DC 2.4 cells were seeded at a density of 2×10^4 cells/ml in 96-well plates and incubated with RPMI-1640 medium containing 10% FBS and antibiotics in the presence of various concentrations of SK-126. After a 24 h incubation, cytotoxicity was assessed by estimation of the viability of DC 2.4 cells by the 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) assay. After completion of drug treatment, 5 mg/ml of MTT solution was added to each well, followed by 100 μl complete medium. After incubation for 3–4 h at 37 °C, the MTT solution was removed. The incorporated formazan crystals in viable cells were solubilized with 100 μl of dimethyl sulfoxide. The absorbance was determined using VICTOR3™ (PerkinElmer, Waltham, MA).

2.9. Immunofluorescence

DC 2.4 cells were plated on poly-L-lysine-coated coverslips in a 24-well plate. Cells were pre-treated with SK-126 for 2 h and stimulated with LPS for 1 h. Cells were then fixed with 4% formaldehyde. Next, cells were permeabilized using phosphate-buffered saline (PBS) containing 0.25% Triton X-100 and blocked with PBS containing 1% BSA and 10% FBS. Cells were subjected to staining with phospho-STAT3 antibody overnight at 4 °C. Phospho-STAT3 in cells was visualized with Alexa 594-conjugated secondary antibody. DAPI was added to stain the nucleus together with mounting medium.

2.10. LPS-induced septic shock model in vivo

Various doses of SK-126 were injected into Balb/c mice. Two hours after SK-126 administration, mice were challenged intraperitoneally with LPS (1 mg/kg). Two or three hours after the challenge, blood serum was collected from the mouse in each treatment group and ELISA was used to measure TNF-α and IL-10 production. On the other hand, SK-126 was orally administrated in different concentrations 1 h before LPS challenge (10 mice/group). Survival of mouse challenged with a lethal dose of LPS (40 mg/kg of LPS, i.p.) was recorded every 6 h.

2.11. Statistical analysis

The paired Student's t-test was used for statistical analysis. It was represented as mean ± S.E. $P < 0.05$ was considered significant.

3. Results

3.1. SK-126 inhibits TNF-α and enhances IL-10 at a transcriptional level in a DC cell line and primary dendritic cells

Exposure to stimuli like lipopolysaccharide (LPS) induces the full maturation of DCs, characterized by strongly up-regulated expression of costimulatory molecules and production of pro-inflammatory cytokines [15,16]. We investigated whether SK-126 (Fig. 1A) was able to affect inflammatory cytokine production at transcriptional levels in the dendritic cell line DC 2.4. As shown in Fig. 1B, the LPS-induced increase in TNF-α mRNA was decreased by SK-126 pretreatment in a dose-dependent manner. In contrast, IL-10 mRNA level was

