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A hydroxyethyl derivative of chrysin exhibits anti-inflammatory activity in dendritic cells and protective effects against dextran sodium salt-induced colitis in mice

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ABSTRACT

Inflammatory bowel disease (IBD) is a chronic disease that occurs in the intestinal tract. Phyto-ingredients have been evaluated for their ability to protect against IBD because of their anti-inflammatory activities. In our previous study, we identified a novel derivative of chrysin (HE-chrysin) using irradiation technology, which exhibited stronger anti-cancer activity in human colorectal cancer cells than the original chrysin. Here, to determine whether HE-chrysin is a new therapeutic candidate for IBD, we investigated the anti-inflammatory effects of HE-chrysin on bone marrow-derived dendritic cells (BMDCs) and dextran sodium salt (DSS)-induced colitis in mice. HE-chrysin more effectively inhibited BMDC maturation compared to chrysin, as demonstrated by the decreased levels of pro-inflammatory cytokines, surface molecules, antigen-presenting ability, and T cell proliferation/activation in lipopolysaccharide-stimulated BMDCs. These anti-inflammatory effects of HE-chrysin were regulated by mitogen-activated protein kinases and nuclear factor-κB. Furthermore, oral administration of HE-chrysin attenuated DSS-induced colitis symptoms and clinical signs in the mouse model. The protective effects of HE-chrysin treatment against colitis were mediated by decreasing Th1- and Th17-type cytokine levels. These results indicate that HE-chrysin is attractive candidate for IBD therapy.

1. Introduction

Inflammatory bowel disease (IBD) has become a worldwide healthcare issue, and numerous studies have been conducted to develop therapeutic agents for IBD [1,2]. IBD can be divided into two broad subtypes, Crohn's disease (CD) and ulcerative colitis (UC), which are marked by chronic, relapsing, and abnormal immune responses [3]. CD is characterized by transmural inflammation involving any part of the gastrointestinal (GI) tract from the mouth to the anus, whereas UC shows localized inflammation of the rectum and large bowel [4]. Both diseases have common symptoms, such as abdominal pain and diarrhea, rectal bleeding, and body weight loss [5]. Although patients with CD typically have more severe symptoms, the worldwide prevalence of UC is much higher [6].

Immunological therapy, including classic anti-inflammatory drugs and biological agents, have been widely used in IBD therapy [7,8]. Aminosalicylates, which are typical anti-inflammatory drugs for UC therapy, moderate disease symptoms by inhibiting *cyclo*-oxygenase, prostaglandins, and nuclear factor-κB (NF-κB) signaling in immune cells [9,10]. Similarly, short-term use of glucocorticoid therapy effectively suppresses pro-inflammatory proteins by inhibiting multiple transcription factors induced by glucocorticoids-receptor complex activation [11]. Anti-tumor necrosis factor (TNF) antibodies are widely used as a biological therapy for IBD and show high clinical efficacy in patients with UC and CD [12,13]. However, these drugs have several limitations and side effects, such as allergic reaction, loss of efficacy on repetitive use, probability of infection, and risk of carcinogenesis [14–16]. Thus, recent studies have focused on developing new medicines from nature-derived materials for treating IBD [17–19].

Polyphenolic compounds belong to a large class of plant secondary metabolites, which have gained attention because of their potential to protective effects against inflammation-related diseases [20]. Among them, chrysin is abundant in propolis and shows strong anti-inflammatory activities [21]. To develop more effective natural-derived

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medicines, some studies have been conducted to improve physiological activities through structural transformation of chrysin. For example, a methylglyoxal derivative of chrysin exhibited enhanced anti-glycation activity and water solubility [22]. Fonseca and coworkers synthesized a *bis*-arylselanyl chrysin derivative by ultrasonic irradiation, which showed improved antioxidant and anticancer activities [23]. Additionally, ruthenium-conjugated chrysin had greater inhibitory effects on platelet function and thrombus formation compared to original chrysin [24]. Recently, we found that chrysin is converted to a new hydroxyethylated product (HE-chrysin) by gamma irradiation, which shows stronger anti-cancer activity than original chrysin [25]. However, no studies have examined the role of HE-chrysin in inflammatory-related diseases. Therefore, the main objectives of this study were to evaluate the anti-inflammatory activity of HE-chrysin and explore whether HE-chrysin is a new candidate for treating IBD.

2. Materials and methods

2.1. Reagents and antibodies

Fluorescein isothiocyanate (FITC)-conjugated anti-CD80, anti-interleukin (IL)-1β, anti-I-Ab-E$\alpha_{52\text{-}68}$, phycoerythrin (PE)-conjugated anti-CD86, and PE-Cy7-conjugated anti-IL-17A monoclonal antibodies (mAbs) were purchased from eBioscience (San Diego, CA, USA). V450-conjugated anti-CD4, anti-CD11c, PE-conjugated anti-interferon (IFN)-γ, allophycocyanin-conjugated anti-TNF-α, PE-Cy7-conjugated anti-major histocompatibility complex (MHC)-II mAbs were purchased from BD Biosciences (San Jose, CA, USA). Anti-phosphorylated extracellular signal-regulated kinase (ERK) 1/2 (p-ERK1/2), p-c-Jun N-terminal kinase (JNK), p-p38, p-IκB mAbs and IκB, NF-κB p65, and β-actin mAbs were purchased from Cell Signaling Technology (Danvers, MA, USA). Chrysin was purchased from Sigma-Aldrich (St-Louis, MO, USA).

