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Lutein Suppresses the Maturation and Function of Bone Marrow-Derived Dendritic Cells

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Abstract: Lutein, nonivamide, and baicalein, organic compounds found in a variety of plants, are known to exert anti-inflammatory effects in animal cells. Dendritic cells (DCs) are professional antigen-presenting cells (APCs) and link the innate and adaptive immune systems. DCs can be directed into fully mature APCs by exposure to bacterial or viral components, resulting in inflammatory situations. The manipulation of DC maturation provides a strategy for the treatment of allergic and inflammatory diseases. In this study, we evaluated the effects of lutein, nonivamide, and baicalein on the maturation and activation of DCs. Compared to nonivamide and baicalein, lutein significantly and dose-dependently reduced the levels of maturation-associated cell surface markers, including CD40, co-stimulatory molecule CD86, and major histocompatibility complex class II (I-A^b) molecule in lipopolysaccharide (LPS)-stimulated DCs. Lutein also decreased IL-12p40 and IL-6 gene expression and secretion in LPS-stimulated DCs. Furthermore, lutein significantly enhanced the endocytic ability of LPS-stimulated DCs. These results demonstrated that lutein may exhibit immunosuppressive activity by inhibiting the phenotypic and functional maturation of DCs, and provide new evidence for the value of lutein in the search for novel therapeutic agents in the treatment of inflammatory diseases.

Keywords: Lutein, Dendritic cells, Maturation, Immunosuppression; SDG 3 Good health and well-being

1. Introduction

Professional antigen-presenting cells (APCs) are immune cells that specialize in capturing, processing and presenting antigens and are very effective in activating T cells. Professional APCs express high levels of antigen presentation molecules, such as MHC class

I and II, as well as co-stimulatory molecules whereas non-professional APCs solely express MHC class I molecules [1]. Dendritic cells (DCs) are effective APCs that play crucial roles in initiating and modulating immune responses [2]. In short, DCs residing in peripheral tissues are poised to capture and process antigens into small peptides, which are presented on their cell surfaces along with appropriate co-stimulatory molecules. Simple molecules and regular patterns of molecular structures known as pathogen-associated molecular patterns (PAMPs) are recognized by DCs through pathogen-recognition receptors (PRPs) [3]. PAMPs, such as lipoteichoic acid, bacterial lipoproteins, double-stranded RNA, and lipopolysaccharides, as well as inflammatory mediators such as TNF-α and IL-1β, are captured by DCs, after which DCs undergo maturation. Mature DCs, with upregulated CD40, CD80, CD86, and MHC molecules on their cell surfaces [4], migrate toward the draining secondary lymphoid organs where they activate antigen-specific T cells, which stimulate cellular immune responses [5]. Thus, DCs link innate and adaptive immunity.

Lutein, an oxygenated carotenoid found in carrots, kale, spinach, and other green leafy vegetables, modulates light energy in plants and its consumption by animals improves visual function [6]. It has other beneficial biological functions, including antioxidant [7, 8], anti-carcinogenic [9], anti-atherogenic [10], anti-osteoporotic [11], and anti-ototoxic [12] activities. Such advantageous properties of lutein have led scientists to investigate its effect on the immune system. Many studies have concluded that lutein has an anti-inflammatory role in a variety of cells. For example, lutein was reported to decrease IL-8 production in LPS-stimulated uveal melanocytes through the reduction of the JNK and NF-κB signaling pathways [13]. Additionally, lutein downregulated pro-inflammatory cytokines, including IL-12 and IL-1β, in LPS-stimulated hepatocytes [14]. Furthermore, lutein decreased neutrophil and inflammatory cell accumulation in sensory nerve cells during acute neurogenic edema [15]. Although previous research illuminates the anti-inflammatory effects of lutein in a variety of cells, its direct effects on DCs have not yet been elucidated.

Nonivamide and baicalein are organic compounds also extracted from plants; both are known to induce numerous positive outcomes in humans. Nonivamide, also known as pelargonic or nonylic acid vanillylamide, is a pungent organic compound found in chili peppers along with other capsaicinoids, including capsaicin and dihydrocapsaicin [16]. Capsaicinoids are commonly used as food additives because of their spicy flavor and are also used in self-defence pepper sprays [17]. Among them, nonivamide is often synthesized and applied topically to the skin for its anaesthetic and anti-inflammatory effects [18]. Nonivamide is also known to be neuroprotective against oxidative stress-associated factors in human neuroblastoma cells [19] and to improve skin elasticity by inducing dermal IGF hormone release [20]. Despite these beneficial properties, the potential impacts of nonivamide on the immune system have yet to be discovered.

