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Formulated extract from multiple citrus peels impairs dendritic cell functions and attenuates allergic contact hypersensitivity

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ABSTRACT

It has been reported that gold lotion (GL), a formulated product made from the peels of six citrus fruits, has many pharmacological properties, such as anti-tumor, antioxidant, and anti-inflammatory activities. In this study, we investigated the immunomodulatory effect of GL on lipopolysaccharide (LPS) stimulated mouse bone marrow-derived DC maturation and function. Our experimental results have shown that GL significantly impaired the pro-inflammatory cytokine and chemokine secretion, suppressed the expression of major histocompatibility complex class I/II and costimulatory molecules (CD40, CD80 and CD86), increased phagocytic capacity, and reduced propensity to stimulate the autologous CD4⁺ and CD8⁺ T cell proliferation of LPS-induced DCs. Furthermore, we found that oral administration of GL attenuated the 2,4-Dinitro-1-fluorobenzene induced contact hypersensitivity (CHS) in animal models. Subsequently, our molecular mechanism studies showed that GL interfered with LPS-induced MAPK–JNK, p38 phosphorylation and nuclear translocation of NF-κB p65. In an essence, these findings are the first report to provide new insight in the immunopharmacological role of GL in terms of its effects on DC.

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1. Introduction

There are many types of citrus in the world, including orange, grapefruit, lemon, lime, etc. The citrus peel has drawn increased attention in recent years because it contains many special phytochemicals associated with health benefits and has the potential for development in functional foods and medicinal agents [1–4]. In oriental countries, citrus peel tinctures are often used as a medicine for phlegm and cough [5]. Recently, there have been reports to exhibit many biological properties such as anti-inflammatory, anticancer, and anti-microbial activities in vitro and in experimental animals [6–9]. In citrus fruits, the citrus peel, a rich natural source of flavonoids and polymethoxyflavones (PMFs), is of particular interest due to their broad spectrum of bioactivities. These compounds play important roles not only in biological

activities, but also in applications of food and pharmaceutical industries [10–15].

Gold lotion (GL), a formulated product made from six different citrus fruit peels (*navel oranges*, *Citrus hassaku*, *Citrus limon*, *Citrus natsudaidai*, *Citrus miyauichi* and *Satsuma*), was initially formulated in cosmetics in Japan to protect skin from ultraviolet (UV) light irradiation. Chemical analysis of GL has revealed an abundant existence of flavonoids with total content measurements of at least 450 ppm or 0.45 mg/ml. The analysis also revealed measurements of PMF content as high as 106 ppm or 0.1 mg/ml as well [16,17]. Therefore, some consumer reports indicated that topical and oral treatments of GL possess anti-cancer property against melanoma, and prostate, lung, and liver cancers. Further research from the Huntingdon Research Center in the UK found GL to be non-toxic. (Reports from Miyauchi Citrus Research Center). A recent study demonstrated the potent anti-cancer effects of citrus peel flavonoids in human prostate xenograft tumors in nude mice [18].

Evidence shows that GL has anti-inflammatory and immunomodulatory effects. Oral administration of GL protected mice against azoxymethane-induced aberrant crypt foci (ACF) in colonic tissues

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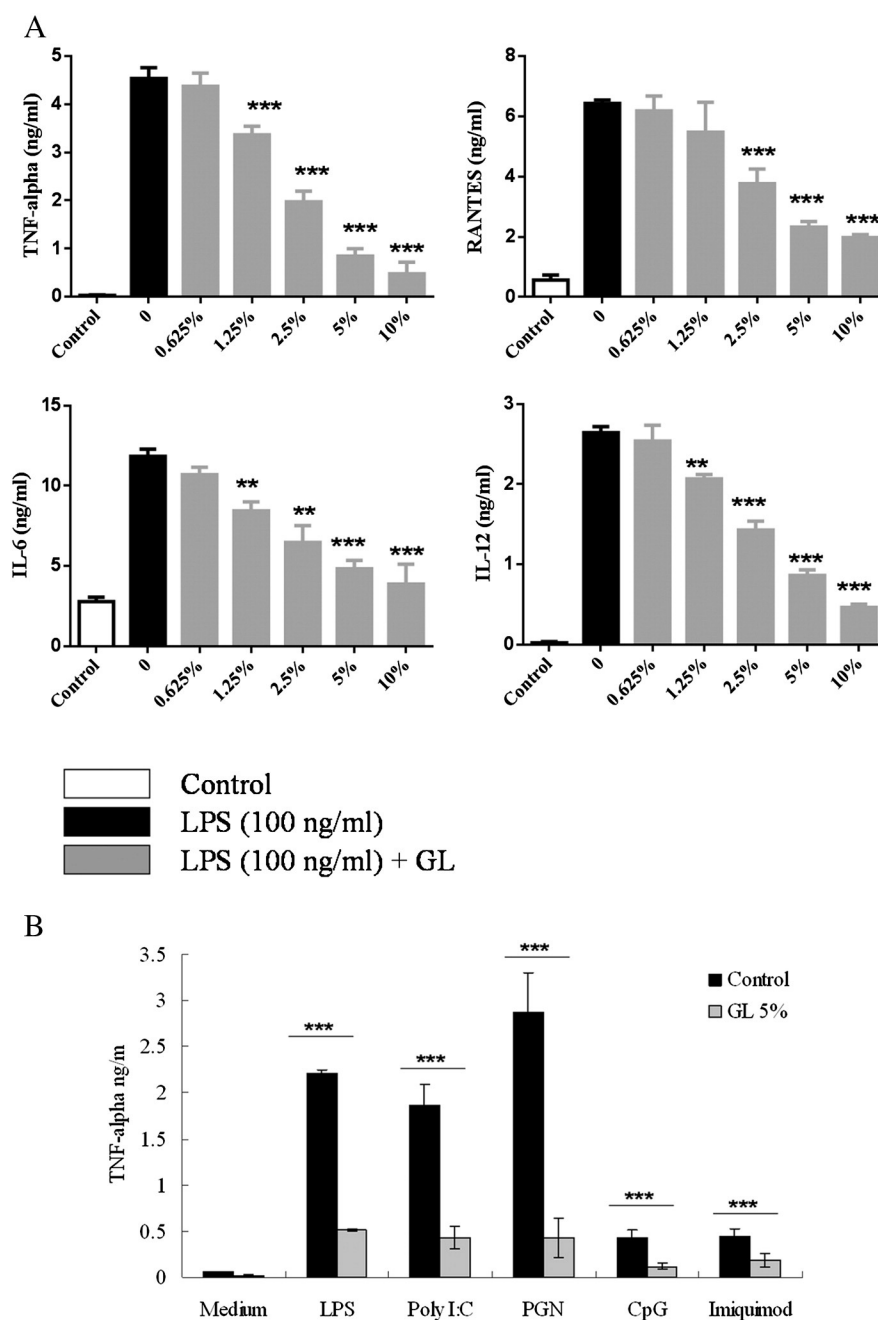


Fig. 1. Cytokines and chemokines released from TLR-stimulated DCs were reduced by GL. A) Immature DCs were treated with 10% GL for 1 h and then stimulated with LPS (100 ng/ml), B) or TLR ligands (including, peptidoglycan (1 µg/ml, TLR1/TLR2), PolyI:C (250 µg/ml, TLR3), CpG ODN 1826 (200 nM TLR-9), and Imiquimod (5 µg/ml TLR-7)) for additional 18 h (TNF-alpha and RANTES for 4 h). After incubation, culture supernatants from each group were collected and subjected to ELISA for determination of the amounts of indicated cytokines. All data on the graph represents the mean ± SD from three wells. **p < 0.01, ***p < 0.001; (ANOVA followed by Tukey post hoc test) versus LPS treatment alone. All results are shown as a representation of three independent experiments yielding similar result.

of mice [16], in which the protection mechanism may be involved in decreasing expression of genes associated with inflammation and tumorigenesis, such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGF), and matrix metalloproteinase 9 (MMP-9) in colonic tissues of mice. In addition, topical application of GL prevents 7,12-dimethylbenz[a]anthracene (DMBA)/TPA-induced skin inflammation and tumor formation [17]. Furthermore, a recent study conducted by Tadahihiro et al. indicated that GL suppresses the inflammation in vitro, and exerts preventive activity through the reduced release of TNF-α

and nitric oxide from macrophage RAW264.7 cells stimulated by LPS [19]. However, to the best of our knowledge, the cellular and molecular targets of GL in the immune system have remained unclear.

