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A novel chrysin derivative produced by gamma irradiation attenuates 2,4-dinitrochlorobenzene-induced atopic dermatitis-like skin lesions in Balb/c mice

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ABSTRACT

Gamma irradiation is a useful technology to change the physical and biological properties of natural molecules. In this study, we investigated whether gamma irradiation improve properties of chrysin as an anti-inflammatory candidates. Chrysin was converted into two compounds (CM1 and CM2) by gamma irradiation. We determined the therapeutic potential of these compounds in bone marrow-derived macrophages and 2,4-dinitrochlorobenzene (DNCB)-induced atopic dermatitis (AD)-like skin lesions in Balb/c mice. The structural changes to chrysin led to the reduction of cytotoxicity without loss of anti-inflammatory properties in BMDMs. Purified CM2 inhibited lipopolysaccharide (LPS)-induced overexpression of nitric oxide, tumor necrosis factor-α, interleukin (IL)-6, and surface molecules without cytotoxicity in BMDMs, while CM1 revealed strong cytotoxicity. Furthermore, treatment with CM2 significantly alleviated AD-like skin symptoms and clinical signs in DNCB-induced AD mice model. The suppression of AD mediated by CM2 treatment was accompanied by decrease inflammatory T cell cytokines (IFN-γ, IL-5, IL-4, and IL-17). The chemical structure of CM2 and structural transformation mechanism were determined by nuclear magnetic resonance and mass spectrometry. Our study findings provide evidence that CM2 produced by gamma irradiation of chrysin can be an attractive therapeutic agent for AD.

1. Introduction

Atopic dermatitis (AD) is a common skin inflammatory disease resulting from abnormal immune response (Biedermann et al., 2015). The hallmark of AD is elevated level of immunoglobulin E (IgE) in blood caused by release of inflammatory cytokines (Lee and Cho, 2011). Patients with AD suffer from skin barrier dysfunctions, including frequent itching, water loss in the epidermis, erythema, and excoriation (Farmer and Marathe, 2017). It is known that some environmental factors (e.g., air pollutants, smoking, skin exposure, and mite dusts) and genetic background (e.g., loss of function of skin barrier function-related genes and immune system genes) cause AD (Kantor and Silverberg, 2017; Weidinger et al., 2007). Cyclosporine is the first chosen immunosuppressive drug for therapy of AD (Megna et al., 2017). Cyclosporine effectively alleviates AD symptoms through reduction of T-helper (Th)2-, Th22-, and some Th17-related molecules (Khattri et al., 2014). Similarly, oral corticosteroids (e.g., prednisolone, dexamethasone, and fludrocortisone) are widely used for short-term therapy of AD (Simon and Bieber, 2014). These corticosteroids inhibit inflammatory actions, including cyclooxygenase induction, nitric oxide (NO) induction, cytokine production, and activation of mast cells, which effectively ameliorate AD symptoms (Ahluwalia, 1998). However, long-term use of these synthetic agents can lead to serious side effects, such as nephrotoxicity, hypertension, osteoporosis, and diabetes (Aulakh and Singh, 2008; Hwang and Weiss, 2014; Otani et al., 2015). Therefore, increasing attention has been focused on scientific approaches to develop alternative agents from nature-derived compounds for the treatment of AD (Jesenak et al., 2016; Kang et al., 2017; Lee et al., 2014).

Phenolic compounds, a large class of plant secondary metabolites,

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have attracted increasing attention in the field of nutrition and medicine because they possess powerful anti-inflammatory property (Kinger et al., 2018). Various researchers have demonstrated that phenolic compounds, such as resveratrol, quercetin, and chlorogenic acid, effectively alleviated AD skin lesions (Karuppagounder et al., 2016; Sozmen et al., 2016; Tsang et al., 2016). Among them, chrysin, an abundant polyphenolic compound in propolis, is known as an effective molecule that inhibits inflammatory response (Ahad et al., 2014; Rauf et al., 2015). Although many previous studies revealed that polyphenolic compound is beneficial to human's health, some reports suggested unexpected effects, e.g., skin irritant, pro-oxidant, and cancerogenic activity, caused by cytotoxicity of polyphenolic compounds (Korkina et al., 2008; Kyselova, 2011). In particular, they can cause mutagenic and genotoxic effect, which lead to serious injury to organs (Ekeanyanwu and Njoku, 2014; Skibola and Smith, 2000). Furthermore, previous report revealed that some polyphenolic compound, including chrysin, can exhibit developmental toxicity (Bugel et al., 2016). Thus, constant research for reducing toxicity of polyphenols is required.

Gamma irradiation is a useful technology for enhancing the safety and storage quality of foods and drugs (Banerjee et al., 2016; Luo et al., 2018). In recent years, gamma irradiation has drawn wide attention owing to its potential to reduce the toxicity of phenolic compounds and to enhance their physiological activities (Alsager et al., 2018). It has been reported that gamma irradiation induced structural modification of genistein, which reduced cell death without reduction in anti-inflammatory effects (Byun et al., 2014). Similarly, gamma irradiation generated new products of rosmarinic acid, which exhibited strong anti-adipogenic activity (Jeong et al., 2018). In our previous study, gamma-irradiated chrysin showed enhanced anti-inflammatory potential than intact-chrysin via conversion of chrysin into new radiolytic molecules (Byun et al., 2016). However, in-depth understanding of the anti-inflammatory potential of radiolytic molecules in animal models and their exact chemical structure will be required for their application in the treatment of inflammatory diseases. Therefore, in the current study, we aimed to determine the chemical structure of radiolytic derivatives of chrysin and to evaluate their therapeutic effect in 2,4-dinitrochlorobenzene (DNCB)-induced AD mice model.

2. Materials and methods

2.1. Materials

Chrysin, 2, 4-dinitrochlorobenzene (DNCB), and dexamethasone (Dexa) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 was purchased from InvivoGen (San Diego, CA, USA). Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (mAbs) to CD80 and CD86 were purchased from BD biosciences (San Diego, CA, USA). Phycoerythrin (PE)-conjugated mAbs to MHC class II; and PE-Cy7-conjugated F4/80 were purchased from eBioscience (San Diego, CA, USA).