Baicalein is a flavonoid originally isolated from the roots of *Scutellaria baicalensis* and is also found in a variety of herbs. This compound is commonly found in traditional Chinese herbal medicine and is used as an anti-bacterial, anti-viral, and anti-inflammatory agent to treat a variety of conditions, such as diarrhoea and hepatitis [21, 22]. Recent studies

have also revealed a wide range of benefits, including anxiolytic [23], anti-carcinogenic [24, 25], and anti-oxidative [26] effects, as well as an ability to alleviate the symptoms of Alzheimer's disease [27, 28], type 2 diabetes [29], and Parkinson's disease [30]. The role of baicalein in immunity has been of interest and its anti-inflammatory [31-33] properties are well-known; however, the direct effects of baicalein treatment on DCs have not been studied.

The anti-inflammatory capacity of plant-based compounds has long been exploited in Chinese medicine, due to the presence of various bioactive components [34]. Lutein, nonivamide, and baicalein have been shown to lower oxidative stress and inflammation [18, 35, 36]. Numerous compounds have been shown to exhibit anti-inflammatory responses by reducing pro-inflammatory cytokines in macrophages [18, 37, 38]. Although DCs are the most prominent APCs in the immune system and inflammatory responses, the effects of lutein, nonivamide, and baicalein on DC functionality have not yet been elucidated.

In this study, we demonstrated that, compared with nonivamide and baicalein, lutein significantly suppressed the phenotypic and functional properties of DCs after maturation induced by LPS (Figure 1). This property was characterized by reduced expression of cell surface molecules and pro-inflammatory cytokines along with an enhanced capacity for antigen uptake. These results indicated that lutein inhibited the functionality of DCs. The inhibitory effects of lutein imply that it could be a potential therapeutic agent for inflammatory diseases.

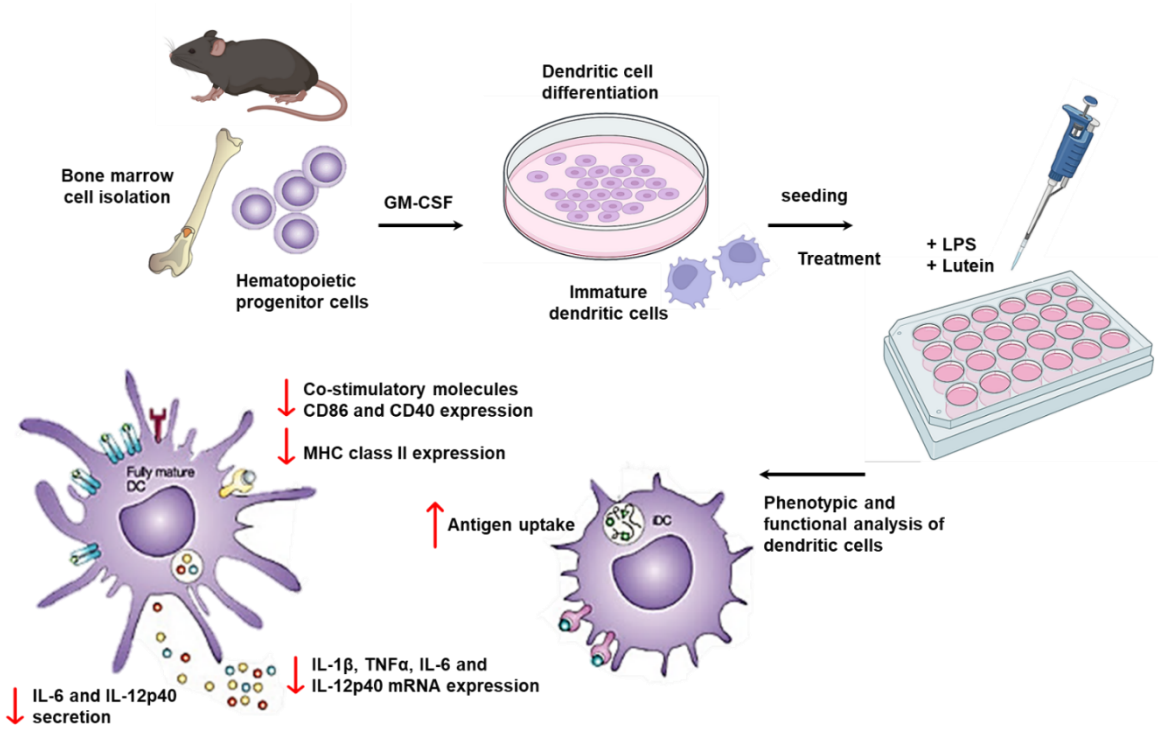

Figure 1. A graphical abstract illustrates lutein suppresses the phenotypic and functional properties of DCs after maturation induced by LPS.

2. Materials and Methods

2.1. Mice

Seven- to 9-week-old female C57BL/6 mice were purchased from OrientBio (Seongnam City, Korea). The mice were maintained under pathogen-free conditions and were treated according to the Korea University Guidelines for the Care and Use of Laboratory Animals (No. of approval: KLG 08-011).