Dendritic cells (DCs) are potent antigen presenting cells that control both innate and adaptive immune systems [20]. Under steady state, immature DCs reside in the peripheral nonlymphoid tissues where they sample antigens, such as those associated with microbial pathogens and tumors, by endocytosis. Upon encountering antigens, DCs undergo the morphological, phenotypic, and functional process termed “maturation”. The common characteristics of DC maturation, include the

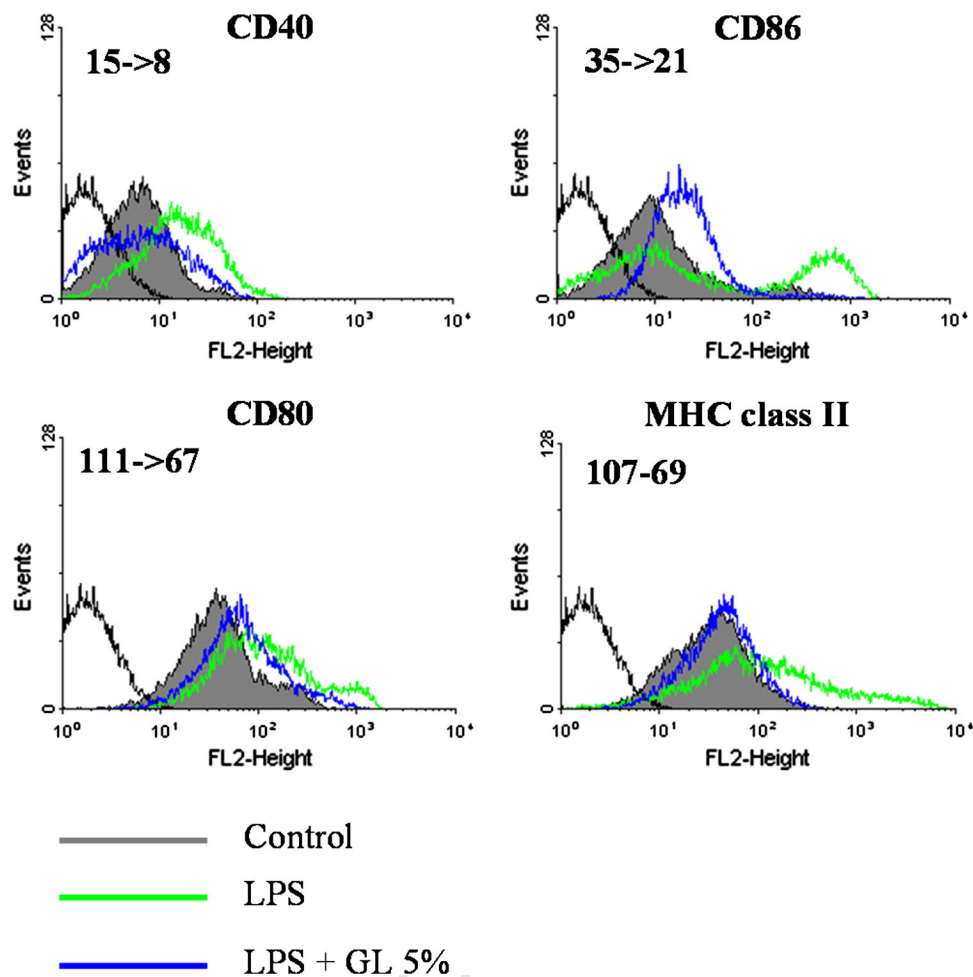


Fig. 2. GL attenuates the expression of co-stimulatory molecules and MHC proteins on DC stimulated with LPS. Immature DCs were pretreated with 10% GL for 1 h and then stimulated with LPS (100 ng/ml) for additional 18 h. After incubation, cells were stained with specific primary antibodies, which was followed and analyzed by flow cytometry. The dotted line indicates the isotype control group. Data was shown as the change of mean fluorescence intensity (MFI) from LPS to LPS + GL 5% was indicated. The histogram graphs are shown as a representation of three independent experiments yielding similar result.

increased surface expression of major histocompatibility complexes (MHCs) and costimulatory molecules (CD40, CD80 and CD86), reduced phagocytic activity, and the production of numerous inflammatory cytokines and chemokines [21,22]. Upon arriving at the secondary lymphoid organs through the afferent lymphatic vessels, mature DCs present antigenic peptide in the context of MHC molecules to stimulate naïve T cells, which is a unique ability that only in mature DC possesses [22,23]. However, in contrast to the enhanced T cell immunity associated with DCs, the role of DC in establishing peripheral T cell tolerance has yet to be convincingly demonstrated [24]. Thus, it is quite important to regulate DC function via the manipulation of exogenous factors.

In this study, we investigated the ability of GL on the maturation and functional properties of dendritic cells (DCs). Our results demonstrated, for the first time, that GL can inhibit the LPS-induced functional maturation of BMDCs and furthermore, can impede the ability to prime and expand Ag-specific T cell proliferation. In addition, oral administration of GL could also prevent 2,4-dinitro-1-fluorobenzene (DNFB)-induced contact hypersensitivity (CHS) in vivo. The inhibition of LPS-induced MAPK–JNK, p38 and NF- κ B signaling pathways may partially explain the suppressive effects of GL on DC activation. Overall, these results indicate that GL impacts the functional properties of DCs and may have potential therapeutic applications against harmful immune responses.

2. Materials and methods

2.1. Mice and preparation of bone marrow derived murine DCs

Female C57BL/6 (H-2b) 4–6 week-old mice were purchased from National Laboratory Animal Center (Taipei, Taiwan). OT-II TCR transgenic mice were provided by Dr. Clivord Lowell (UCSF, San Francisco, CA). They were housed at the facility of Taichung Veterans General Hospital (Taiwan). This study was carried out in strict accordance and with the recommendations found in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the Institutional Animal Care and Use Committee of National Chung Hsing University (permit number: 101-106). All efforts were made to minimize pain and suffering. Mouse bone marrow derived DCs were generated using a previously described method [25]. Briefly, bone marrow cells were isolated from mice and cultured with 20 ng/ml recombinant mouse granulocyte macrophage colony-stimulating factor (PeproTech, Rocky Hill, NJ, USA) and 20 ng/ml recombinant mouse IL-4. Every 2 days, half of the supernatant was gently removed and replaced with the same volume of fresh culture medium. On day 7, non-adherent or loosely adherent cells were collected and used as immature DCs. More than 80% of the cells expressed CD11c, as determined by flow cytometry (data not shown). CD11c⁺ DCs were purified by positive selection using anti-