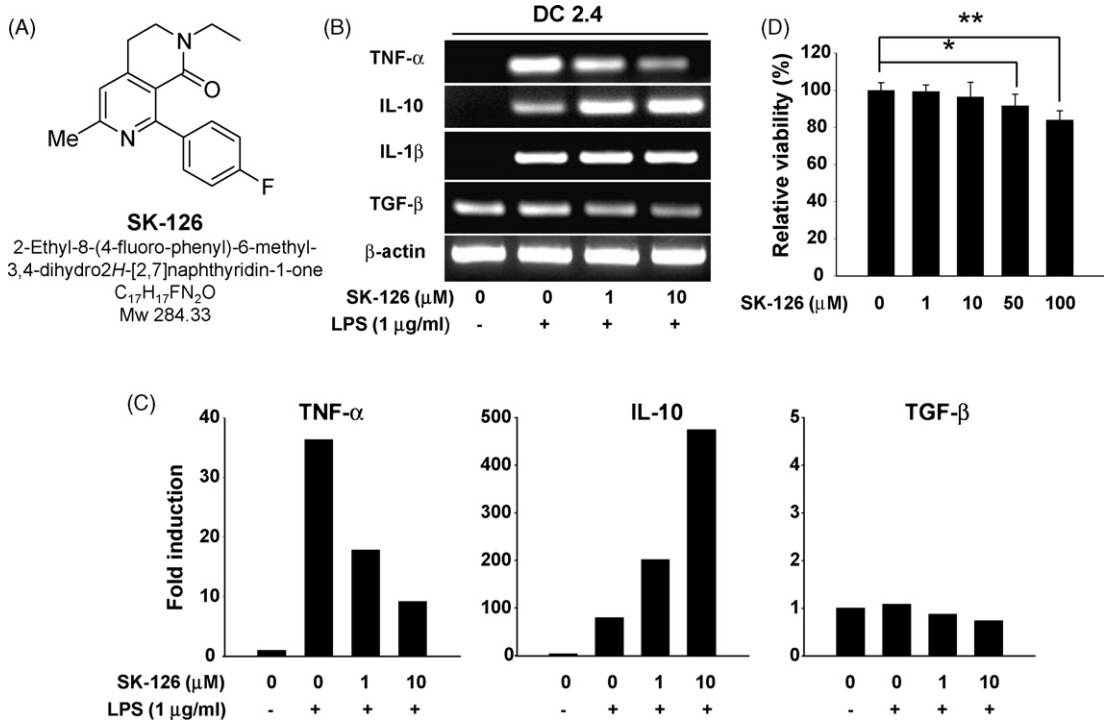

Fig. 1 – Inhibition of TNF-α and enhancement of IL-10 by SK-126 in DC 2.4. Structure of synthetic compound, SK-126 (A). DC 2.4 cell line was pre-treated with SK-126 for 2 h and stimulated with 1 μg/ml of LPS for 2 h. Cells were harvested for RNA preparation. Transcriptional levels of cytokines were detected using RT-PCR (B) and real-time PCR (C). For determining cell viability of DC 2.4 after treatment with SK-126, cells were treated with SK-126 for 24 h and cell viability was determined using MTT assay (D). Data represent the mean ± S.E. of triplicates. $^{*}P < 0.05$ and $^{**}P < 0.01$, control *versus* treated.

significantly enhanced by SK-126 pretreatment. The level of IL-1β or TGF-β transcription was not affected by the same procedure. Similar to these results, a decrease in TNF-α and an increase in IL-10 at mRNA levels were confirmed using real-time PCR (Fig. 1C). Again, no difference in TGF-β mRNA level was observed via real-time PCR. To examine whether SK-126 affects cell viability, we performed MTT assays after treating DC 2.4 cells with several concentrations of SK-126. As a result, cell viability was not affected at the concentrations below 10 μM, but was slightly decreased at higher concentrations (Fig. 1D). In order to investigate whether it had the same effect on primary DCs, we performed a similar experiment using bone marrow-derived primary DCs. As shown in Fig. 2A, LPS-induced increase of TNF-α mRNA in primary DC was also decreased by SK-126 pretreatment, while IL-10 mRNA was significantly increased. The mRNA level of other cytokines including TGF-β, IL-1β, IL-12p35, and IL-12p40, was not changed by SK-126 treatment. These results were again confirmed by real-time PCR using fold change to illustrate changes in mRNA levels (Fig. 2B). To investigate the effects of SK-126 on TNF-α and IL-10 expression induced by other TLR ligands, primary DCs were treated with CpG or poly I:C. As shown in Fig. 2C, SK-126 again exhibited significant modulation of cytokine expression in DCs stimulated with CpG or poly I:C. Collectively, these data demonstrated that SK-126 was able to inhibit pro-inflammatory cytokine, TNF-α, and enhance anti-inflammatory cytokine, IL-10, at transcriptional levels in dendritic cells.

3.2. *SK-126 inhibits LPS-induced TNF-α secretion, whereas it enhances LPS-induced IL-10 secretion*

We next examined the possibility that SK-126 can modulate the secretion of the soluble form of cytokines from DCs. BMDCs and DC 2.4 were pre-treated with SK-126 for 2 h and stimulated with 1 μg/ml of LPS for 18 or 24 h. Activation of BMDCs and DC 2.4 with LPS resulted in a significant production of TNF-α and LPS-induced TNF-α production was markedly inhibited by pretreatment with SK-126 (Fig. 3A). In contrast, the pretreatment of DCs with SK-126 enhanced LPS-induced IL-10 in both primary DCs and the cell line DC 2.4 (Fig. 3B). These results suggest that SK-126 has the ability to modulate the transcription of cytokine genes and production of cytokines linked to the LPS-induced inflammatory response in DCs.

3.3. *Effect of SK-126 on DC differentiation and proliferation*

DCs have been known to express diverse surface markers. In mouse, CD11c has been acknowledged as a typical DC marker. When DCs mature, they express high levels of MHC molecules, CD40, CD80, CD83, CD86, and so on [3]. In order to examine the phenotypic changes of DCs following treatment with SK-126, SK-126 was in along with GM-CSF and IL-4 during DC differentiation or added for 2 h after full differentiation. DCs expressed high levels of surface antigens as shown in Fig. 4A.