2.2. Proteins and reagents

The plant compounds, nonivamide (HPLC grade ≥97%), baicalein (HPLC grade ≥97.5%), and lutein (HPLC grade ≥96%) were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF) was purchased from Prospec (Rehovot, Israel). APC-conjugated anti-mouse CD11c and FITC-conjugated anti-mouse MHC class II were purchased from eBioscience (San Diego, CA). PE-conjugated anti-mouse CD40 and PE-conjugated anti-mouse CD86 were purchased from BD Bioscience (San Diego, CA). LPS (from *Escherichia coli* 0111:B4) and Dextran-FITC (average molecular weight: 40000) were purchased from Sigma-Aldrich (St. Louis, MO).

2.3. Generation of bone marrow-derived dendritic cells (BMDCs)

Bone marrow-derived dendritic cells (BMDCs) were generated as described [39]. Briefly, tibiae and femurs of C57BL/6 mice were isolated and the BM cells were flushed out from the bones with RPMI 1640 medium by using a syringe. The cell clusters were then suspended by pipetting and red blood cells were removed by RBC lysis buffer containing 0.15 M of NH_4Cl, 1 mM of $KHCO_3$, and 0.1 mM of EDTA. Then, the cells were suspended in RPMI 1640 medium containing 10% heat-inactivated FBS (Capricorn Scientific), 50 μM of 2-ME (Sigma-Aldrich, St. Louis, MO), 10 mM of HEPES, 10 ng/ml GM-CSF (ProSpec, Rehovot, Israel) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin, Life Technologies BRL, Rockville, MD). The number of cells was set to 5×10^6 cells/ml and this cell culture was filled up to 10 ml in culture dishes. The culture dishes were maintained at 37°C in a humidified atmosphere with 5% carbon dioxide (CO_2) for 7 d. Fresh culture medium was provided every 2 d. Loosely adherent clustered cells were harvested on day 7 and used as immature DCs. BMDCs were first treated with 10 ng/ml LPS, followed by the addition of each test compound (nonivamide, baicalein, and lutein) at the indicated concentration for 20 h.

2.4. Flow cytometric analysis

To investigate the expression of surface markers on BMDCs, BMDCs (1.5×10^6 cells/well) were harvested and resuspended in FACS buffer (0.5% FBS and 0.05% sodium azide in PBS). Next, cells were stained for 15 min at 4°C with APC-conjugated anti-CD11c (N418) along with one of the following: PE-conjugated anti-CD40 (3/23), PE-conjugated

anti-CD86 (GL1), and FITC-conjugated anti-MHC class II (NIMR-4). For apoptosis analysis, the phosphatidylserine translocation in the cells was stained with the annexin V-FITC/propidium iodide (PI) detection kit (Biobud, Seoul, Korea) according to the manufacturer's instructions. The stained cells were subjected to FACS analysis. Flow cytometry was performed on a FACSCalibur with CellQuest software (BD Biosciences).

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

BMDCs (2×10^6 cells/well) were harvested and washed twice with PBS. Then, total mRNA extracted from the BMDCs was reverse transcribed into cDNA, followed by PCR amplification using a thermal cycler from MJ Research (Watertown, MA). The sequences of the primers in this study were as follows: IL-6 (F), tgaacaacgatgatgcactt, IL-6 (R), cgtagagaacaacataagtc; IL-1β (F), ctgaagcagctatggcaact, IL-1β (R), acaggacaggtatagattc; TNF-α (F), ggcaggtctactttggagtcattg, TNF-α (R), acattcgaggctccagtgaatttcgg; IL-12p40 (F), cagaagctaaccatctcctggtttg, IL-12p40 (R), tccggagtaatttggtgcttcacac. After amplification, the products were electrophoresed in a 1.5% agarose gel stained with ethidium bromide (EtBr) and photographed under UV exposure.

2.6. Cytokine assays

The quantities of IL-12p40 and IL-6 in the culture supernatants were determined by using mouse IL-12/IL-23 total p40 Ready-SET-Go! ELISA kit (eBioscience, San Diego, CA) and OptEIA™ IL-6 ELISA kit (BD Bioscience, San Diego, CA).

2.7. Quantification of antigen uptake

To measure the endocytic ability of the BMDCs, BMDCs (2×10^5 cells/well) were equilibrated at 37°C or 4°C for 30 min and further incubated for 40 min with media containing 0.5 mg/ml dextran-FITC at 37°C. As a negative control, BMDCs were incubated at 4°C. Cells were then washed with FACS buffer and stained for 10 min with APC-conjugated CD11c mAb. Afterwards, the stained cells were analyzed by FACSCalibur (BD Biosciences).