2.2. Sample preparation

Chrysin methanol solution (1 mg/mL) was irradiated at 100 kGy in a cobalt-60 irradiator (point source AECL, IR-79; MDS Nordion International Co., Ltd, Ottawa, Ontario, Canada) equipped with a 11.1 PBq source strength in the Advanced Radiation Technology Institute (ARTI), a branch of the Korea Atomic Energy Institute (KAERI, Jeongeup, Republic of Korea). Dosimetry was calibrated using 5-mm diameter alanine dosimeters (Bruker Instruments, Rheinstetten, Germany). Methanol was removed using a rotary evaporator (Tokyo Rikakikai Co., Ltd., Japan). Then, both intact- and gamma-irradiated chrysin were freeze dried.

2.3. Structural analysis of derivatives of chrysin

Agilent HPLC system 1260 (Agilent Technologies, Inc., Santa Clara, CA, USA) with a Diode Array Detector (DAD) was used for the chromatographic separation of gamma-irradiated chrysin. The injection volume was 20 μL. The brief conditions of the HPLC system are as follows: reverse phase: Agilent Eclipse XDB–C18 column (5 μM pore size and length I.D., 4.6 mm × 250 mm); mobile phase: A: 0.1% formic acid in water; B: methanol. The separation was performed using the following gradient program: 0–3 min (20% B), 10 min (50%), 20 min (50%), 35 min (80%), 45 min (80%). The flow rate was set at 1 mL/min, and the DAD detector was set at 280 nm. The isolation of CM1 and CM2 was performed using a preparative HPLC 1260 infinity system (Agilent Technologies). The gradient program was 0–5 min (20% B), 12 min (40%), 25 min (55%), 30 min (80%) 38 min (80%). The chemical structure of isolated CM1 and CM2 was characterized using LC-MS, ^{1}H NMR, and ^{13}C-NMR.

2.4. Generation and culture of macrophages

Murine bone marrow-derived macrophages (BMDMs) were separated from female C57BL/6 mice according to an established protocol (Zhang et al., 2008). Briefly, bone marrow cells were plated in petri dishes and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco BRL), 100 units/mL of penicillin/streptomycin (Gibco BRL), and 20 ng/mL recombinant mouse macrophage colony-stimulating factor (R&D systems, Minneapolis, MN, USA) at 37 °C under 5% CO_2. The cells were harvested at 6 days, and the population of macrophages was assessed by the expression of F4/80 surface molecule by FACS analysis. The BMDMs were washed and cultured for 24 h in DMEM containing the different reagents as indicated (i. e., LPS, chrysin, CM1, and CM2).

2.5. Cell viability assay

Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, BMDMs were seeded in a 24-well plate (2×10^5 cells/well) and incubated with DMEM containing the indicated reagents for 24 h. After incubation, the medium in the plate was removed, and the cells were treated with MTT solution (0.5 mg/mL in DMEM) for 2 h. The insoluble formazan crystals were dissolved in dimethyl sulfoxide (DMSO). The absorbance was measured at 570 nm using a SpectraMax M3 multi-detect microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.6. Measurement of nitric oxide and cytokine production

Cell culture supernatants were collected and stored at −80 °C until use. Nitrite accumulation in culture supernatants was determined by Griess method (Benevides Bahiense et al., 2017). The culture supernatant (100 μL) was mixed with 100 μL of Griess reagent (1% sulfanilamide in 5% H_3PO_4 and 0.1% *N*-(1-naphthyl)ethylenediamine in distilled water) and incubated for 10 min. The absorbance was measured at 540 nm using $NaNO_2$ as a standard. The level of cytokines was determined by ELISA using commercial reagent kits (BD Biosciences) according to the manufacturer's instructions.

2.7. Measurement of cell surface molecules by flow cytometry

To analyze the surface molecules, the cells were harvested and pre-incubated with 0.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min. Then, the cells were washed and stained with anti-CD80, anti-CD86, anti-MHC-II, and anti-F4/80 for 15 min at room temperature (RT). The resulting fluorescence was measured on a flow cytometer and analyzed using FlowJo software.

2.8. Animals

Six-week-old female BALB/c mice were purchased from Orient Bio Inc., Seoul, Korea. All animals were kept under specific pathogen-free environment and acclimatized to controlled conditions of temperature (23 ± 2 °C), humidity (55 ± 5%), and light (12 h light/dark cycle) at the Central Animal Research Laboratory at ARTI, KAERI. The mice were allowed free access to food and water. All procedures performed on mice were approved by Korean Institutional Animal Ethical Committee (Approval No. KAERI-IACUC-2017-032).

2.9. Induction of AD-like skin lesions in BALB/c mice and topical application of CM2

The schematic experimental procedure is described in Fig. 3. The mice were divided into seven groups (n = 6 per group) as follows: (1) Normal group; (2) DNCB + vehicle (control); (3) DNCB + 0.005% Dexa-emulsion (positive control); (4) DNCB + 0.05% CM1 emulsion; (5) DNCB + 0.2% CM1 emulsion; (6) DNCB + 0.05% CM2 emulsion; and (7) DNCB + 0.2% CM2 emulsion. DNCB was used to induce AD-like skin lesions according to a previously described method (Kang et al., 2018). Briefly, the hair at the back of the mice was removed using an electric shaver. On the following day, the mice were sensitized with 200 μL of 1% DNCB (w/v) in acetone/olive oil (3:1) two times in the first week on the dorsal skin and each ear. The Dexa-, CM1 or CM2 emulsion did not treat during first week. During second to fourth week, the mice were sensitized with 0.4% DNCB (w/v) on the dorsal skin and ears repeatedly two times in a week. After 4 h of sensitization, 200 μL of each emulsion was topically applied on the back skin and ears. Base emulsion (vehicle) was manufactured by a previously described method (Trang and Son, 2017) with slight modifications. Vehicle comprised distilled water, olive oil, cetyl alcohol, and polyglyceryl-3-methylglucose distearate. Dexa, CM1 or CM2 solution was mixed with vehicle using a homogenizer.

2.10. Evaluation of ear thickness, skin severity score, and lymph node weight

Ear thickness was measured using a micrometer (Mitutoyo Corp., Kawasaki, Japan) on the right ear of each mouse. The severity of dermatitis on the dorsal skin and ear lesions was evaluated as previously described (Yoon et al., 2015). Dermatitis score was defined as the sum of individual scores (0, no symptom; 1, mild; 2, moderate; 3, severe) for the following five signs and symptoms: (1) erythema/hemorrhage, (2) dryness, (3) edema, (4) excoriation/erosion, and (5) lichenification. The lymph nodes were collected from sacrificed mice, and the weights of lymph node were measured using an electronic balance.