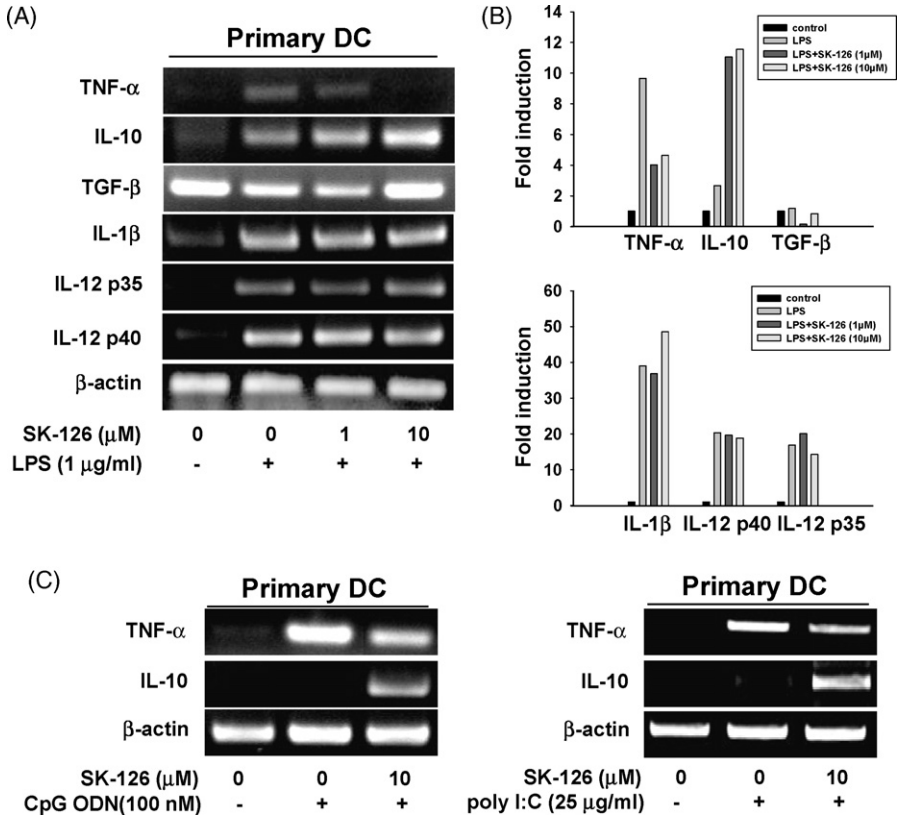

Fig. 2 – Effect of SK-126 on TNF-α and IL-10 mRNA expression in primary dendritic cells. DCs were pre-treated with the indicated concentration of SK-126 for 2 h and stimulated with LPS (A and B), CpG or poly I:C (C) for 2 h. Cells were harvested for RNA preparation. Transcriptional levels of cytokines were detected using RT-PCR (A and C) and real-time PCR (B).

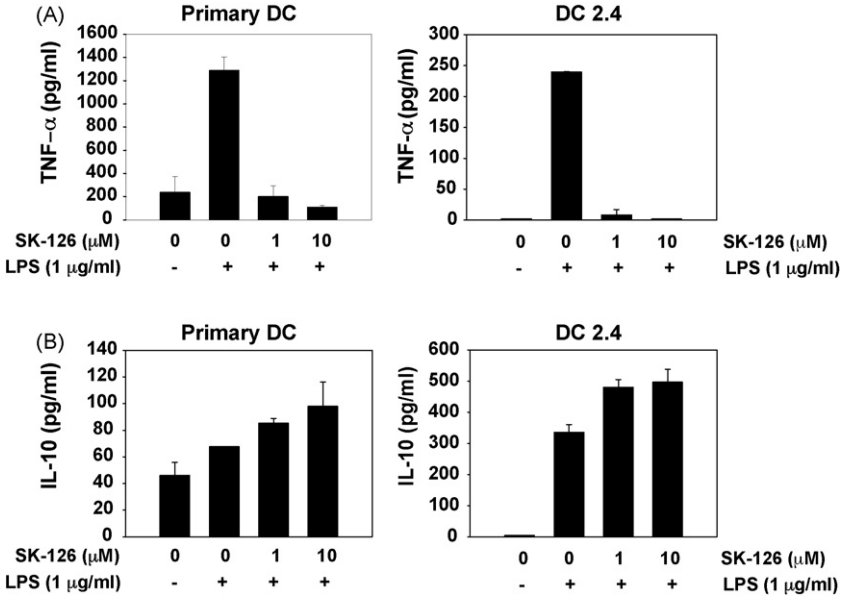

Fig. 3 – Inhibition of TNF-α and enhancement of IL-10 secretion by SK-126 in primary dendritic cells and DC 2.4 cell line. DCs and DC 2.4 were pre-treated with SK-126 for 2 h and stimulated with 1 μg/ml of LPS for 18 h (A) or 24 h (B). The levels of TNF-α (A) and IL-10 (B) in culture supernatants were determined by ELISA. Data represent the mean ± S.E. of duplicates.