2.8. Statistical analysis

All experiments were repeated at least three times. The results were calculated as the mean ± standard deviation (SD) and the statistical significance between the control group and the experimental groups was analyzed by the student *t*-test. The p values indicated as *p <0.05, **p <0.01 and ***p <0.001 are considered statistically significant.

3. Results

3.1. Nonivamide, baicalein, and lutein differentially affected the expression of CD86 on LPS-stimulated DCs

LPS are endotoxins found in gram-negative bacteria that induce strong immune responses in animals, including the maturation of DCs. Therefore, LPS-stimulated DCs were used as positive controls for identifying the effects of nonivamide, baicalein, and lutein on suppressing DC maturation. In order to determine the influence of the three plant-derived compounds on DC maturation, immature DCs (iDCs) were cultured with 10 ng/ml LPS for 20 h in the absence or presence of 50 µM of each test compound. As depicted in Figure 2A and Figure 2B, LPS-stimulated DCs treated with lutein displayed a 40% decrease in CD86 expression, whereas those treated with nonivamide and baicalein did not exhibit a significant change. CD86 is a co-stimulatory molecule associated with the maturation of DCs and is known to bind to regulatory receptors on T cells [40]. The cytotoxicity of lutein in DCs was determined by annexin V-FITC and propidium iodide (PI) staining. As shown in Figure 2C, even in the presence of 50 µM lutein, the percentages of DCs not stained with either dye remained constant, indicating that lutein did not affect cell viability.

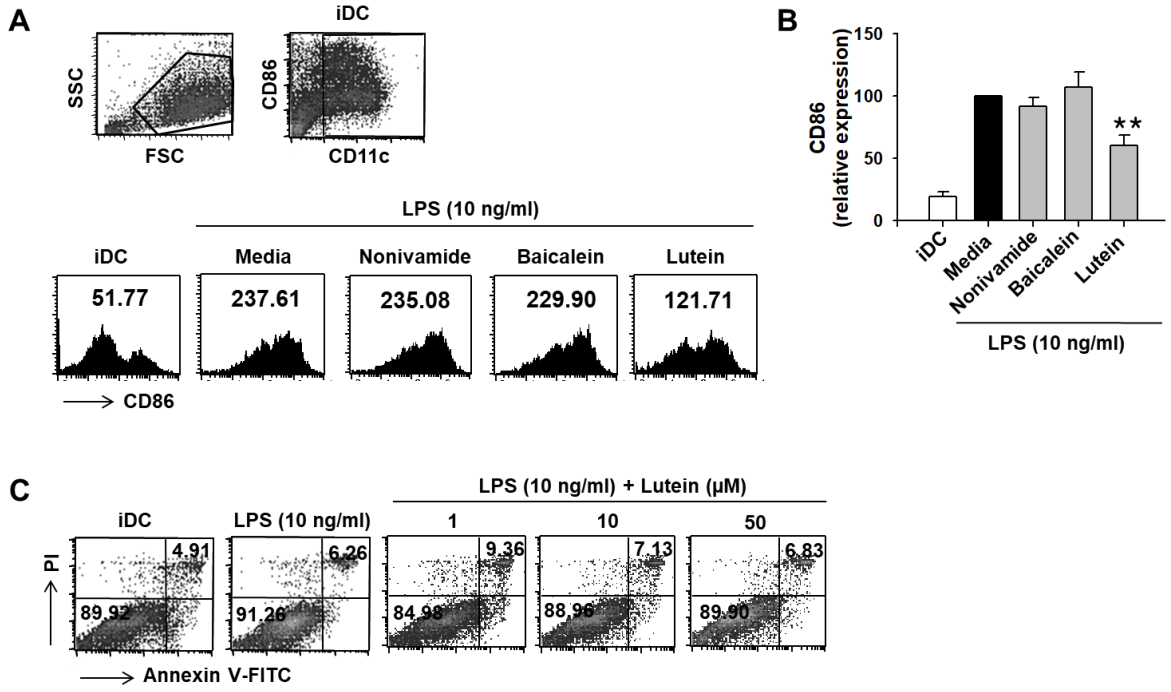

Figure 2. Differential effects of nonivamide, baicalein, and lutein on CD86 expression in LPS-stimulated DCs. Bone marrow cells were isolated and differentiated into iDCs by GM-CSF (10 ng/ml) treatment for 7 d. (A) iDCs were treated with LPS (10 ng/ml) in the absence and presence of nonivamide, baicalein, and lutein (50 µM) and cultured for 20 h. The untreated LPS-stimulated DCs were considered a positive control for DC maturation. The expression of CD86 was analyzed by flow cytometry and represented by histograms gated for $CD11c^+$ cells. The numbers in the histograms represent the mean fluorescence intensity (MFI) and the data are representative of three independent experiments. (B) Expression of CD86 was plotted relative to that in untreated LPS-stimulated DCs (100%, black bar). The data are expressed as mean ± SD from three independent experiments. **p<0.01 compared to the untreated LPS-stimulated group by Student *t*-test. (C) iDCs were

treated with LPS (10 ng/ml) in the absence and presence of lutein (50 μM) and cultured for 20 h. The cells were then stained with propidium iodide (PI) and annexin V-FITC and analyzed using flow cytometry. The data are representative of three independent experiments.