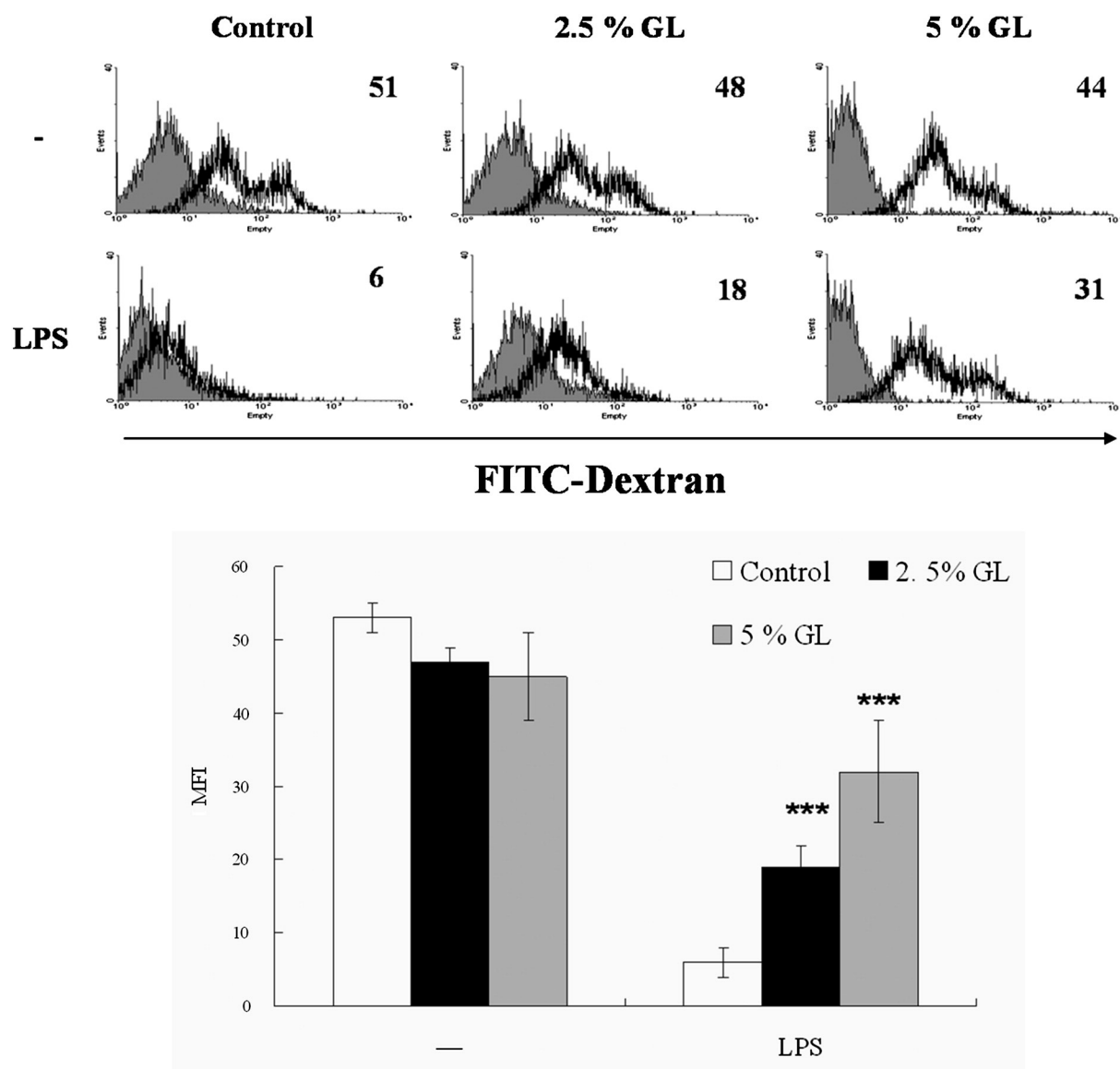


Fig. 3. GL restores the attenuation of phagocytic capacity of DCs at maturation induced by LPS. Immature DCs were pretreated with the 2.5, and 5% GL for 1 h and then stimulated with LPS for additional 18 h. Cells were then exposed to 500 $\mu\text{g/ml}$ dextran-FITC for 1 h at 4 °C (gray filled area) or 37 °C (black dot line). After washing, cell-associated fluorescence was analyzed by flow cytometry. Data was shown as mean fluorescence intensity (MFI) of each histogram (MFI at 37 °C). The graphs that are shown represent the mean $\pm$ SD from three independent experiments. *** $p < 0.001$; (ANOVA followed by Tukey post hoc test) versus untreated control groups in the presence or absence LPS respectively.

CD11-coated beads (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions, and these cells were used for MAPK and NF- κ B activity assay and T cell proliferation analysis. Purity of CD11c⁺ cells was confirmed >90% by flow cytometry (data not shown).

2.2. Preparation of extract from multiple citrus peels (Gold lotion, GL)

In this study, GL was provided by Miyauchi Citrus Research Center, Ltd. (Japan) and was stored at 4 °C prior to use. The natural process of GL extracting from six citrus peels with water/ethanol was conducted without fermentation. Once extracts were bottled, they were maintained in a natural environment for over two years before use. The chemical composition of flavonoids and PMFs in the GL was as previously described [16,17]. According to the dose of the in vitro experiment, the experimental group was treated with 10% GL and contained 100 μl of extract at a final concentration of medium (1 ml). Previous

studies have suggested that the concentrations of flavonoids and PMFs in 10% GL were equivalent to 46.44 $\mu\text{g/ml}$ and 10.61 $\mu\text{g/ml}$, respectively. In addition, the flavonoids contained eight major chemical components and the concentrations of these chemicals were 5.8 $\mu\text{g/ml}$ (nobiletin), 2.13 $\mu\text{g/ml}$ (sinesetin), 1.92 $\mu\text{g/ml}$ (3,5,6,7,8,3',4'-Hepta-methoxyflavone), 1.06 $\mu\text{g/ml}$ (Tangeretin), 0.31 $\mu\text{g/ml}$ (3,5,6,7,3',4'-Hexamethoxy-flavone), 0.11 $\mu\text{g/ml}$ (5,6,7,4'-Tetramethoxy-flavone), 25.36 $\mu\text{g/ml}$ (Naringin) and 10.47 $\mu\text{g/ml}$ (Hesperidin).

2.3. Effect of GL in LPS-induced BMDC maturation

Immature BMDCs in RPMI supplemented with 10% FBS were incubated with medium or various concentrations of GL for 1 h followed by stimulation with or without 100 ng/ml lipopolysaccharide (LPS) or different TLR ligands that included Poly I:C (250 $\mu\text{g/ml}$, TLR-3), PGN (1 $\mu\text{g/ml}$, TLR-2), CpG (200 nM, TLR9), and Imiquimod (3 $\mu\text{g/ml}$, TLR-

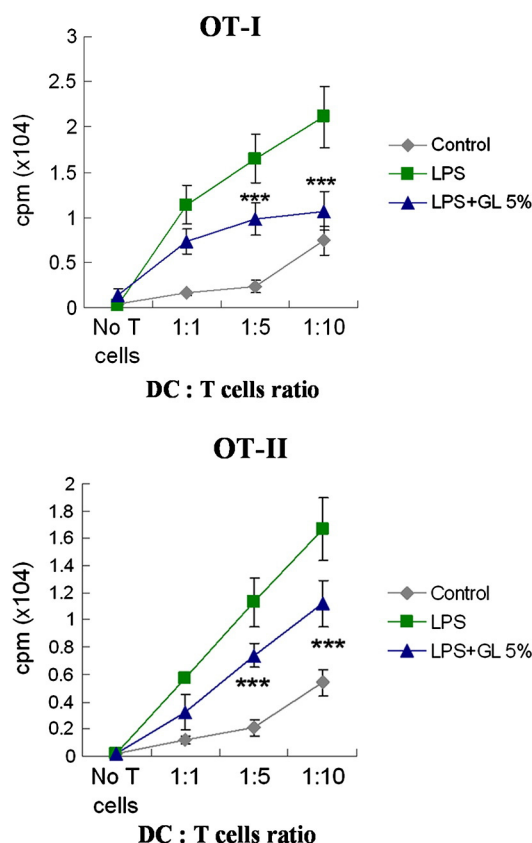


Fig. 4. GL decreases the Ag-specific CD4⁺ and CD8⁺ T cell activation induced by LPS-stimulated DCs. Immature DCs were stimulated with the OVA peptide and treated with or without 5% of GL for 1 h and followed by stimulation with 100 ng/ml LPS for additional 18 h. After incubation, the DC cells were washed and co-cultured with either containing OT-I CD8⁺ or OT-II CD4⁺ T cells at the indicated ratio of DC:T cell for additional 4 days. To analyze T cell proliferation, the cells were incorporated with [³H]-thymidine for 18 h prior to stopping incubation. Cell-associated radioactivity was then analyzed. All data on the graph represents the mean $\pm$ SD from three wells. **p < 0.01 (ANOVA followed by Tukey post hoc test) versus LPS treatment alone. All results are shown as a representation of three independent experiments yielding similar result.

7) (All from Invivogen, San Diego, CA, USA) for additional 18 h (4 h for TNF-alpha and RANTES cytokine expression). After incubation, the supernatant was collected to measure TNF-alpha, IL-12, IL-6, and RANTES production by ELISA (PeproTech, Rocky Hill, NJ, USA). On the other hand, the cells were also harvested and their surface molecular expression was characterized by flow cytometry (BD Bioscience) using specific antibodies against CD11c⁺, CD40, CD80, CD86, MHC class I and MHC class II conjugated to FITC- or phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs) (All from Biolegend, San Diego, CA, USA), as well as a control isotype-match antibody. The data was analyzed using WINMDI software (Scripps, La Jolla, CA, USA).