2.11. Measurement of serum IgE

Blood samples were collected from the abdominal vein using syringe under anesthesia, and transported to blood tube (MiniCollect 0.8 mL Z Sep, Greiner Bio-One, Frickenhausen, Germany). Serum was separated by centrifugation at 12000 rpm, 15 min, and stored at −80 °C until use. The serum IgE was measured using mouse IgE ELISA kits (BD biosciences), according to the manufacturer's instruction.

2.12. Measurement of Th1, Th2, and Th17 cytokine production

After the experiment, the mice were sacrificed and $CD3^+$ T cells were isolated from the spleens by magnetic-activated cell sorting (MACS) separation (Miltenyi Biotec, Bergisch Gladbach, Germany). The isolated cells were incubated on 48-well plates that were pre-coated with anti-mouse CD3/CD28 antibody (BD biosciences) and incubated for 48 h at 37 °C. The levels of IFN-γ, IL-5, and IL-17 in the supernatant were measured using ELISA kits (eBioscience), according to the manufacturer's instructions. To determine cell viability of splenocyte, lactate dehydrogenase (LDH) was measured using automated biochemical analyzer (Dri-Chem 7000, FUJIFILM, Tokyo, Japan).

2.13. Histopathological studies

A piece of dorsal skin and ear tissue was fixed in 10% formalin for histopathological analysis. The fixed tissues were washed and embedded in paraffin. Sections (5 μm thick) were cut using a microtome (Leica Microsystems, Wetzlar, Germany). The sections were de-waxed and stained with hematoxylin and eosin (H&E) to evaluate epidermal hyperplasia and with toluidine blue to evaluate infiltration of mast cells, which was observed under a microscope and photographed.

2.14. Statistical analysis

All experiments were repeated at least three times. Significant differences were analyzed with one-way ANOVA followed by Tukey's multiple comparison test using the GraphPad Prism program (San Diego, CA, USA). The data in our graphs are expressed as the means. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ were considered statistically significant.

3. Results

3.1. Effects of gamma-irradiated chrysin on cytotoxicity and cytokine production in LPS-stimulated BMDMs

To confirm the structural modification of chrysin induced by gamma irradiation, we first analyzed chromatogram using HPLC. We observed that two major radiolytic derivatives (CM1 and CM2) were generated from chrysin solution (Fig. 1A). To evaluate the changes of gamma-irradiated chrysin on cytotoxicity and anti-inflammatory effect, BMDMs were treated with various doses (ranging from 10 to 80 μM) of both gamma-irradiated chrysin and intact-chrysin. Gamma-irradiated chrysin showed lower cytotoxicity than intact-chrysin (Fig. 1B). Although structural changes reduced cytotoxicity, gamma-irradiated chrysin effectively inhibited the overproduction of mediators, including TNF-α, IL-6, and NO, in LPS-stimulated BMDMs (Fig. 1C and D). We hypothesize that the anti-inflammatory effect of gamma-irradiated chrysin is responsible for the production of new radiolytic derivatives (CM1 and CM2). Thus, we next investigated the anti-inflammatory effect of main derivatives of chrysin (CM1 and CM2).

3.2. Effects of chrysin derivatives on cell viability and pro-inflammatory mediators in LPS-stimulated BMDMs

To evaluate the anti-inflammatory effect of chrysin derivatives (CM1 and CM2), we isolated each compound using preparative-HPLC. To determine the appropriate concentration for subsequent experiments, cell viability was tested by MTT assay. Treatment with CM2 did not exert cytotoxicity at doses ranging from 8.7 to 70 μM, while CM1 showed high cytotoxicity even at low concentration treatment (Fig. 2A). Due to the high cytotoxicity of CM1, the anti-inflammatory effect of CM1 was not determined. We next examined the inhibitory effects of CM2 on LPS-induced inflammation in BMDMs. Overproduction of NO and pro-inflammatory cytokines (TNF-α and IL-6) was observed in LPS-treated group. However, LPS-induced overproduction of NO and IL-6 was significantly inhibited by CM2 treatment (Fig. 2B). We subsequently examined the inhibitory effect of CM2 on co-stimulatory molecules and MHC-II. High expression of surface molecules (CD80, CD86, and MHC-II) was observed in LPS-treated group. Notably, CM2 strongly inhibited the increased expression of CD80 and MHC-II in LPS-treated BMDMs, while it did not affect the expression of CD86 (Fig. 2C). Taken together, these results indicate that CM2 has potential in regulating LPS-induced overexpression of inflammatory