3.2. Lutein suppressed the expression of co-stimulatory molecules and major histocompatibility complex class (MHC) class II molecules on LPS-stimulated DCs

An increase in co-stimulatory molecules and MHC class II molecule expression on DC cell surfaces signifies the cell's maturation. These DC surface markers together integrate signals to activate T cells [41]. In order to analyze the ability of lutein to inhibit DC maturation, iDCs were cultured with 10 ng/ml LPS for 20 h in the absence or presence of various concentrations of lutein (1, 10, 25, and 50 μM). The expression of maturation-associated cell surface molecules was then investigated using flow cytometry. As illustrated in Figure 3A and Figure 3B, lutein significantly and dose-dependently suppressed the expression of CD40, CD86, and MHCII on LPS-stimulated DCs. These results indicated that lutein inhibited the phenotypic maturation of DCs induced by LPS.

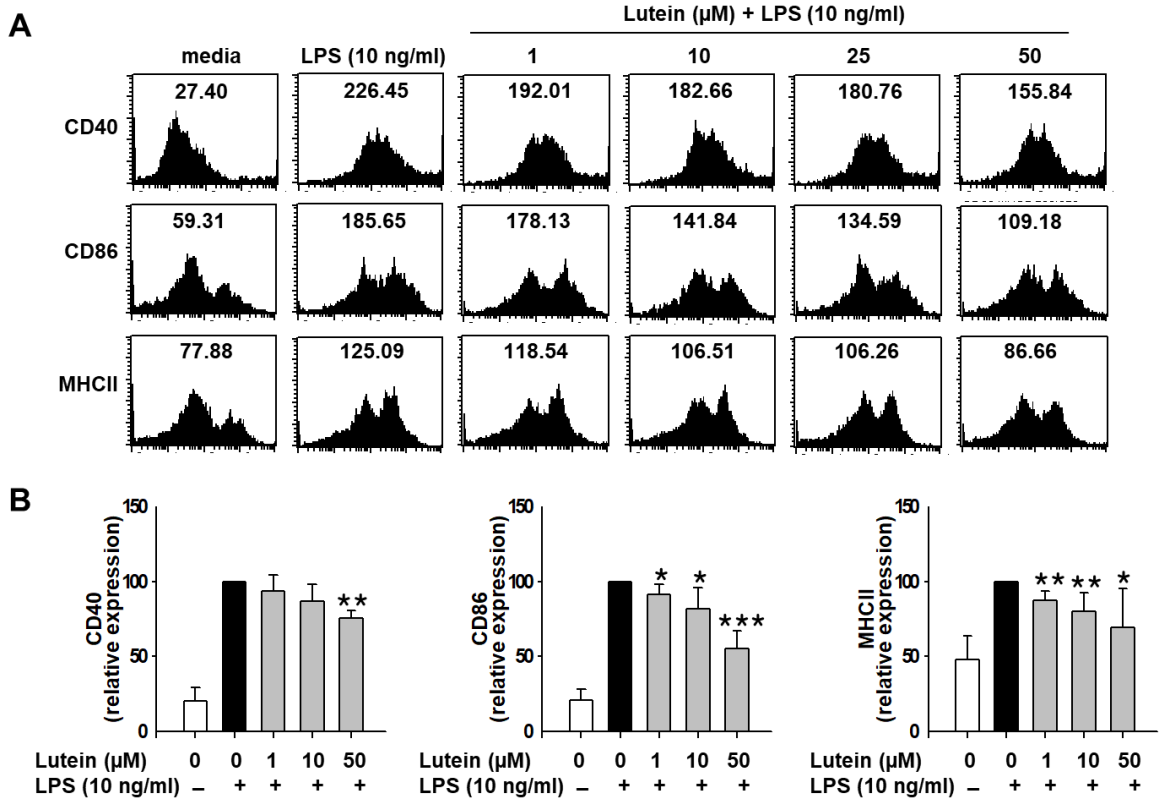

Figure 3. Lutein suppresses the expression of maturation-associated cell surface molecules on LPS-treated DCs. (A) iDCs were treated with LPS (10 ng/ml) in the absence and presence of lutein (1, 10, 25, and 50 μM) and cultured for 20 h. The cells were then stained with PE-conjugated anti-CD40, anti-CD86, APC-conjugated anti-I-A^b, and FITC-conjugated anti-CD11c mAb, and analyzed using flow cytometry. The numbers in the histograms represent the MFI and the data are representative of three independent experiments. (B) Expression of surface molecules was plotted compared to that in untreated LPS-stimulated DCs (100%, black bars). The

data are expressed as mean ± SD from three independent experiments. *p <0.05, **p <0.01, ***p <0.001 compared to the untreated LPS-stimulated DCs by Student t-test.