2.4. OVA-specific T cell activation

The experimental protocol was modified from our previous reports [25,26]. DCs were pretreated with or without GL for 1 h and subsequently, DCs were stimulated by LPS (100 ng/ml) for additional 18 h. After incubation, the cells were harvested and washed with PBS. OVAP2 specific CD4⁺ and OVAP1 CD8⁺ T cells were positively enriched from OT-2 and OT-I mice by MACS cell separation, according to the manufacturer's instruction (Miltenyi Biotec, Auburn, CA, USA). To perform in vitro co-culture assay, isolated T cells were cultured with different treated DCs in the presence of OVA_{323–339} (OVAP2) and OVA_{257–264} (OVAP1) (synthesized by Echo Chemical Co., Taiwan) at DC:T cell ratios of 1:2.5 and 1:10 in a total volume of 200 μ l per condition. After 3 days,

cells were pulsed with 1 μ l [³H] thymidine per well overnight. Incorporation of radioactivity was determined using a beta-counter (Beckman).

2.5. Antigen uptake assay

Antigen uptake assays were performed, as a previously described method [27]. Briefly, enriched CD11c⁺ positive mBMDCs were pretreated with or without GL (10%) and then stimulated with LPS (100 ng/ml) for additional 18 h. Cells were then harvested, and mannose receptor (MR) mediated endocytosis was examined by uptake of 500 μ g/ml dextran-FITC (42,000 molecular weight; Sigma) at 37 $^{\circ}$ C for 2 h. After incubation, the cells were washed and then analyzed using a FACSCalibur flow cytometer. In addition, nonspecific binding of uptake of dextran-FITC to DCs was performed by examining dextran-FITC at 4 $^{\circ}$ C.

2.6. Preparation of nuclear extracts and western blotting

Briefly, CD11c⁺ enriched DC were pretreated with or without GL for 1 h and subsequently stimulated with LPS (100 ng/ml). Cells were harvested and whole cell lysates were prepared at the indicate time points, as described previously [25,27]. Nuclear protein fractions were obtained by NE-PER Nuclear and Cytoplasmic extraction reagents (Pierce), as recommended by the manufacturer's recommendations. Sample protein concentrations were examined using Bicinchoninic Acid (BCA) Protein Assay Kit. For protein detection, protein extracts (50 mg/ml) were boiled, and resolved in a 10% SDS-polyacrylamide gels and electrotransferred onto nitrocellulose membranes. The blots were blocked with 5% skim milk in TBS with 0.05% Tween 20 for 1 h and incubated of a primary antibody at 4 $^{\circ}$ C overnight. The membranes were then washed and incubated with horseradish peroxidase-labeled secondary Abs (Jackson ImmunoResearch, West Grove, PA) at room temperature for 2 h at 4 $^{\circ}$ C overnight and blots were developed by enhanced chemoluminescence (Amersham) and analyzed with the LAS3000 system (Fujifilm, Tokyo, Japan). The primary antibodies used in this experiment included anti-phospho-p38, anti-total p38, anti-phospho-ERK, anti-total ERK, anti-phospho JNK, anti-JNK and NF- κ B p65. They were all purchased from Cell signaling and anti-Histone H4 was purchased from Santa Cruz Biotechnology for overnight. Densitometric analysis was performed with ImageJ software (National Institutes of Health, Bethesda, MD, USA). Phosphorylated forms of pErk, p38 and JNK were normalized with corresponding total nonphosphorylated forms.

2.7. 2,4-Dinitro-1-fluorobenzene-induced contact hypersensitivity assay

2,4-Dinitro-1-fluorobenzene (DNFB; Sigma-Aldrich)-induced contact hypersensitivity (CHS) was modified from described previously [27]. The shaved belly of mice were painted with 20 μ l 0.5% (w/v) DNFB in acetone:olive oil (at a ratio of 4:1) twice (on days – 5 and – 4). Vehicle (acetone/olive oil)-painted mice were used as controls. In the experimental group, mice were fed a 200 μ l daily dose of GL, which contained 92.8 μ g of flavonoids and 21.34 μ g/ml of PMFs. The concentration of major chemical components was 10.16 μ g (nobiletin), 4.26 μ g (sinesetin), 3.84 μ g (3,5,6,7,8,3',4'-Hepta-methoxyflavone), 2.12 μ g (Tangeretin), 0.62 μ g (3,5,6,7,3',4'-Hexamethoxy-flavone), 0.22 μ g (5,6,7,4'-Tetramethoxy-flavone), 50.72 μ g (Naringin), and 20.94 μ g (Hesperidin). After a 5 day (day 0) treatment, all mice were painted with 20 μ l DNFB (0.2%) in 4:1 acetone/olive oil solution distributed equally on both sides of the right ear. The CHS response was evaluated by measuring the increase thickness of the ear [thickness of the right (challenged) ear – thickness of the left (unchallenged) ear] using an engineer's spring-loaded micrometer (Mitutoyo, Tokyo, Japan) in the indicated times and determined 24 h later by histological analysis using H&E staining.