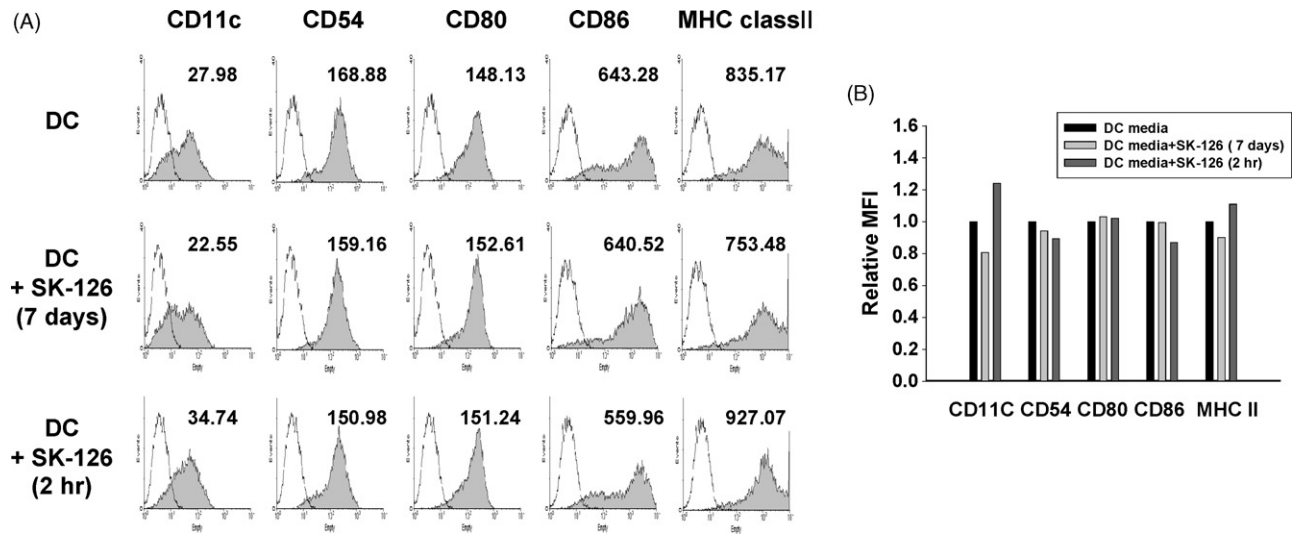

Fig. 4 – DC differentiation and proliferation were not affected by treatment with SK-126. Bone marrow-derived DCs were generated by culturing bone marrow cells with 10 ng/ml of GM-CSF and 10 ng/ml of IL-4 for 7 days. Thereafter, cells were treated with 1 μM of SK-126 for 2 h (DC + SK-126 (2 h)). On the other hand, bone marrow-derived cells were cultured with 10 ng/ml of GM-CSF, 10 ng/ml of IL-4, and 1 μM of SK-126 for 7 days (DC + SK-126 (7 days)). Cells were allowed to react with appropriate antibodies at 4 °C for 30 min for the detection of CD11c, CD54, CD80, CD86 and MHC class II. Cells were analyzed using a FACSCalibur flow cytometer (A). The numbers indicate mean fluorescence intensity. The data are also represented as relative Mean Fluorescence Intensity (MFI) (B).