3.3. Lutein inhibited gene expression and protein secretion of pro-inflammatory cytokines in LPS-stimulated DCs.

Mature DCs produce a variety of pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α, and IL-12p40. The effect of lutein on mRNA expression (IL-1β, TNF-α, IL-6, and IL-12p40) and protein secretion (IL-12p40 and IL-6) in LPS-treated DCs was identified. To assess mRNA levels by RT-PCR analysis, iDCs were cultured in 10 ng/ml LPS for 6 h in the presence and absence of lutein (1, 10, and 50 μM). As shown in Figure 4A, IL-1β gene expression was unaffected, but TNF-α, IL-6 and IL-12p40 gene expression was slightly inhibited by lutein treatment compared to those of untreated DCs stimulated with LPS.

To assess protein levels using sandwich ELISA, iDCs were cultured in 10 ng/ml LPS for 24 h in the presence and absence of lutein (1, 10, and 50 μM) and the culture supernatants were evaluated. As indicated in Figure 4B, lutein significantly and dose-dependently suppressed IL-12p40 and IL-6 secretion from LPS-stimulated DCs. These results indicated that lutein suppressed the functional maturation of DCs induced by LPS.

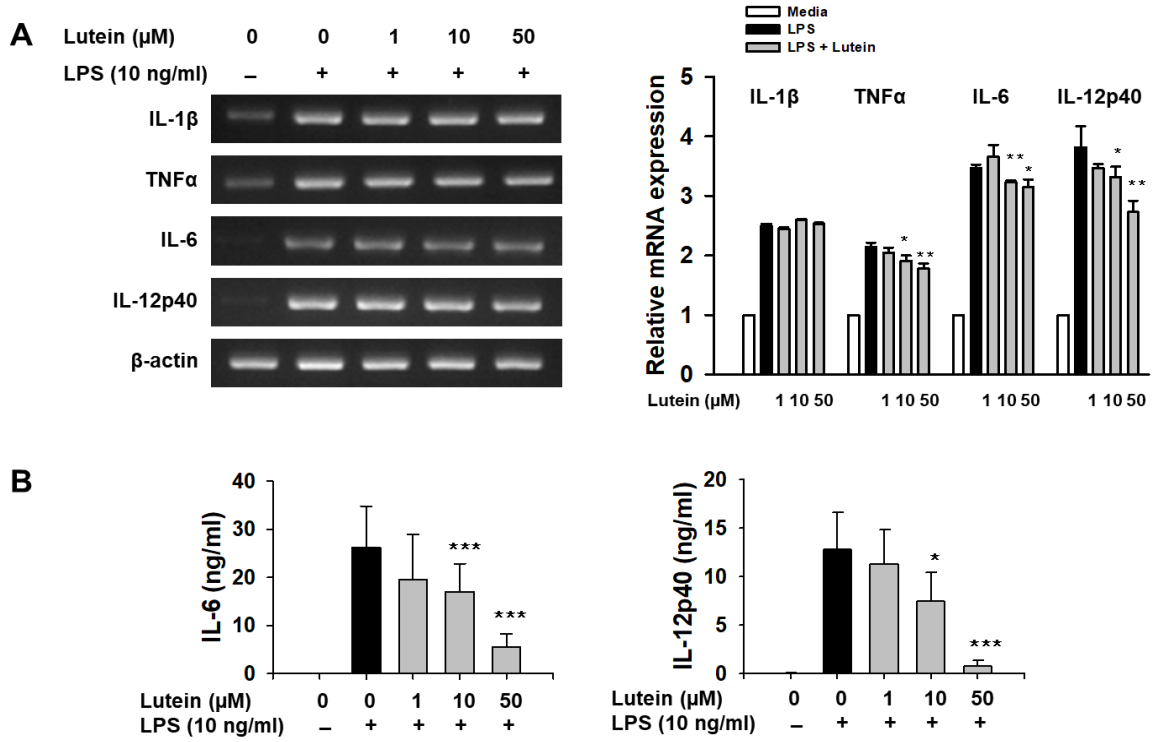

Figure 4. The effect of lutein on the expression of pro-inflammatory cytokines in LPS-stimulated DCs. (A) iDCs were treated with LPS (10 ng/ml) in the absence and presence of lutein (1, 10, and 50 μM) and cultured for 6 h. The mRNA levels of IL-1β, TNF-α, IL-6, IL-12p40, and β-actin were determined by RT-PCR. The relative mRNA expression of IL-1β, TNFα, IL-6, and IL-12p40 were quantified using the ImageJ program. Data are mean ± SD from three independent experiments. (B) iDCs were treated with LPS (10 ng/ml) in the absence and presence of lutein (1, 10, and 50 μM) and cultured for 20 h. The protein

levels of IL-6, and IL-12p40 in the culture supernatants were measured by ELISA. The data are expressed as mean ± SD from three independent experiments which were done in triplicates. *p <0.05, ***p <0.001 compared to the untreated LPS-stimulated DCs by Student t-test.