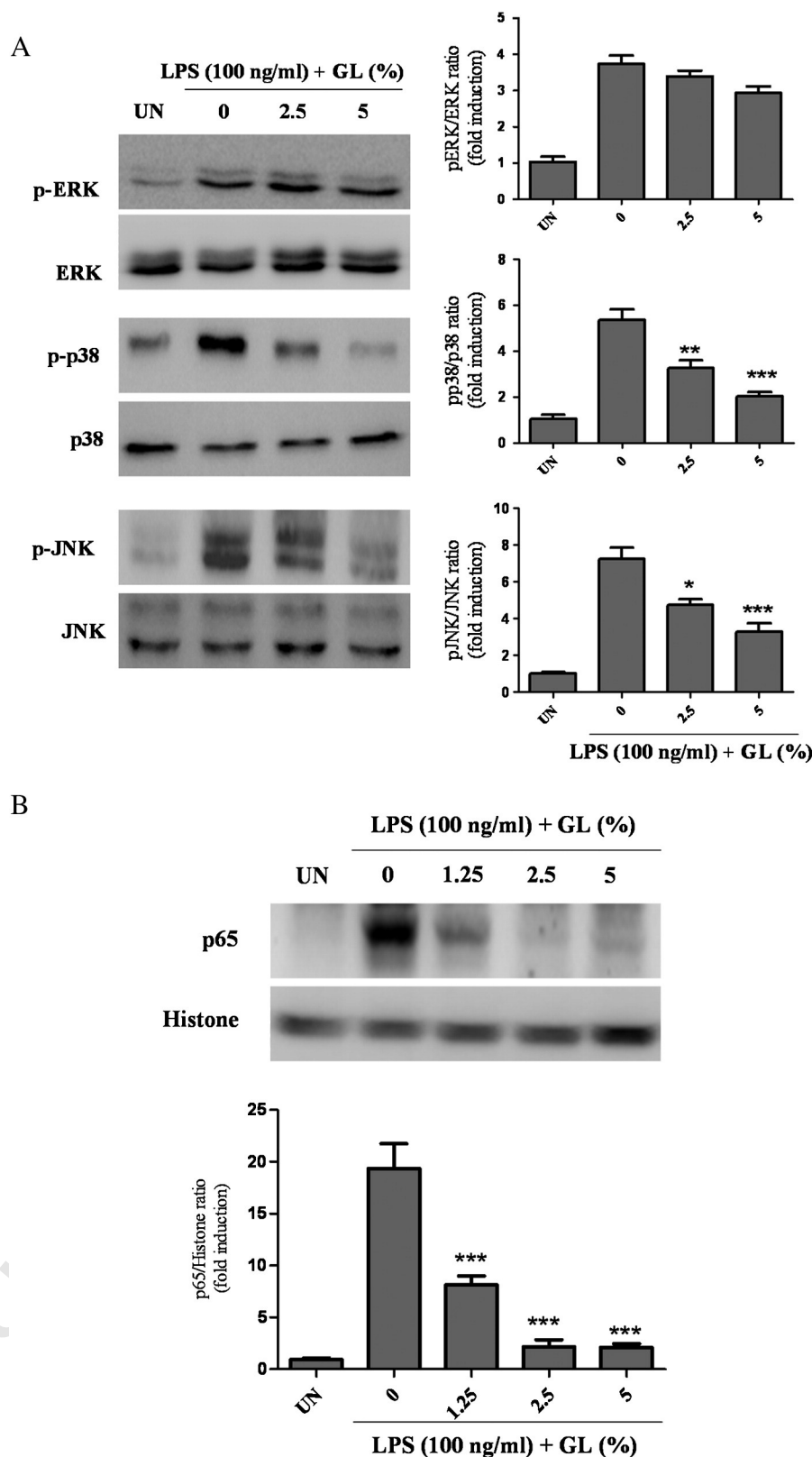


Fig. 5. GL decreased LPS-induced MAPK–p38 phosphorylation and NF- κ B p65 nuclear translocation in BMDCs. Immature DCs were treated with or without GL at 1.25, 2.5 or 5% for 1 h, followed by LPS treatment at 100 ng/ml and whole cell lysates or nuclear fraction of lysates were collected at indicated time points. A) Western blotting was performed to determine phosphorylated or non-phosphorylated Erk, JNK, and p38 using phosphospecific Abs respectively. The graphs are shown as a representation of three independent experiments yielding similar result. pERK, pp38, and pJNK levels were normalized with total ERK, p38 and JNK levels and then compared with the untreated control group arranged as one unit. The graphs shown represent the mean $\pm$ SD from three independent experiments with similar results. *** p < 0.001; (ANOVA followed by Tukey post hoc test) versus LPS alone groups. B) NF- κ B p65 in nuclear extracts as mentioned was performed as described in the [Material and methods](#) section. Histone H4 loading controls are shown to demonstrate relatively equal protein load across lanes. p65 levels were normalized with Histone H4 levels and then compared with the untreated control group arranged as one unit. The graphs that are shown represent the mean $\pm$ SD from three independent experiments with similar results. *** p < 0.001; (ANOVA followed by Tukey post hoc test) versus LPS alone groups.

2.8. Quantitative RT-PCR

6, 12, and 24 h after DNFB challenge, the total RNA was extracted from ear tissues using the RNAspin Mini kit (GE). One microgram of total RNA was used in each reaction by using the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's instructions. cDNA was used as template for real time-PCR using SYBR Green Master Mix (Roche) with primers described as below: IFN- γ , F: 5'-GCAACAGCAAGGCGAAAAAG-3' and R: 5'-CTGGACCTGTGGTGTGTTGAC-3'; F: 5'-ACTGGCAAAGGATGGTGAC-3'; IL-1 beta, F: 5'-TCGTGCTGTCGGACCCATA-3' and R: 5'-TTGTTGGTTGA ATTCTGCCATTG-3'; Ccl2, F: 5'-TTGAATGTGAAGTTGACCCGTAA-3' and R: 5'-TGAGGTGGTTGTGGAAAAGGT-3'; IL-6, F: 5'-TCGGAGGCTTAATTACACATTGTTC-3' and R: 5'-TGCCATTGCACA ACTCTTTTCT-3'; IL-10, F: 5'-AGCATGGCCCAGAAATCAAG-3' and R: 5'-CGCATCTGAGGGTCTTCAG-3'; TNF-alpha, F: 5'-GGCTGCCCGACTACGT-3' and R: 5'-CTCCTGGTATGAGATAGCAAATCG-3'; hypoxanthine guanine phosphoribosyl transferase 1 (HPRT), F: 5'-GTTGGATAAGGCCAGACTTGTG-3' and R: 5'-GATTCACTTGGCCATCTTAGGC-3'; in an Eco Real-Time PCR System (Illumina). The data were normalized using the expression level against HPRT. The relative expression level of the gene in the experimental group was compared with that of the vehicle (acetone/olive oil)-painted but was unchallenged with DNFB mice group.

2.9. Histology

The ears were removed and sliced longitudinally into 6 μ M-thick sections 2 days after the DNFB (0.2%) painting. Tissue sections were stained with hematoxylin and eosin to study their microarchitecture. Immunohistochemistry was performed as follows: sections were fixed

with formalin; then sections were pre-treated using heat mediated antigen retrieval with EDTA buffer (pH 9, epitope retrieval solution) for 20 min; endogenous peroxidase was removed with 0.02% hydrogen peroxide; sections were washed with PBS five times and incubated overnight at 4 °C with primary rabbit antibodies against mouse CD4⁺ and CD8⁺ in PBS/0.1% BSA. The next day, HRP-conjugated goat anti-rabbit secondary antibody was applied in PBS/0.1% BSA for 30 min at room temperature. Enzyme activity was detected by using a DAB substrate kit (Leica Biosystems). The section was then counterstained with hematoxylin. Staining and analyzed independently in blind fashion by two observers. For the quantification of immune infiltrating cells, a light microscope with a 10 $\times$ eyepiece and a 40 $\times$ objective lens (400 $\times$) was used.

2.10. Statistical analysis

The data of samples were compared using the one-way ANOVA followed by Tukey's post hoc test. A p value less than 0.05 was considered to be statistically significant. All statistical analyses were performed using GraphPad Prism software package version 4.0 (GraphPad Software; San Diego, CA, USA).

3. Results

3.1. GL decreased pro-inflammatory cytokines and chemokine production by TLR-stimulated DC

It has been hypothesized that DCs and macrophages can produce inflammatory cytokines such as TNF-alpha, and IL-12 resulting in regulation of the subsequent adaptive immunity [21–23]. Thus, in the first

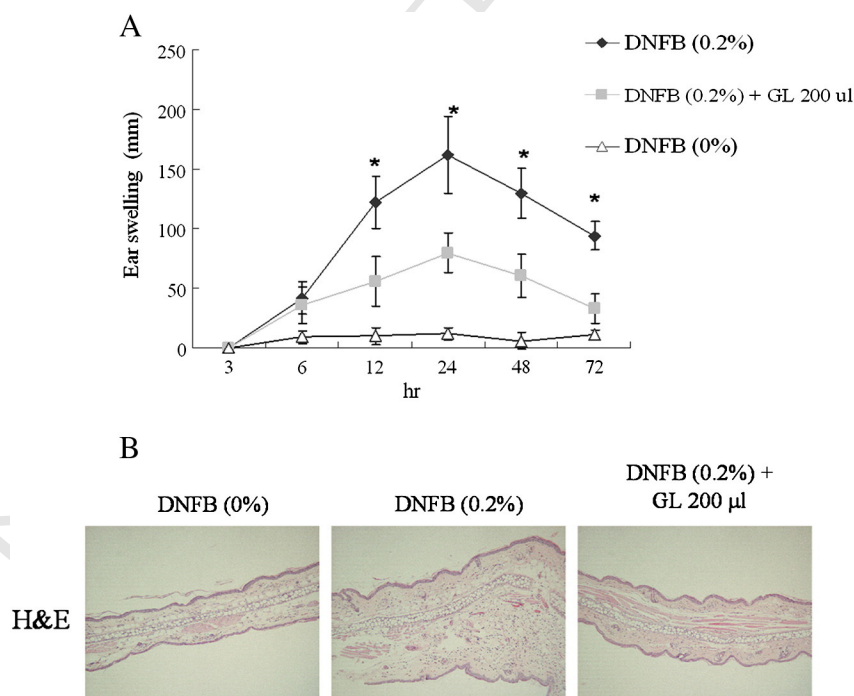


Fig. 6. Oral administration of GL reduced the DNFB-induced contact hypersensitivity (CHS) response in mice. DNFB-induced CHS was described in the Materials and methods section. A) Increases in ear thicknesses were measured using a spring-loaded micrometer during the indicated times. The data is shown as mean $\pm$ SD from four mice and as a representation of two independent experiments yielding similar result, * $p < 0.05$ (ANOVA followed by Tukey post hoc test) versus DNFB (0.5%) sensitized, DNFB (0.2%) challenged mice group. B) Representative histological patterns showing inhibitory effects of GL on DNFB induced ear swelling. Cryostat sections of ears from different treated mice prepared at 24 h after hapten challenge were stained with hematoxylin and eosin (H&E), original magnification $\times 100$. A higher number of leukocytes with infiltrated dermis due to the DNFB (0.2%) application as compared with the DNFB (0.2%) plus GL (200 μ l) group. C) Cryostat sections of the ears were immunostained with anti-mouse CD4 antibody or anti-mouse CD8 antibody, respectively. The number of cells was determined by examining 10 fields under a microscope at $\times 400$ original magnification. The skin from vesicle sensitized, DNFB (0.2%) challenged mice was excised as a control. Arrows indicate examples of positive immunohistochemical staining. D) Ear was excised at 24 h after hapten challenge and RNA was prepared and used to examine mRNA expression of IL-1-beta, IL-6, IFN-gamma, Ccl2, IL-10 and TNF-alpha by qRT-PCR. The data was normalized to HPRT expression in each sample. All data on the graph represent the mean $\pm$ SD from three wells. * $p < 0.05$; (ANOVA followed by Tukey post hoc test) versus untreated, DNFB-sensitized mice group (DNFB 0.2%). All results are shown as a representation of two independent experiments yielding similar results.