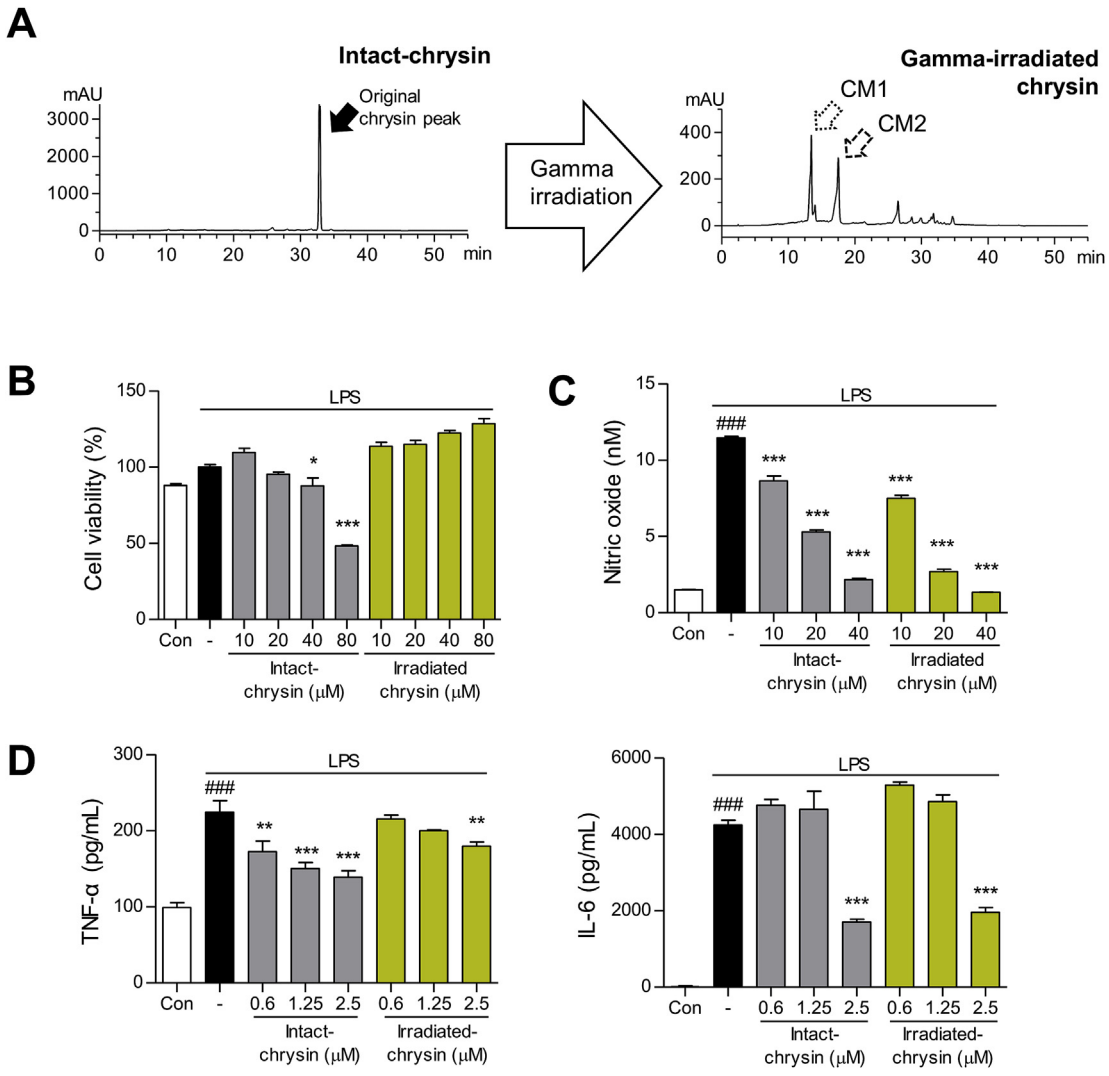

Fig. 1. Effects of gamma irradiation on cytotoxicity and anti-inflammatory properties of chrysin in LPS-stimulated bone marrow-derived macrophages. (A) Structural transformation was determined by HPLC analysis. (B) Cell viability was measured by MTT assay (C) Level of nitric oxide in culture medium measured by Griess assay. (D) Level of cytokine in culture medium measured by ELISA. The data represent the mean ± SEM (n = 3). $^{###}p < 0.01$ represents significant difference compared to control group. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ represent significant differences compared to only LPS-treated group.

mediators in macrophages.

3.3. Effects of CM2 treatment on DNCB-induced AD-like symptoms in BALB/c mice

The DNCB-induced AD mice model was used to investigate the therapeutic effect of CM2 on AD. DNCB sensitization and topical application of CM1 or CM2 cream were performed for 30 days according to the schedule summarized in Fig. 3. Repeated sensitization of DNCB to the back skin and ears induced the development of AD-like lesions. To investigate the therapeutic effects of CM1 or CM2 emulsion on DNCB-induced AD-like symptoms in BALB/c mice, we assessed ear thickness, severity of skin lesions, and serum IgE level. For dermatitis score, all the Dexa-treated (15 days, 22 days, 29 days; $p < 0.05$), 0.05% CM2-treated (15 days, 22 days, 29 days; $p < 0.05$) and 0.2% CM2-treated (15 days, 22 days, 29 days; $p < 0.05$) groups showed lower dermatitis score compared with the control group (Fig. 4A and B). Furthermore, Dexa-treated (15 days, 22 days, 29 days; $p < 0.05$), 0.05% CM2-treated (15 days, 22 days; $p < 0.01$), and 0.2% CM2-treated (15 days, 22 days, 29 days; $p < 0.01$) groups reduced ear thickness compared with the control group. Both Dexa- and 0.2% CM2-treated groups reduced serum IgE level compared with the control group. CM1-treated group did not alleviate DNCB-induced AD-like skin lesions. These results indicate that CM2 can reduce AD-like symptoms in mice.

3.4. Effects of CM2 treatment on histological changes in DNCB-treated mice

A previous report revealed that infiltration of mast cells into the dermis and dermal thickening are commonly observed in AD (Yoshihisa et al., 2016). To examine the effect of CM2 treatment on histological change, the tissue sections were stained with H&E staining and toluidine blue staining. As shown in Fig. 5A and B, repeated DNCB treatment induced hypertrophy and edema in the epidermis, while application of CM2 emulsion effectively reduced epidermal thickness in the ear and back skin. DNCB treatment resulted in infiltration of mast cells into the dermis, but treatment of CM2 emulsion significantly inhibited infiltration of mast cells into the dermis (Fig. 5C and D). However, treatment with CM2 significantly reduced DNCB-induced inflammatory symptoms in the ear and back skin. Histological analysis revealed that CM2 effectively inhibited DNCB-induced AD-like skin thickening and infiltration of mast cells.