3.4. Lutein enhanced the endocytic ability of LPS-stimulated DCs

iDCs actively engulf external molecules by receptor-mediated endocytosis and macropinocytosis, processing and presenting them as antigens. Macropinocytosis is responsible for the continual internalization of solutes dissolved in the fluid surrounding DCs. The capacity of DCs to capture antigens decreases as DCs mature [42]. In order to identify the effect of lutein on the endocytic ability of DCs, iDCs were cultured in 10 ng/ml LPS for 20 h in the presence and absence of lutein (50 μM). To exclude the non-specific binding and uptake of FITC-labeled dextran, experiments were also carried out at 4°C. As presented in Figure 5A and Figure 5B, lutein restored endocytosis in cells that showed poor endocytic activity owing to LPS stimulation. These results indicated that lutein inhibited the functional maturation of DCs induced by LPS.

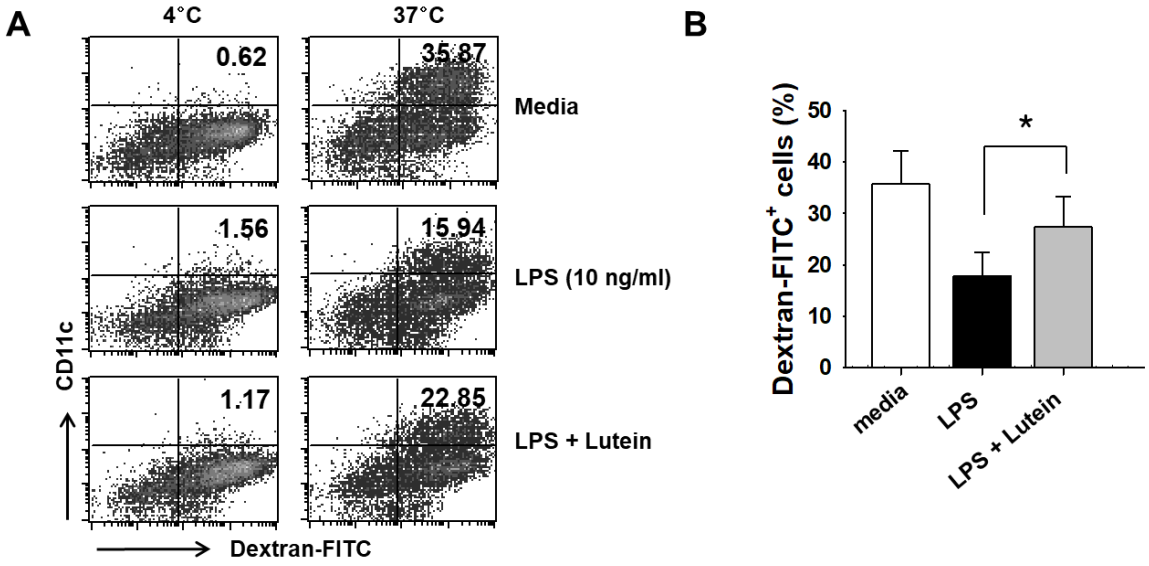

Figure 5. Lutein enhances the endocytic ability of LPS-stimulated DCs. iDCs were treated with LPS (10 ng/ml) in the absence and presence of lutein (1, 10, and 50 μM) and cultured for 20 h. (A) The cells were harvested and stained with FITC-dextran (0.5 mg/ml) for 40 min at 37°C and 4°C, respectively. The cells were further stained with APC-conjugated CD11c mAb for 10 min and the amount of FITC-dextran in $CD11c^+$ cells was determined by flow cytometry. The numbers in the plots represent the percentage of $CD11c^+Dextran^+$ cells and the data are representative of three independent experiments. (B) The percentages of $CD11c^+Dextran^+$ cells were expressed as mean ± SD from three independent experiments. * p < 0.05 compared to the untreated LPS-stimulated DCs by Student t-test

4. Discussion

Carotenoids are fat-soluble, organic pigments primarily found in fruits and vegetables included in the human diet. Their consumption is considered essential because they are known to contribute to normal skin color and ultraviolet sensitivity [43]. One of the most common dietary carotenoids is lutein, which is found abundantly in egg yolks, spinach, kale, zucchini, and carrots. Lutein consumption and accumulation in the lens and retina of the human eye is also necessary because it protects the ocular tissue against oxidative damage [44]. Therefore, it is important to identify the effects of lutein on immune responses. DCs are professional APCs that play important roles in inflammatory responses. During activation, DCs produce a variety of cytokines and chemokines to promote adaptive responses. Consequently, the effects of lutein on LPS-stimulated DCs were investigated to reveal the distinct roles of lutein in regulating the immune response.