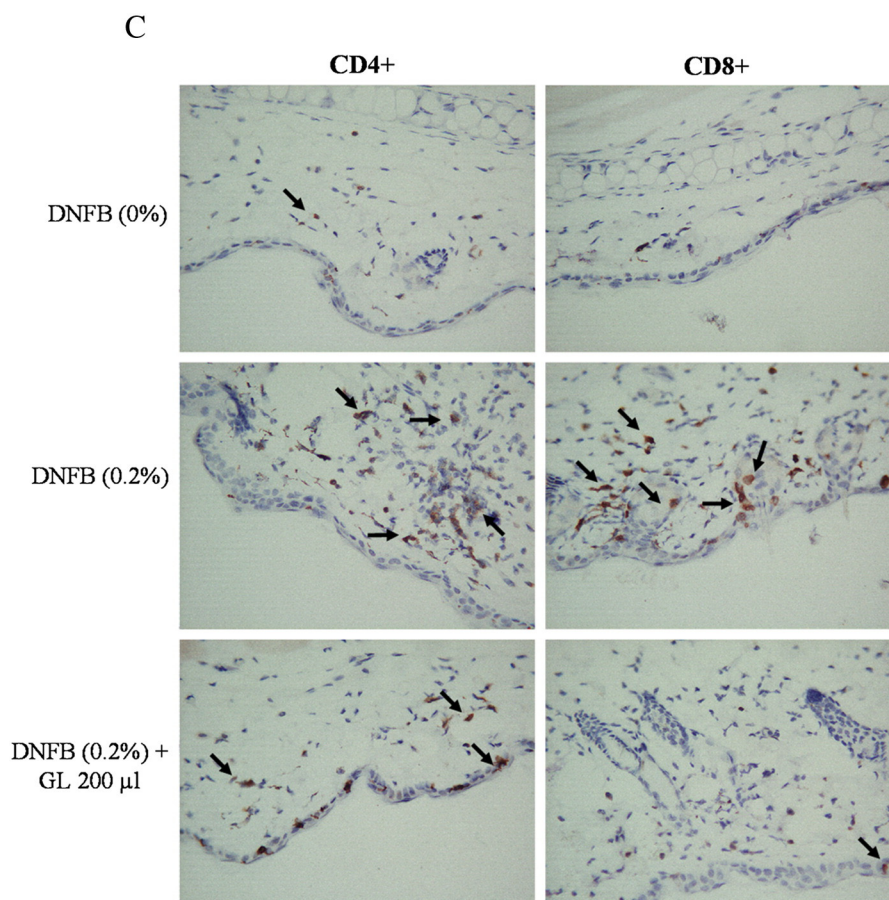


Fig. 6 (continued).

set of experiments, we determined the effect of GL on the production of cytokine/chemokine expressions in LPS-stimulated BMDCs. As shown in Fig. 1A, large amounts of expression of TNF-alpha, IL-6, and IL-12 cytokines and RANTES chemokine, were observed in LPS-stimulated BMDCs. However, upon exposure of LPS-stimulated DCs to GL, these cytokines were down-regulated in a dose-dependent manner. Furthermore, we also stimulated with various TLR ligands, including Poly I:C (TLR-3), PGN (TLR-2), CpG (TLR-9), and Imiquimod (TLR-7), and found that 5% GL significantly reduced TNF-alpha production in all conditions (Fig. 1B).

3.2. GL suppressed LPS-induced surface molecule expression on BMDCs

It has been known that during maturation, DCs up-regulate the expression of co-stimulatory and MHC molecules [21–23]. We therefore determined the influence of GL on the expression of co-stimulatory and MHC molecules. As shown in Fig. 2, following LPS stimulation, DCs highly expressed the costimulatory molecules (CD80, CD86 and CD40) and major histocompatibility complex molecules (MHC class II, MHC class I). However, the GL treatment reduced the expression of these maturation markers, suggesting that GL suppresses the functional and phenotypic maturation of LPS-stimulated DC.

3.3. GL prevented the LPS-induced attenuation of phagocytic capacity in DCs

Immature DCs are highly phagocytic in nature, but the phagocytic capacity is reduced in mature state [23]. We attempted to test whether GL changes the ability of immature DC to capture FITC-conjugated dextran antigen in vitro. As shown in Fig. 3, both untreated DCs and GL-treated group showed high phagocytic capacity. However, LPS-

stimulated DC reduced phagocytic activity. In contrast, the cells pretreated with GL displayed an increased and higher degree of phagocytic capacity in LPS-stimulated DC, again suggesting that GL can enhance the immature state of DCs.

3.4. GL attenuated the OVA specific T-cell proliferation induced by LPS-stimulated DCs

Induction of T lymphocyte proliferation via mature DCs is an important step in the activation of adaptive immune response. We examined whether GL affects the activity of LPS-treated DCs to stimulate the proliferation of antigen specific T-cells [21–23]. OVA_{323–329} peptide or OVA_{257–264} peptide loaded with immature DCs was treated with or without GL respectively, stimulated with LPS, and examined for their stimulating ability to allogeneic OVA-specific CD4⁺ and CD8⁺ T cells from OT-II and OT-I transgenic mice. The T cell proliferation was determined by [³H] thymidine incorporating into new strands of chromosomal DNA in the cell proliferating stage. As shown in Fig. 4, co-culture with LPS-stimulated DCs effectively induced CD4⁺ OT-II and CD8⁺ OT-I T cell proliferation, whereas, GL reduced this stimulatory ability of DCs and inhibited the cell proliferation of the challenged T cells. These results suggested that GL attenuated DC maturation and led to the reduction of their ability to activate Ag-specific T cells.

3.5. GL inhibited MAPK-p38, JNK and NF- κ B p65 activation in LPS-stimulated DC

Activation of the MAPKs and NF- κ B signaling pathways is responsible for the production of cytokines and co-stimulatory molecule expression [28,29]. Therefore, we investigated whether the GL-mediated inhibitory effect of BMDC maturation was due to the difference in