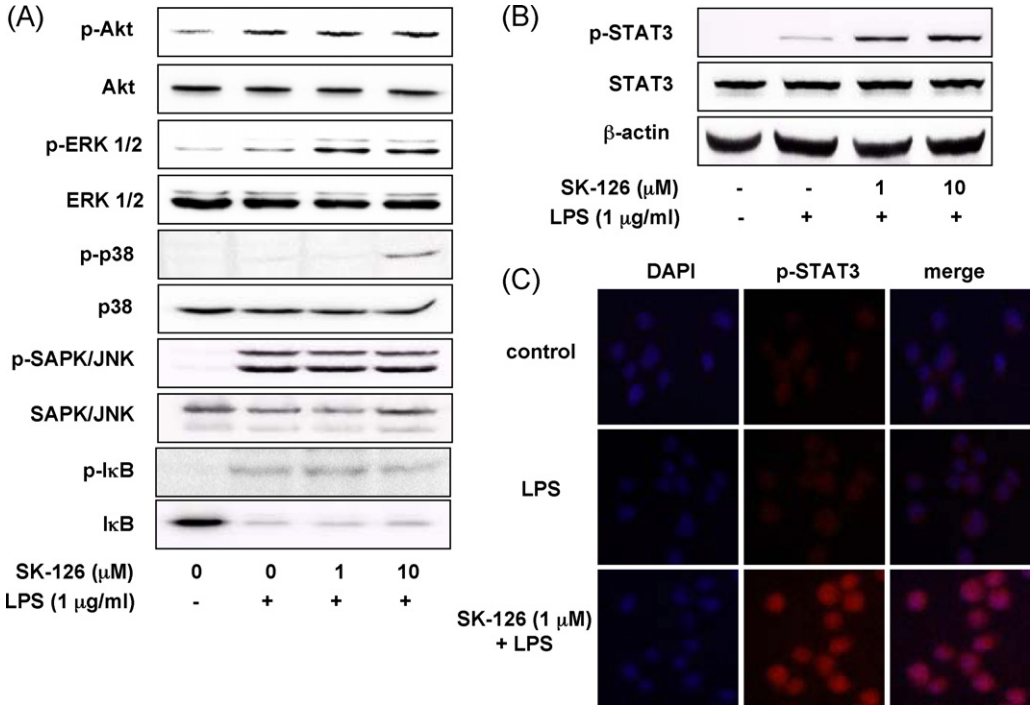

Fig. 5 – Regulation of LPS-induced Akt, MAPK, IκB and STAT3 by SK-126. DC 2.4 cells were pre-treated with SK-126 for 2 h and stimulated with 1 μg/ml of LPS for 30 min. Equal amounts of whole-cell lysates were subjected to electrophoresis on a SDS-PAGE and Western blot analysis was performed using specific Abs, respectively (A). DC 2.4 cells were pre-treated with SK-126 for 2 h and stimulated with 1 μg/ml of LPS for 1 h. Cell lysates were subjected to electrophoresis on a SDS-PAGE and Western blots were performed using specific Abs to p-STAT3 or STAT3 (B). Immunochemical staining of p-STAT3 in DC 2.4 cells treated with or without LPS (C). Cells were fixed, permeabilized with 0.5% Triton X-100, and stained for p-STAT3 and DAPI. DAPI staining identified the nuclei of cells.

However, in both cases, SK-126 itself did not have any effect on the differentiation of BMDCs. This was also observed when antigen expression was presented as mean fluorescence intensity (MFI) (Fig. 4B). Furthermore, the cell count of DCs derived from bone marrow did not vary among the three populations (data not shown). Thus, we concluded that DC differentiation and proliferation are not affected by treatment with SK-126 and the regulation of cytokine production in DCs by SK-126 is not due to the effect on DC differentiation efficiency from bone marrow.

3.4. SK-126 enhances ERK1/2 and p38 activation in dendritic cells

Because MAPK and NF-κB are critical factors mediating cellular responses to external stimuli such as LPS, we next examined MAPK and I-κB activation by SK-126 in response to LPS in DC 2.4. Cells were pre-treated with SK-126 for 2 h and stimulated with 1 μg/ml LPS for 30 min. As a result, SK-126 significantly enhanced ERK1/2 and p38 phosphorylation (Fig. 5A). However, in the presence of LPS, it did not induce any additional phosphorylation of Akt, SAPK/JNK, or IκB. Thus, these data revealed that SK-126 may suppress TNF-α production and enhance IL-10 production via the regulation of ERK1/2 and p38 activation. It has been reported that signal transducer and activator of transcription 3 (STAT3) is essential for the function of IL-10 and activation of STAT3 induces IL-10 production [17]. In addition, activation of p38 induces phosphorylation of STAT3 [18,19]. We then evaluated the activation of STAT3 by SK-126 in the presence or absence of LPS. DC 2.4 cells were pre-treated with SK-126 for 2 h and stimulated with 1 μg/ml of LPS for 1 h. As shown in Fig. 5B, phosphorylation of STAT3 was increased by LPS stimulation and was additionally enhanced in the presence of SK-126. Moreover, the translocation of the active form of STAT3 into the nucleus was more efficient in SK-126-treated cells than in cells treated with LPS alone (Fig. 5C). Therefore, the data suggest that strong IL-10 production after treatment with LPS in combination with SK-126 is induced by both MAPK and STAT3 activation.

3.5. SK-126 regulates cytokine production via the p38 and STAT3 pathway rather than ERK1/2 in dendritic cells

Next, we investigated the signal transduction pathway leading to cytokine production by comparing the regulation of cytokine production upon LPS stimulation in the presence of MAPK and STAT3 inhibitors. DC 2.4 cells were pre-treated with each inhibitor for 1 h and followed by treatment with SK-126 for 2 h. Cells were then stimulated with LPS for 2 h. As shown in Fig. 6A, we re-confirmed that SK-126 inhibited TNF-α and enhanced IL-10 production upon LPS stimulation. However, SK-126 did not modulate cytokine production in the absence of LPS stimulation. Although PD98059 effect on TNF-α mRNA was relatively weak in real-time PCR experiment, TNF-α mRNA expression that was suppressed by SK-126 was recovered by PD98059 and SB203580 treatment. On the other hands, as expected, IL-10 production enhanced by SK-126 was greatly reduced by PD98059 and SB203580 treatment (Fig. 6B). The effect was greater with p38 inhibitors compared to ERK1/2 inhibitors. Therefore, p38 MAPK, and to a lesser extent ERK, play an important role in cytokine modulation of SK-126 after LPS stimulation. Interestingly, whereas PI3 kinase inhibitor, wortmannin, abrogated SK-126-induced repression of TNF-α production in the presence of LPS, it did not interrupt SK-126-induced enhancement of IL-10 production (Fig. 7A and B).