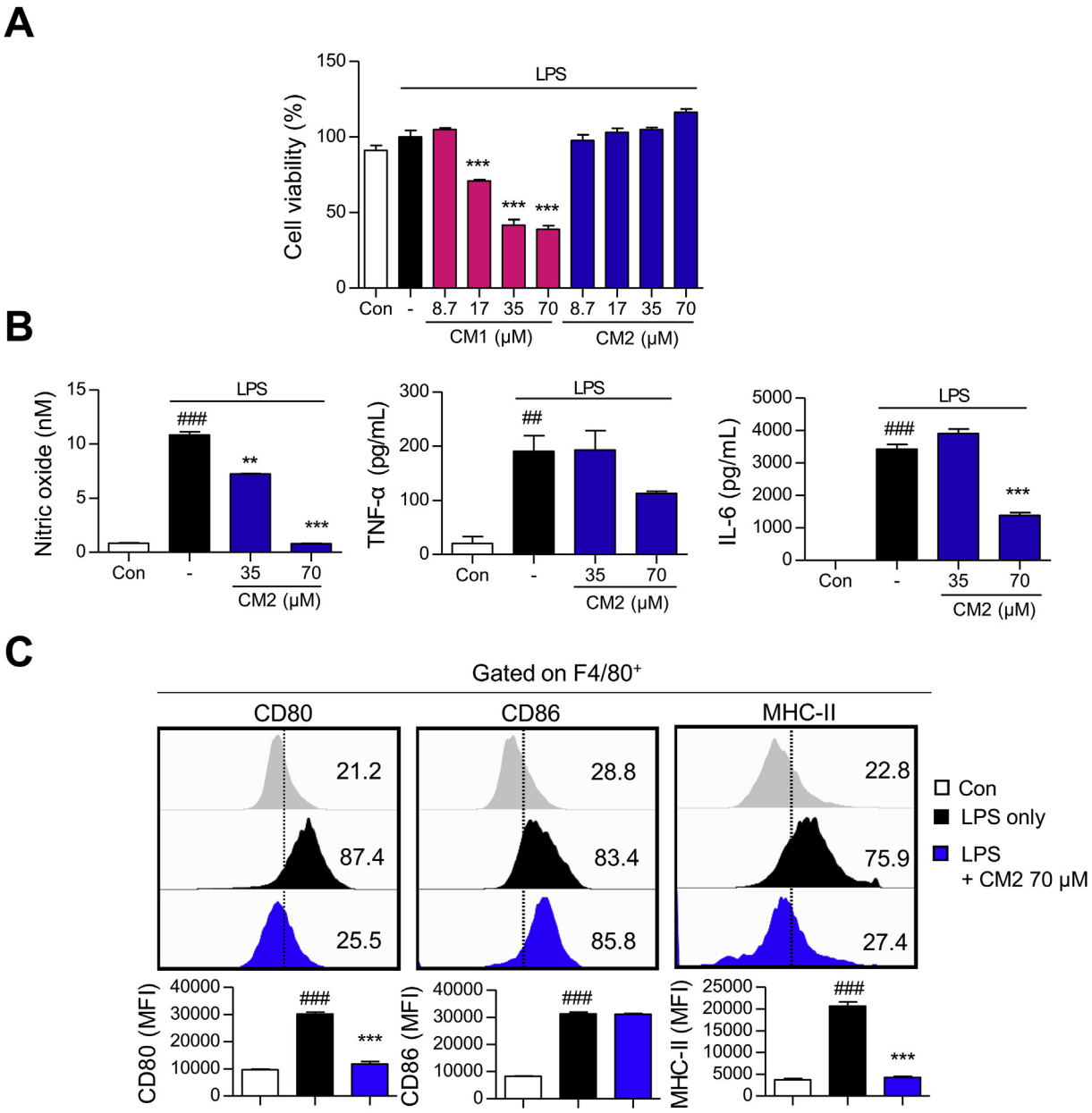

Fig. 2. Effects of purified chrysin derivatives on cell viability and inflammatory mediators in LPS-stimulated bone marrow-derived macrophages. (A) Cell viability was measured by MTT assay. (B) Levels of nitric oxide, TNF-α, and IL-6 in culture medium were measured by ELISA. (C) Surface molecules are analyzed by flow cytometry. The cells were gated on F4/80$^+$ cells. Bar graphs show the mean ± SEM of median fluorescence intensity (MFI). $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ represent significant differences compared to control group. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ represent significant differences compared to only LPS-treated group.

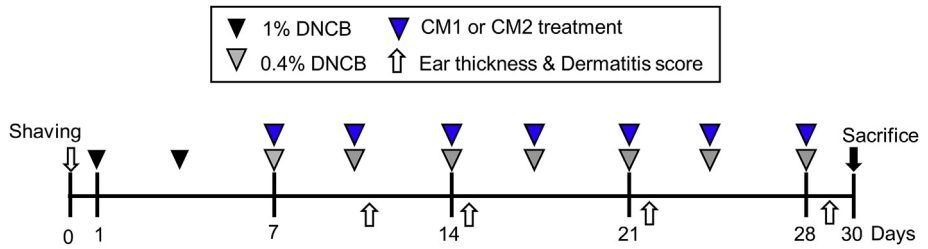

Fig. 3. Experimental schedules for developing 2,4-dinitrochlorobenzene (DNCB)-induced atopic dermatitis (AD)-like lesions and treatment with CM1 or CM2 in Balb/c mice.