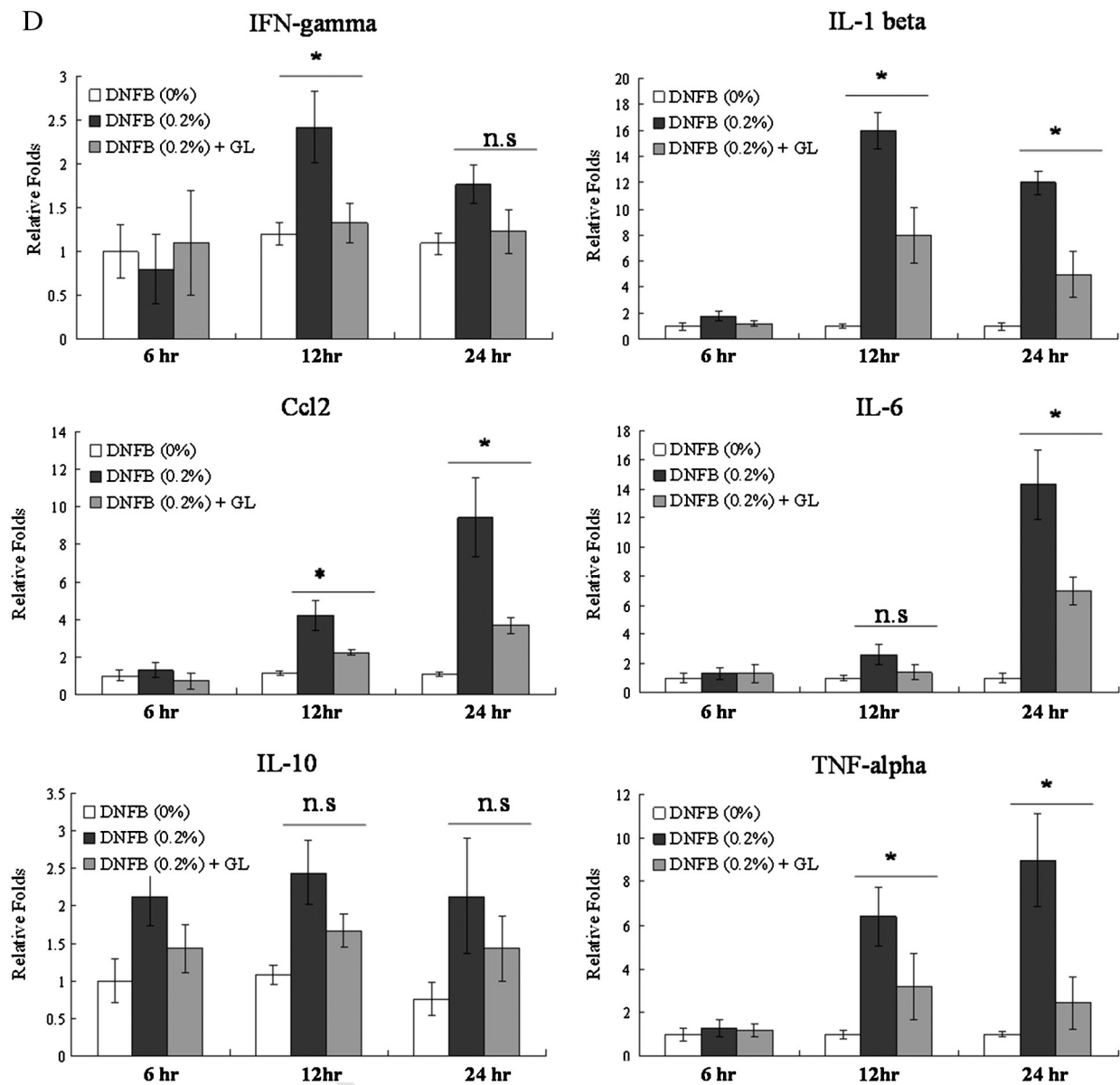


Fig. 6 (continued).

these signaling pathways. Immature DCs were treated with GL before LPS stimulation. As shown in Fig. 5A, LPS increased the phosphorylation of MAPK-ERK, p38 and JNK, which occurred at 30 min in BMDC. However, the pretreatment of immature DCs with 10% GL did not significantly affect phosphorylation of ERK, but did show the inhibition of the phosphorylation of JNK, p38 and MAPK. To further investigate whether NF- κ B activity plays an important role in downstream TLR-4 signaling of GL on DCs, an NF- κ B p65 translocation was performed. As shown in Fig. 5B, GL decreased the NF- κ B p65 subunit nuclear translocation in LPS-treated DCs. These results suggest that GL attenuates the MAPK-JNK, p38 and NF- κ B signaling pathways in LPS-treated DCs.

3.6. GL weakened contact hypersensitivity (CHS) responses

Contact hypersensitivity (CHS) is a typical dendritic cell mediated T cell-dependent cutaneous immune response to reactive haptens [30]. CHS can be induced by epicutaneous immune sensitization and becomes subsequently challenged with haptens such as DNFB. We used this model to examine the in vivo effect of GL on DNFB hapten-loaded DC-mediated CHS immune responses. Our result showed that

oral administration of 200 μ l GL showed significantly less DNFB-induced ear swelling (Fig. 6A, B), less CD4⁺ and CD8⁺ T cell infiltrating (Fig. 6C, Table 1), and less expression levels of inflammatory markers including IL-1 beta, IL-6, TNF-alpha, IFN-gamma, Ccl2 (a monocyte-attracting chemokine) and IFN-gamma cytokine production in the ear (Fig. 6D) than that of DNFB (0.2%) challenged alone group, suggesting that GL can attenuate the DC-mediated sensitization in CHS reaction.

4. Discussions

In this study, we demonstrated that GL is a potent inhibitor of DC maturation based on the finding that GL inhibits the following: (i) the production of cytokines and chemokines, (ii) the expression of costimulatory and MHC molecules, (iii) the phagocytic capacity of DCs, (iv) the ability of DC to stimulate antigen specific T cells proliferation, and (v) the CHS responses under in vivo conditions. This data provides us scientific evidence and rationale to understand the immunopharmacological aspects of GL application against harmful immune responses such as chronic inflammation, autoimmunity, and transplantation.

Table 1

Infiltrated immune cells at ear sites.

Group	CD4+ T cells (mean $\pm$ SD)	CD8+ T cells (mean $\pm$ SD)
Control	10 $\pm$ 4	6 $\pm$ 3
DNFB 0.2%	76 $\pm$ 14**	66 $\pm$ 12
DNFB 0.2% + GL 200 μ l	36 $\pm$ 8***	23 $\pm$ 7***

Cell count was performed at magnification $\times 400$, and three samples and three randomly chosen fields/samples were evaluated. Results are expressed as mean $\pm$ standard deviation (SD) of immunohistochemically positive cells in the cryosection.

** $p < 0.01$.*** $p < 0.001$.

In this study, we observed that GL can inhibit the capacity of LPS-stimulated DCs to induce allogeneic CD4⁺ and CD8⁺ T cell proliferation

in vitro (Fig. 4). Although, the underlying mechanisms still remain elusive, we suggest that the inhibitory effect may result in following findings: Firstly, it reduced the LPS induced costimulatory (CD40, CD80, and CD86) expression (Fig. 2). Previous studies have showed that the interaction of CD40 on DC with CD40L on T cells induces interleukin-12 (IL-12) production by the DC, which leads to promote the T helper type 1 (Th1) differentiation [31]. Similarly, ligation of CD80 and CD86 on DC and CD28 on T cells is known to induce the secretion of cytokines by DCs and proliferation and differentiation T cells [32]. Secondly, GL suppressed the expression of MHC molecules (MHC class I/II) on the surface of the DCs, which are essential for the presentation of antigen for CD8⁺ and CD4⁺ T cells. Thirdly, it reduced the cytokine reduction in LPS-stimulated DCs. Remarkably, the decreased production of IL-6 and IL-12 by GL could potentially weaken the ability of DC to stimulate

Table 2

Flavonoid and PMF contents in citrus peel extract (GL).

No.	PMFs and flavonoids	Conc. (μ g/ml)	Compound structure
1	Nobiletin	50.8	
2	Sinisetin	21.3	
3	Tangeretin	10.6	
4	3,5,6,7,8,3',4'-Hepta-methoxyflavone	19.2	
5	3,5,6,7,3',4'-Hexamethoxy-flavone	3.1	
6	5,6,7,4'-Tetramethoxy-flavone	1.1	
Total PMFs in GL		106.1	
7	Naringin	253.6	
8	Hesperidin	104.7	
Two measured flavonoids in GL		358.3	
Total measured flavonoids in GL		464.4	

Q27 T cell proliferation and differentiation. IL-6 has such anti-apoptotic properties as it prolongs CD4 T cell survival in vitro most likely by retaining Bcl-2 expression in the isolated T cells [33]. In addition, a study conducted by Lotz et al. indicated that IL-6 was described as a costimulatory molecule for T cell activation enhancing proliferation independently of IL-2 gene expression [34]. Furthermore, Yoo's study indicated that IL-12 provides proliferation and survival signals to CD4⁺ T cells through the phosphatidylinositol 3-kinase/Akt signaling pathway [35]. There have also been several studies that have reported that IL-12 can suppress the apoptotic cell death of activated T cells [36,37].