In this study, the inhibitory effects of lutein on the maturation and activation of DCs were determined for the first time. First, lutein significantly and dose-dependently suppressed the expressions of three maturation-associated surface markers (CD40, co-stimulatory molecule CD86, and MHC class II molecule) in LPS-stimulated DCs (Figure 3). Among these phenotypic changes caused by lutein exposure, CD86 expression was most substantially affected (Figure 3).

Moreover, DCs secrete large amounts of inflammatory cytokines during maturation, which are considered functional markers of DC maturation. After lutein treatment, the production of pro-inflammatory cytokines IL-12p40 and IL-6 efficiently decreased in LPS-stimulated DCs (Figure 4). IL-12 is mainly produced by phagocytes and activated DCs, which promote Th1 differentiation, and efficiently induce $CD4^+$ T cells to produce IFN-γ [45]. IL-6 is a multifunctional cytokine secreted by many cell types that induces B-cell proliferation [46] and Th17 differentiation [47]. IL-6 is produced at the site of inflammation and IL-6-induced activation of TH-17 cells promotes the production of pro-inflammatory cytokines, which can exacerbate inflammation and tissue damage [48, 49]. Suppression of IL-6 expression [50] and IL-6 receptors [51] is emerging as a potential therapeutic method for reducing inflammation and modulating immune responses.

Furthermore, lutein restored the endocytic activity of LPS-treated DCs (Figure 5). Immature DCs actively internalize antigens and solutes through mannose receptor-mediated endocytosis and macropinocytosis [52], whereas endocytosis is downregulated in mature DCs [53]. Lutein suppressed DC maturation by upregulating its endocytic capacity (Figure 5). These results clearly indicated that lutein inhibited the phenotypic and functional maturation of LPS-stimulated DCs.

DCs are well-known key mediators of immune responses during inflammation. Upon maturation, DCs secrete distinct types of cytokine profiles and trigger antigen-specific immune responses by proliferating and polarizing Th cells. Therefore, the suppression of DC maturation provides an effective solution for reducing the symptoms of inflammatory diseases. Many previously studied substances that inhibit DC maturation may become potent anti-inflammatory drugs. For example, retinoic acid (RA), a metabolite derived from

carotenoids, negatively regulates the initiation of the allergic airway inflammatory response. RA blocks the IL-1β-mediated recruitment of NF-κB to promoters of NF-κB-regulated genes, thereby suppressing DC maturation and inflammation [54]. Accumulating evidence, including that presented in the aforementioned report, indicates that lutein inhibits NF-κB signaling. Lutein induced anti-inflammatory effects in macrophages by disrupting NF-κB activation [55, 56]. Lutein also inhibited LPS-induced IL-8 secretion in uveal melanocytes via the reduction of NF-κB and JNK signals [13]. In LPS-stimulated microglia, lutein suppressed inflammatory responses through NF-κB inactivation [57]. Therefore, we speculated that lutein suppressed LPS-treated DC maturation mainly by interrupting NF-κB signaling. However, the exact molecular mechanism involved in the inhibition of DC maturation remains to be elucidated.

The capacity of lutein to reduce inflammation has long been investigated. In addition to its ability to protect the eye from inflammatory damage resulting from oxidative stress, lutein has been reported to inhibit chronic inflammation and alleviate the symptoms of various conditions, such as diabetes [58], bone fracture [59], and coronary disease [60]. Likewise, treatment of inflammation mostly focuses on inhibiting the production of pro-inflammatory mediators. Anti-inflammatory properties of lutein may contribute to the development of precision nutrition, resulting in a healthier gut microbiome and helping with the disease process [61].

5. Conclusions

This study demonstrated that lutein phenotypically and functionally inhibited the maturation of LPS-stimulated DCs. Therefore, lutein may be a useful therapeutic agent, especially for the treatment of mature DC-mediated inflammatory diseases. However, the exact molecular mechanism relevant to the lutein-mediated suppression of DC activation remains to be elucidated.

Author Contributions: Conceptualization, TSK and HXL; investigation and data analysis, SC and HXL; writing-original draft, SC and HXL; resources, TSK; writing-review and editing, TSK. All authors have read and agreed to the published version.

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Conflicts of Interest: The authors declare that there are no conflicts of interest.

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