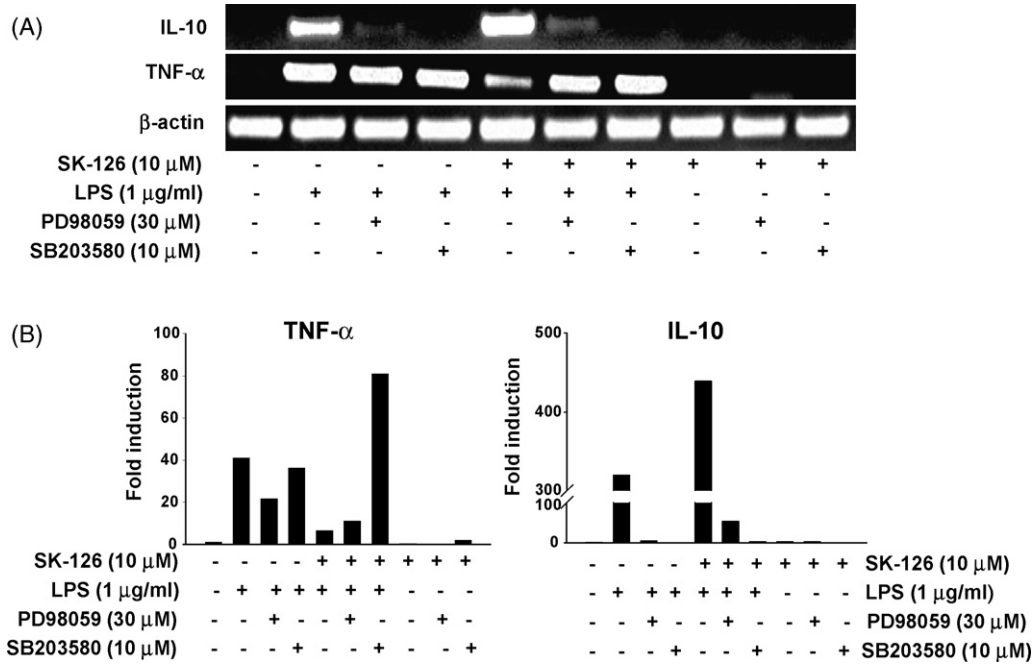

Fig. 6 – Involvement of p38 and ERK1/2 pathway in the effect of SK-126. DC 2.4 cells were pre-treated for 1 h with each kinase inhibitor and further incubated for 2 h in the presence or absence of SK-126. Finally, cells were stimulated for 2 h with LPS and harvested for RNA preparation. Transcriptional levels of cytokines were detected using RT-PCR (A) and real-time PCR (B).

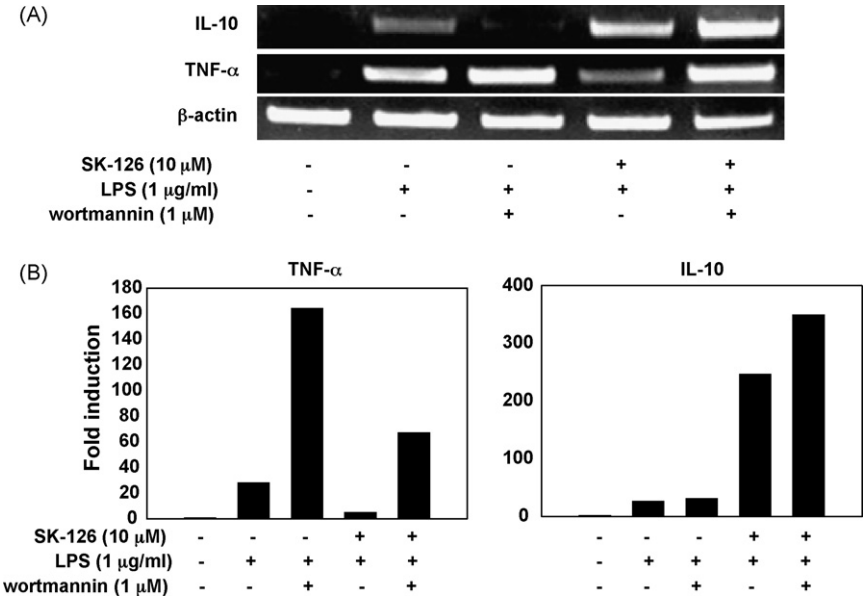

Fig. 7 – Involvement of PI3K/Akt pathway in the inhibition of TNF-α, but not in the enhancement of IL-10 production by SK-126. DC 2.4 cells were pre-treated for 1 h with wortmannin and further incubated for 2 h in the presence or absence of SK-126. Finally, cells were stimulated for 2 h with LPS and harvested for RNA preparation. Transcriptional levels of cytokines were detected using RT-PCR (A) and real-time PCR (B).

These data suggest that the PI3K/Akt pathway plays an essential role in SK-126-mediated inhibition of TNF-α production, but not in SK-126-mediated IL-10 induction in the presence of LPS. However, as expected, IL-10 mRNA expression was drastically inhibited by cucurbitacin I, a specific STAT3 inhibitor (data not shown).