Numerous evidences from preclinical and clinical studies have shown that NF- κ B and MAPK, including Erk, p38 and JNK, play central roles in the inflammatory response and DC maturation [38,39]. In addition, the activation of macrophages or DCs by LPS has to be preceded by activating the NF- κ B and MAPK pathways [40,41]. Our mechanistic studies using western blotting demonstrated that GL can inhibit the nuclear translocation of NF- κ B into the nucleus (Fig. 5b), supporting the findings that GL suppressed the I κ B-alpha and translocation of NF- κ B in RAW 264.7 cells stimulated by LPS [19]. In addition, we also observed that GL decreased JNK, and p38 but did not change ERK activation in LPS-treated BMDCs suggesting that niclosamide inhibits TLR-related signaling. This could include the NF- κ B and MAPK–JNK, p38 signaling

Q28 pathways (Fig. 5A). However, whether or not other pathways are involved in the inhibitory effect of GL remains unclear. Therefore, to further elucidate the other signaling pathways responsible for DC maturation such as PI3K/AKT, TBK-1/IRF-3, or STAT6 mediated signaling pathways are needed.

DCs coordinate T-cell response, and the type of cytokines release from activated dendritic cells, is believed to play a crucial role in manipulating the differentiation of T helper (TH) cells into TH1-, TH2- or TH17-cell subsets [42]. Especially, IL-12 is commonly known to induce Th1 cell differentiation and suppresses Th2 differentiation [43]. Our present study showed for the first time that GL can significantly impair the ability of LPS stimulated IL-12 production in BMDCs (Fig. 1). In addition, hapten induced contact hypersensitivity (CHS) in the animal model was mainly as Th1-type reaction [44]. Therefore, our results also found that oral GL reduces systemic Th1 immune responses and attenuate CHS response. The in vitro and in vivo data provided further evidence that GL may be effective in several Th1 dominant chronic inflammatory diseases such as allergic contact dermatitis, multiple sclerosis (MS), diabetes, and rheumatoid arthritis (RA) [44]. However, we also cannot exclude that GL may modulate the ability of DC to polarize other different types of T lymphocyte responses, such as TH2 or TH17. Thus, it will be important to further explore the effect of GL in allergen or fungus induced DC-mediated Th2 or TH17 responses in vitro and in vivo.

On the functional level, DC exposed to pathogen mostly via TLR ligands, can produce an array of pro-inflammatory cytokines which leads to an environment allowing for DC activation [45]. In this study, we also examine whether GL could modulate the cytokine release from DCs treated with different TLR ligands. The presence of each substance promotes TNF-alpha secretion, as compared with untreated DC. Interestingly, these secretions were completely inhibited by 5% GL (Fig. 1B). Although the mechanism of GL suppresses the TNF-alpha production of DCs after different TLR ligand stimulation is not yet fully understood, we suggest that it may be related to the inhibitory effect of GL on TLR-linked MAPK–JNK, p38 or NF- κ B signaling pathway. Further investigation of the effect of GL on DC activation through other non-TLR pathways such as cytosolic DNA- or RNA-mediated STING-dependent innate signaling should be also justified [46].

Previous study reported that chemical analysis on GL revealed its richness of flavonoids and PMF including nobiletin, sinasetin, 3,5,6,7,8,3',4'-Heptamethoxyflavone, Tangeretin, 3,5,6,7,3',4'-Hexamethoxy-flavone, 5,6,7,4'-Tetramethoxy-flavone, Naringin and Hesperidin (Table 2). In the present study, we further examined the effects of these compounds

on the activation of DCs. We initially tested whether these compounds could affect the production of TNF-alpha in LPS-stimulated DCs, which is a hallmark of DC activation. The concentration of each compound was equivalent to their concentrations contained in 10% GL. Our results show that nobiletin, sinasetin, Tangeretin, 3,5,6,7,8,3',4'-Heptamethoxyflavone and Naringin can significantly down-regulate TNF-alpha production in LPS stimulated DCs (Supplemental Fig. 1). Among them, sinasetin and Naringin had better inhibitory effects on DC activation compared to other compounds. However, the inhibitory effect of 10% GL on DC activation was significantly more effective than either sinasetin or Naringin (Supplemental Fig. 1). These results suggested that the inhibitory effect of GL on DC activation could be caused by the synergistic effect of flavonoids or from other unidentified components in the extracts.

In summary, our study suggests that GL significantly influences the important pro-inflammatory signaling cascades and impairs the phenotype and function of mouse DCs, making it as an attractive therapeutic agent in chronic inflammatory and autoimmune disease. Furthermore, we also consider it necessary and pivotal to have future investigations on additional issues associated with GL property in biological pathways. In particular, the exact cellular effects and the detailed molecular mechanism that are critical for the ability of GL to suppress DC function are not yet well understood. In addition, since Th1 cells are either functionally immunogenic or provide protection against invading pathogens and killing cancer, the need for a close monitoring of immune function in persons who are at high risk for bacterial or virus infection is extremely important.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.intimp.2014.02.005>.

Conflict of interests

The authors declare that they have no conflicts of interest.

Acknowledgments

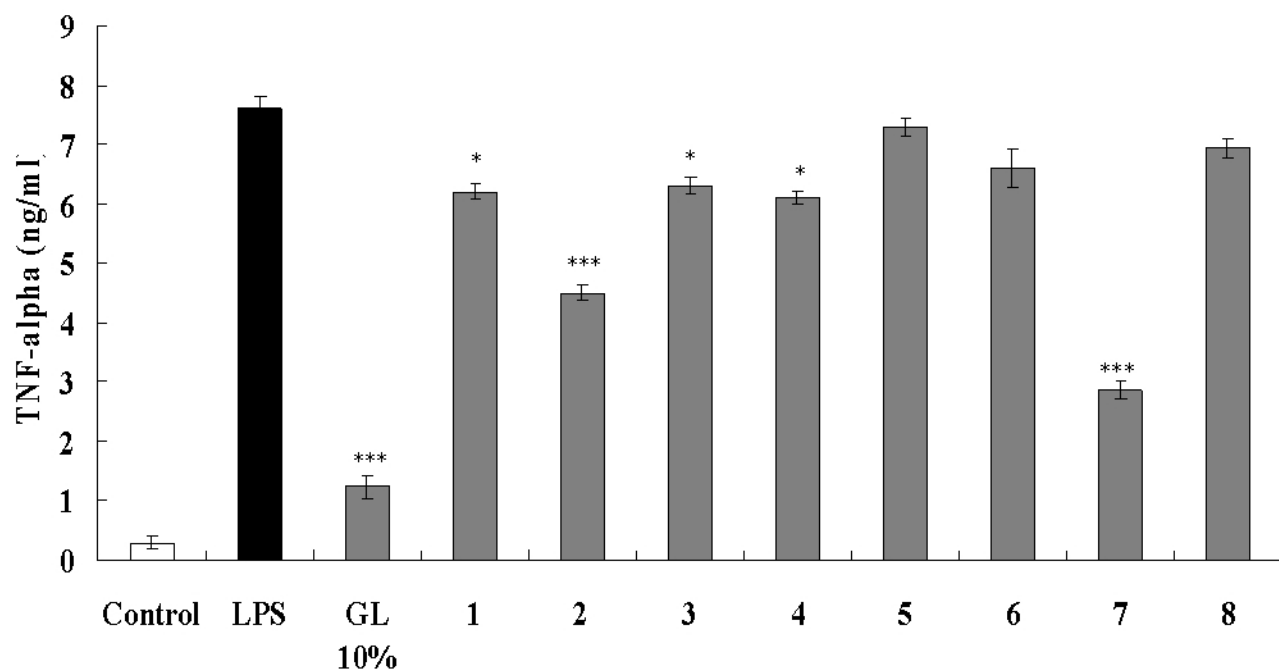
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Supplemental figure 1



1. Nobiletin
2. Sinesetin
3. Tangeretin
4. 3,5,6,7,8,3',4'-Hepta-methoxyflavone
5. 3,5,6,7,3',4'-Hexamethoxy-flavone
6. 5,6,7,4'-Tetramethoxy-flavone
7. Naringin
8. Hesperidin