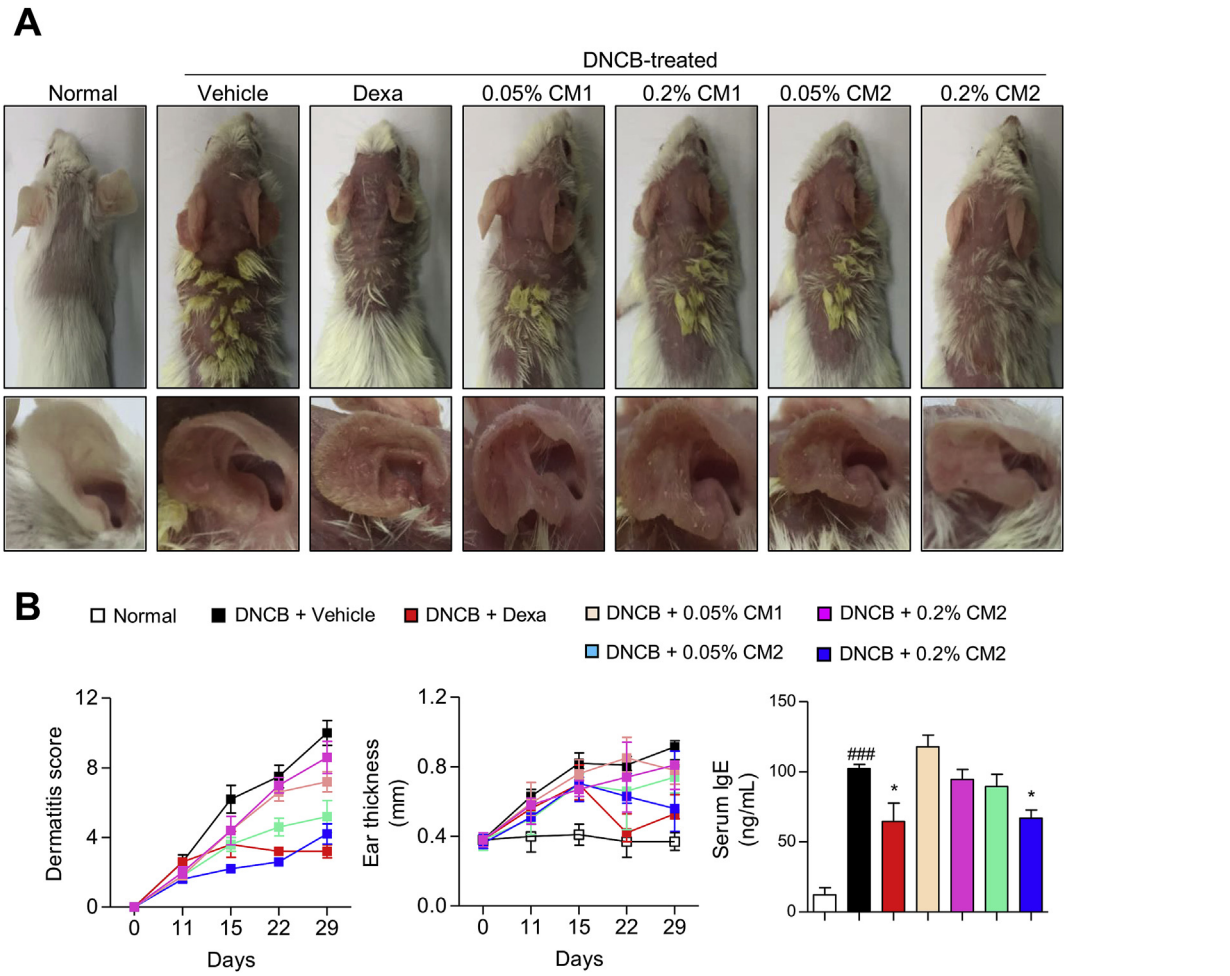

Fig. 4. Topical application of CM2 alleviated 2,4-dinitrochlorobenzene (DNCB)-induced AD-like symptoms. (A) Representative photograph images of back skin (top images) and ear (bottom images) in AD-like skin lesion-induced mice. (B) Changes in dermatitis score, ear thickness, and serum IgE level during topical application of CM1 or CM2 emulsion. The values are expressed as mean ± SD of five mice in each group. $^{###}p < 0.001$ represents significant difference compared to normal group. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ represent significant differences compared to control (DNCB + vehicle) group.

3.5. Effects of CM2 treatment on lymph node weight and T cell cytokines

In the chronic phase of AD, $CD4^+$ T cell population markedly increased, and they polarized into Th1- and Th17 cells (Koga et al., 2008; Yamanaka and Mizutani, 2011). These activated T cells are then accumulated into immune organs, such as the spleen and lymph node (Arts et al., 1996). First, to determine whether CM2 could suppress the increase in lymph node weight resulting from infiltration of activated T cells, we evaluated the change of auricular lymph node weight. Extremely swollen lymph nodes were observed in control group compared with those in normal group. However, Dexa-treated and 0.2% CM2-treated groups showed smaller size and decreased weight of lymph node (Fig. 6A). We next determined the effect of CM2 treatment on T cell cytokine production. Following DNCB sensitization for 30 days, splenocytes were stimulated *in vitro* with CD3/CD28 antibodies, and the cytokine level in culture supernatant was measured by ELISA. As shown in Fig. 6B, treatment with CM2 significantly reduced the production of IFN-γ, IL-4, IL-5, and IL-17 A compared with the control group, while Dexa-treated group only reduced IL-5 production. The LDH level, a released enzyme by damage of cell membrane, did not increased in Dexa- or CM2-treated group compared with normal group. However, the CM1-treated group did not reduce IFN- IFN-γ, IL-4, IL-5, and IL-17 A (Supple. Fig. 1). These results showed that CM2 effectively reduces Th1-, Th2-, and Th17-type cytokine in spleen isolated from atopic dermatitis-induced mice.

3.6. Identification of CM2

The chemical structure of CM2 was clarified using LC-MS and NMR. The identification of CM2; ^{1}H NMR (500 MHz, DMSO-d_6): 11.7 (1H, s), 10.7 (1H, s), 7.3 (2H, d, J = 8.6 Hz), 7.2 (2H, d, J = 8.6 Hz), 7.2 (1H, m, H-2) 6.0 (1H, s), 5.7(1H, s), 5.4 (1H, m, H-2), 3.4 (1H, m, H-2), 3.4 (1H, d, J = 8.6 Hz), 3.2 (1H, t, J = 8.9 Hz), and 3.1 (1H, d, J = 8.9 Hz; Supplemental Fig. 2A). ^{13}C NMR (125 MHz, DMSO-d_6): 196.2, 167.5, 163.4, 162.8, 140.5, 128.9, 128.3, 126.6, 102.2, 96.2, 86.1, 68.9, 36.3, 31.3 (Supplemental Fig. 2B). The positive HR-MS spectra of CM2 showed molecular ion peaks at m/z 287.0996 $[M + H]^+$ (Supplemental Fig. 3B). The chemical structure and possible mechanism of structural transformation of CM2 are shown in Fig. 7. The positive HR-MS spectra of CM1 showed molecular ion peaks at m/z 299.0833 $[M + H]^+$ (Data not shown).

4. Discussion

Flavonoids have been known to be an attractive source of anti-inflammatory agents (Serafini et al., 2010). However, most flavonoids have strong toxicity, which can induce side effects (Wang et al., 2007). In this study, we found a novel radiolytic derivative from chrysin that was abundant in propolis and has lower cytotoxicity than intact-chrysin. Thus, we aimed to investigate the therapeutic potential of the radiolytic chrysin derivative, named as CM2, in *in vitro* and *in vivo* AD