3.6. *SK-126 inhibits TNF-α expression in Balb/c mice injected with LPS and protects mice against a lethal dose of LPS*

Finally, we examined regulation of TNF-α expression by SK-126 *in vivo*. Balb/c mice were given SK-126 in different

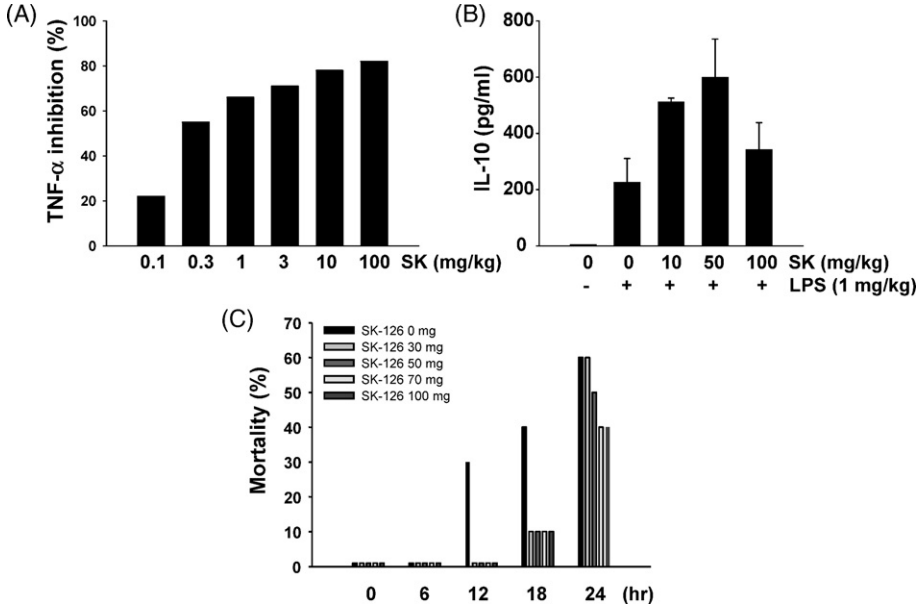

Fig. 8 – Effect of SK-126 on TNF-α expression in Balb/c mice injected with LPS. Balb/c mice were given SK-126 in different concentrations. Two hours after SK-126 administration, mice were challenged intraperitoneally with LPS (1 mg/kg). Two or three hours after the challenge, blood serum was separately collected from the mice in each treatment group and TNF-α (A) or IL-10 (B) production was measured by ELISA. SK-126 was orally administrated in different concentrations 1 h before LPS challenge (10 mice/group). The life span of mice challenged with a lethal dose of LPS (40 mg/kg of LPS, i.p.) was recorded every 6 h (C).

concentrations. Two hours after SK-126 administration, mice were challenged intraperitoneally with LPS (1 mg/kg). Blood was collected from the mouse in each treatment group and sera for an ELISA were prepared. The experimental results showed that SK-126 has the ability to inhibit TNF-α expression in the mouse endotoxin shock model similar to the observations *in vitro* (Fig. 8A). Moreover, SK-126 could increase IL-10 production *in vivo* up to the concentration of 50 mg/kg in response to LPS (Fig. 8B). When we investigated the protective effects of SK-126 against a lethal challenge of LPS, the survival time of mice challenged with a lethal dose of LPS (40 mg/kg of LPS, i.p.) was, to a certain extent, extended by oral administration of SK-126 1 h before LPS challenge, especially between 12 and 18 h after LPS challenge (Fig. 8C). Taken together, these data provide *in vivo* evidence that SK-126 may provide a protective effect against LPS-mediated lethal shock by modulating inflammatory cytokine production.

4. Discussion

Dendritic cells play a critical role as sentinels in the immune systems against infection [1]. Exposure to stimuli like lipopolysaccharide (LPS) induces the full maturation of DCs, characterized by strong up-regulation of costimulatory molecules and the production of pro-inflammatory cytokines [6,15,16]. In the present study, we determined whether a synthetic compound, SK-126, regulates inflammatory cytokine production in dendritic cell. Real-time PCR analysis revealed that SK-126, at the concentration of 1 μM, inhibits TNF-α production in DC 2.4 up to half that of LPS alone group and induces more than a two-fold increase in IL-10 production. Moreover, fold changes in mRNA expression of the two cytokines were much greater in bone marrow-derived DCs. On the other hand, the results indicated that SK-126 alone does not provide cells with enough stimulus to regulate TNF-α and IL-10 production in DCs. However, other inflammatory cytokines such as IL-1α, IL-1β, TGF-β, IL-12p35, and IL-12p40 were not affected by SK-126. IL-12 secretion in primary DCs was weakly inhibited by treatment with SK-126 (data not shown), but it was not evident in DC 2.4 cells because these cells are not able to produce IL-12, even after LPS treatment. It is well known that IL-10 is involved in negative autocrine regulation of IL-12 production [15]. However, our data demonstrate that IL-10 production following SK-126 treatment does not modulate the transcription level of IL-12. These observations collectively suggest that SK-126 inhibits pro-inflammatory cytokine, TNF-α, and enhances anti-inflammatory cytokine, IL-10, at the transcriptional level in dendritic cells. In our preliminary experiment where DC 2.4 cells treated with IL-10-specific siRNA were compared with control DC 2.4 cells, the significant inhibition of TNF-α mRNA expression by SK-126 was also observed in DC 2.4-siIL-10 cells, indicating that the ability of this compound to inhibit TNF-α is not simply a consequence of increased IL-10 secretion (data not shown).