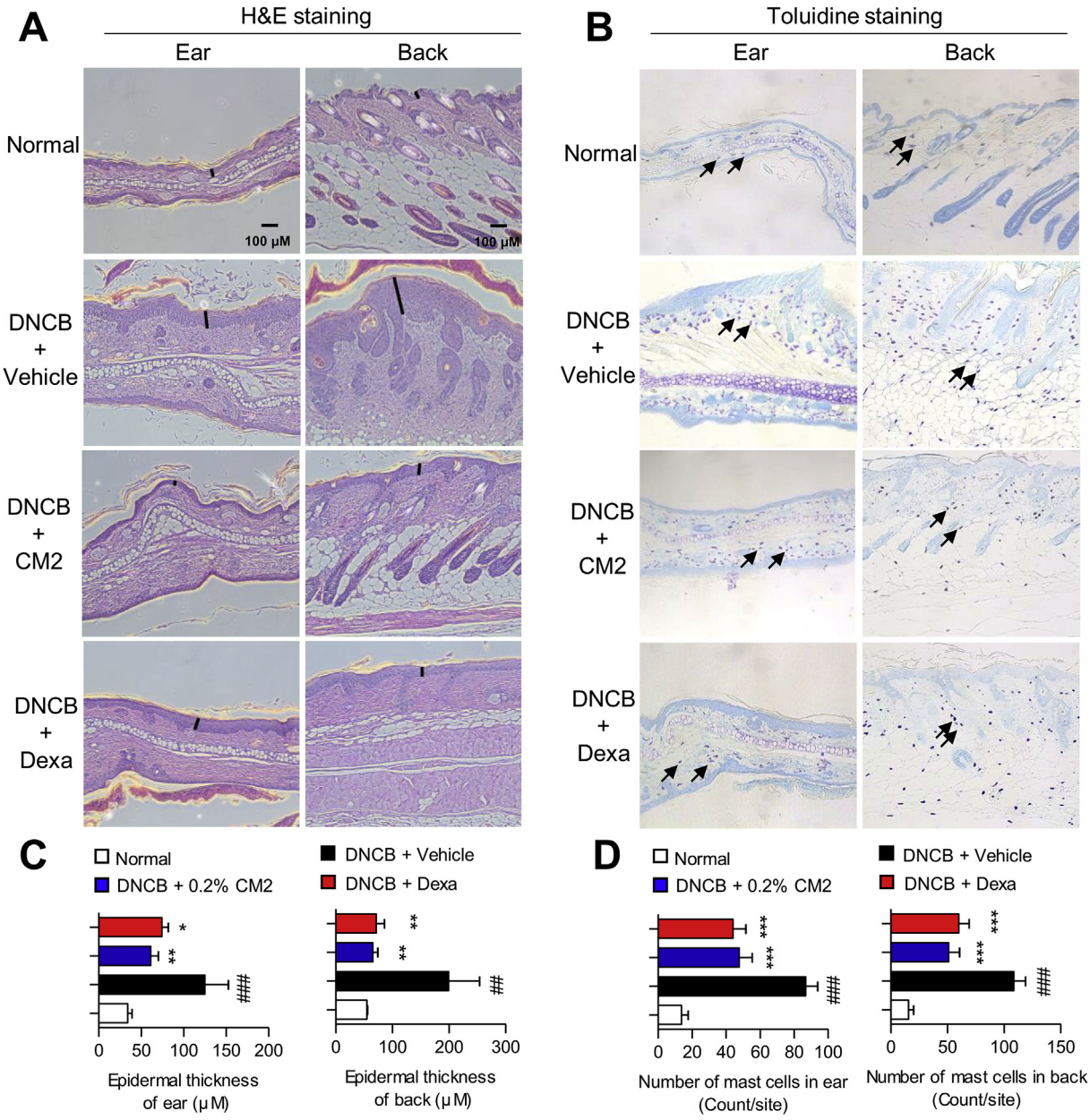

Fig. 5. Topical application of CM2 alleviated 2,4-dinitrochlorobenzene (DNCB)-induced hypertrophy and infiltration of inflammatory cells. The skin sections were stained with H&E (A) or toluidine blue (B), 100 x magnification. Bar graphs show the mean ± SD of epidermal thickness in H&E staining (C) and number of mast cells in toluidine blue staining (D). $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ represent significant differences compared to normal group. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ represent significant differences compared to control (DNCB + vehicle) group.

model.

When a pathogen invades into the skin or mucosal surface, innate cells recognize the pathogen and rapidly initiate inflammatory response by releasing soluble mediators and activating cellular innate mechanisms (Newton and Dixit, 2012). Macrophages are one of the representative innate cells, which strongly release pro-inflammatory molecules such as NO and pro-inflammatory cytokines after phagocytosis (Lin and Karin, 2007). The activated macrophages show inflammatory activity by presenting antigens to $CD4^+$ T cells through overexpression of MHC class and co-stimulatory molecules (CD80 and CD86), which in turn leads to chronic inflammation (Holling et al., 2004; Kim et al., 2015). Macrophages are importantly involved in skin inflammation. Kasraie et al. demonstrated that macrophages are accumulated in inflamed skin in AD (Kasraie and Werfel, 2013). Thus, if CM2 inhibits the inflammatory action mediated by macrophages, it could be an attractive agent for the treatment of AD. To demonstrate this hypothesis, we investigated whether CM2 inhibits inflammatory action in LPS-stimulated BMDMs. Our results showed that CM2 effectively inhibited LPS-induced overexpression of inflammatory mediators, including NO, pro-inflammatory cytokines, and surface molecules without cytotoxicity. Subsequently, to investigate the therapeutic efficacy of CM2 on AD-like skin lesions, we employed DNCB as a sensitizer, because the symptoms of skin inflammation and pathological features in mice treated with DNCB are similar to those in patients with AD (Yu et al., 2015). AD is characterized by itching, erythema, increase in serum IgE level, and infiltration of immune cells into the dermis (Li et al., 2010). We found that application of CM2 emulsion effectively reduced typical AD-like symptoms, such as dryness, edema, excoriation/erosion, and increase in serum IgE level. Furthermore, histological examination showed that CM2 application effectively reduced DNCB-induced typical AD features, including hypertrophy of ear and back skin tissue and dermal infiltration of mast cells (Saba et al., 2016).