SK-126 did not show any substantial effect on differentiation and maturation of BMDCs. It has been reported that phosphatidylinositol 3-kinase (PI3K)/Akt regulates DC activation and survival [20,21]. When we investigated the expression kinetics of Akt, a key downstream effector of PI3K signaling, phosphorylated Akt was enhanced by stimulation with LPS, but LPS-induced phosphorylation of Akt was not modulated by a treatment with SK-126. MAPK and NF-κB are critical factors that mediate cellular responses to external stimuli such as LPS. MAPKs are ubiquitous protein kinases involved in signal transduction [8]. The MAPK cascades are composed of three groups of kinases: ERK, stress-activated protein kinase/c-JNK, and p38 [12,22]. Bacterial LPS leads to the activation of all three MAPKs and activation of MAPK regulates cytokine production in dendritic cells [9]. In our work, SK-126 enhanced activation of ERK and p38 MAPKs induced by the exposure to LPS. Through the activation of ERK and p38, SK-126 could differentially modulate TNF-α and IL-10 production of LPS-treated DCs. Accordingly, pharmacological inhibition of these two MAPKs in combination with SK-126 resulted in the reversal of cytokine production where TNF-α production is recovered and IL-10 up-regulation is inhibited. Importantly, the reversion was more substantially induced by inhibition of p38 MAPK as compared with ERK inhibition (Fig. 6). Moreover, experiments using PI3K inhibitors revealed that the TNF-α inhibitory effect of SK-126 occurs in a PI3K-dependent manner. On the contrary, IL-10 up-regulation by SK-126 takes place in a PI3K-independent manner. Thus, the activity of p38 MAPK may play a more important role in IL-10 up-regulation by SK-126 after LPS stimulation. It has also been reported that signal transducer and activator of transcription (STAT) 3 is essential for the function of IL-10 and activation of STAT3 induces IL-10 production [17]. In addition, activation of p38 induces phosphorylation of STAT3 [18,19]. In line with this, we observed more potent STAT3 activation in DCs treated with LPS plus SK-126 than LPS alone. The ability of SK-126 to promote IL-10 synthesis by LPS is, therefore, associated with the activation of STAT3 by SK-126.

Interleukin 10 is an immunosuppressive cytokine produced by T cells, B cells, dendritic cells, and monocytes/macrophages [23–25]. The immunosuppressive activity of IL-10 is highlighted by the findings that IL-10 knockout mice show increased autoimmune disease and increased resistance to infection [26]. IL-10 acts by binding to the IL-10R1/IL-10R2 receptor complex that recruits Jak1 and Tyk2, which then phosphorylate and activate the transcription factor, STAT3 [25]. Although IL-10 activates STAT3, there has been evidence that IL-10 itself may be controlled by STAT3 binding to a cognate motif in the IL-10 promoter [25]. Thus, IL-10 has been suggested as a therapeutic agent for inflammatory conditions such as rheumatoid arthritis and inflammatory bowel disease and clinical trials to test this hypothesis are in progress [10,27].

DCs play a central role in the establishment of tolerance/immunity because they activate naive T cells. Therefore, pharmacological modulation of DCs has become a major field of interest in immunology. A large body of literature has arisen from the studies of DC biology during immunosuppressive drug treatment. Immunosuppressive drugs have significantly improved the therapeutic management of allograft organ transplantation and autoimmune diseases. There is now strong evidence that DCs might be the key for antigen specific tolerance induction [28]. Several DC subsets with distinct phenotypes and functions have been identified. In mice, CD11c has been acknowledged as a useful marker for DCs. DC subsets of myeloid origin (mDC) mature in response to

bacterial products and pro-inflammatory cytokines and fully mature mDCs are inducers of strong immune responses. However, following treatment with anti-inflammatory cytokines (IL-10 and transforming growth factor-β [TGF-β]) or pharmacologic agents like dexamethasone (DEX) and vitamin D_3, mDCs enter a tolerogenic state [29–32].

Taken together, the ability of SK-126 to reduce TNF-α synthesis and to promote IL-10 induction after exposure to LPS could account for its anti-inflammatory effect *in vitro* and *in vivo* where a DC cell line and primary DCs are used as experimental target cells and elongation of survival time is determined in LPS-induced septic shock mouse model, respectively. Therefore, the signal transduction pathway responsible for the anti-inflammatory action of SK-126 needs to be further assessed in other models of inflammatory diseases that also have dysregulation of pro- and anti-inflammatory cytokine production.

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