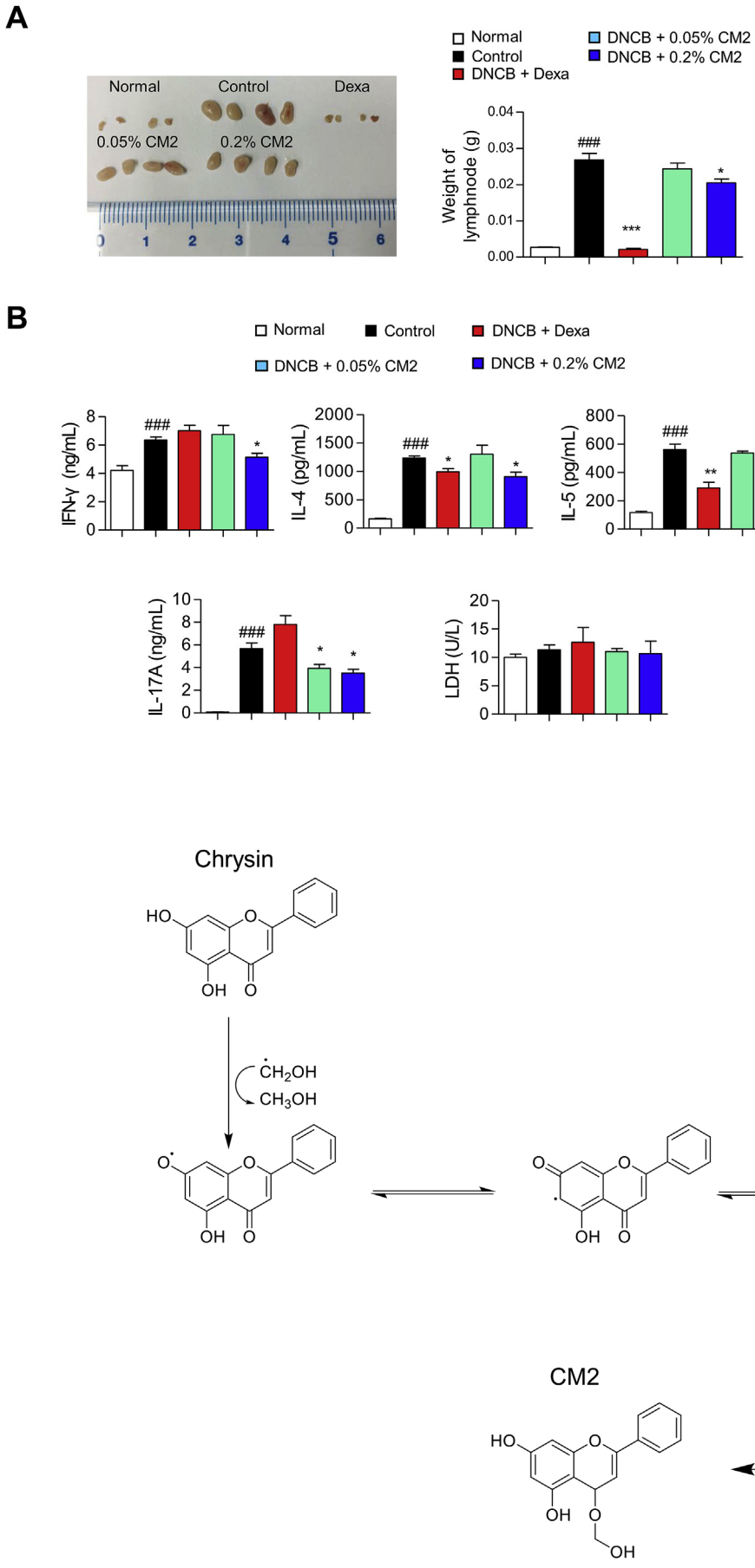

Fig. 6. Topical application of CM2 reduced 2,4-dinitrochlorobenzene (DNCB)-induced recruitment and activation of T cells in immune organs of Balb/c mice. (A) The auricular lymph node was photographed to record morphological changes, and then weighed. (B) Splenocytes isolated from the spleen were stimulated with anti-CD3/CD28 Abs for 48 h. Cytokine was measured by ELISA using a supernatant. The LDH was measured by automated biochemical analyzer using a supernatant. The values are expressed as mean ± SD of five mice in each group. Statistical analysis was performed by Tukey's multiple comparison test. $^{###}p < 0.001$ represents significant difference compared to normal group. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ represent significant differences compared to control (DNCB + vehicle) group.

Fig. 7. Identification of chemical structure of CM2 and structural modification mechanism in chrysin methanolic solution.

The pathogenesis of AD is related to an imbalance between Th1-type and Th2-type immune response. In the onset of AD, predominant systemic Th2 cytokines (e. g., IL-4, IL-5, and IL-13) are observed, which strongly induce the switching of activated B cells to IgE-producing cells (Homey et al., 2006). In contrast, the chronic phase of AD is associated with excessive Th1 response characterized by high levels of IFN-γ and IL-12 (Yamanaka and Mizutani, 2011). These cytokines are importantly involved in skin barrier dysfunctions, including dermal thickening and infiltration of immune cells into the dermis (Kosaka et al., 2008). Several reports have demonstrated that Th17 cells exacerbate certain skin disorders, such as psoriasis and epidermal hyperplasia (Koga et al., 2008; Zheng et al., 2007). Thus, excessive proliferation and activation of T cells were observed in chronic AD (Esaki et al., 2016). Notably, we observed that continuous application of CM2 reduced swelling of auricular lymph node as well as production of splenic Th1 (IFN-γ)-, Th2 (IL-5)-, and Th17 (IL-17 A) cytokines. These results strongly indicate that CM2 may inhibit T cell-based progression of AD. Although speculative, our results suggest that therapeutic effect of CM2 on DNCB-induced AD-like skin lesions is a result of inhibition of inflammatory mediators produced by innate immune cells in development of AD. Consequently, these findings suggest that CM2 can be an attractive medicinal candidate for the treatment of AD. Finally, for facilitating the application of CM2 as a therapeutic agent for AD, we identified the chemical structure of CM2 and mechanism of structural transformation mediated by gamma irradiation. However, further clinical studies are need to confirm the anti-atopic effects of CM2 on human skin.

5. Conclusion

Gamma irradiation produced novel derivatives of chrysin by inducing structural modification. The main derivatives, named as CM2, revealed lower cytotoxicity compared with intact-chrysin in BMDMs, and it has strong anti-inflammatory properties in LPS-stimulated BMDMs. Topical application of CM2 alleviated DNCB-induced AD-like skin lesions of Balb/c mice. Taken together, CM2 can be an attractive candidate for the treatment of AD.

Conflicts of interest

The authors have declared no conflicts of interest.

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Transparency document

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.03.048.

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