2.2. Experimental animals

Specific pathogen-free, female, 6–8-week-old of C57BL/6 or BALB/c mice were purchased from Orient Bio, Inc. (Seoul, Korea). The mice were kept in a specific pathogen-free environment and acclimatized to following the controlled conditions; temperature (23 ± 2 °C), humidity (55 ± 5%), and light (12-h light/dark cycle) at the Central Animal Research Laboratory at Korea Atomic Energy Research Institute (KAERI, Jeongeup, Korea). All procedures were approved by the Institutional Animal Care and Use Committee of KAERI (KAERI-IACUC-2018-016).

2.3. Preparation of HE-chrysin

Chrysin solution (1 mg/mL in methanol) was irradiated at dose of 100 kGy in a cobalt-60 irradiator (IR-79, MDS Nordion International Co., Ltd., Ottawa, Ontario, Canada), and HE-chrysin was isolated using a preparative-high performance liquid chromatography (HPLC) 1260 infinity system (Agilent Technologies, Santa Clara, CA, USA) from crude-irradiated chrysin solution. The gradient method for isolation was performed as previous described [25]. The purity of HE-chrysin was at least 95%, as confirmed by HPLC.

2.4. Differentiation of bone marrow-derived dendritic cells (BMDCs)

Whole bone marrow cells were isolated from C57BL/6 mice following an established protocol [26]. Red blood cells (RBCs) in the whole bone marrow cells were lysed with RBC lysing buffer (Sigma-Aldrich), and then the lysed cells (1×10^6 per 100-mm plate) were incubated in 10 mL complete-RPMI (c-RPMI) 1640 medium (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO), 100 U/mL of penicillin/streptomycin (GIBCO), 20 ng/mL of GM-CSF (JW CreaGene, Daegu, Korea), and 0.5 ng/mL of IL-4 (JW CreaGene) at 37 °C in a 5% CO_2 incubator. On days 3 and 6 of incubation, 10 mL c-RPMI 1640 medium was added, and all experiments were performed after 8 days of culture. Nonadherent cells and loosely adherent cells were harvested after 8 days of culture, and then the cells were stained with fluorescein-conjugated anti-CD11c mAb (BD Biosciences). CD11c-positive cells were obtained at over 90%.

2.5. Cytotoxicity measurement

Cytotoxicity was analyzed using an Ez-cytox cell viability assay kit (Daeil Lab Service, Seoul, Korea) and Annexin V/propidium iodide (PI) apoptosis detection kit (BD Biosciences). Briefly, the BMDCs were seeded into a 48-well plate (2.5×10^5 cells/well) and treated with lipopolysaccharide (LPS; 100 ng/mL), LPS and chrysin (2 and 10 μM), or LPS and HE-chrysin (2 and 10 μM) for 24 h. The media were replaced with 10% EZ-cytox containing RPMI-1640 and incubated at 37 °C for 1 h. The absorbance was measured at 450 nm using a microplate reader (SpectraMax M3, Molecular Devices, Sunnyvale, CA, USA). For the Annexin V/PI assay, the cells were harvested and washed with phosphate-buffered saline (PBS), and then stained with FITC-annexin V and PI according to the manufacturer's protocol. Cytotoxicity was analyzed by flow cytometry (MACSQuaunt VYB, Miltenyi Biotech, Bergisch Gladbach, Germany).

2.6. Cytokine analysis in BMDCs

The levels of TNF-α, IL-12p70, and IL-1β were determined in the BMDC culture supernatant using commercial enzyme-linked immunosorbent assay (ELISA) kits (eBioscience) following the manufacturer's protocol. For intracellular cytokine staining, the DCs were treated with LPS (100 ng/mL), LPS and chrysin (10 μM), or LPS and HE-chrysin (10 μM) in the presence of each 1 μg/mL of GolgiPlug (BD Biosciences) for 12 h. The cells were stained with a Live/Dead cell staining kit (Invitrogen, Carlsbad, CA, USA) using anti-CD11c antibody for 30 min at 4 °C, and then fixed and permeabilized with the Cytofix/Cytoperm kit (BD Biosciences) for 20 min at 4 °C. For intracellular IL-1β staining, the cells were treated with the indicated reagents without GolgiPlug for 18 h, and then stained with anti-CD11c and a Live/dead cell staining kit. The cells were fixed and permeabilized using a Cytofix/Cytoperm Kit and stained with anti-IL-1β antibody. The intracellular levels of TNF-α, IL-12p70, and IL-1β were investigated using fluorescein-conjugated secondary antibodies by flow cytometry. The data were analyzed by FlowJo software (Tree Star, Inc., Ashland, OR, USA).

2.7. Analysis of surface molecule expression

The cells were treated with LPS (100 ng/mL), LPS and chrysin (10 μM), or LPS and HE-chrysin (10 μM) for 24 h and then stained with fluorescent-conjugated Abs (anti-CD80, anti-CD86, anti-MHC-I, and anti-MHC-II) for 30 min at 4 °C. The expression levels of surface molecules were determined by flow cytometry.

2.8. Measurement of antigen-presenting ability

The E$\alpha_{44\text{-}76}$ peptide (RLEEFAKFASFEAQGALANIAVDKANLDVMKKR; the underlined sequence binds to MHC-II) and E$\alpha_{52\text{-}68}$ peptide (ASFEAQGALANIAVDKA) were purchased from AbFrontier (Seoul, Korea). The cells were treated with HE-chrysin (10 μM) or chrysin (10 μM) in the presence of 25 μg/mL of E$\alpha_{44\text{-}76}$ peptide for 24 h. The E$\alpha_{52\text{-}68}$ peptide was used as a positive control. After 24 h, the cells were harvested and stained with anti-CD11c and anti-I-Ab-E$\alpha_{52\text{-}68}$ Ab (Y-Ae; ASFEAQGALANIAVDKA, eBioscience) mAbs for 30 min at 4 °C. The stained cells were analyzed by flow cytometry.

2.9. Immunoblotting analysis

BMDCs were treated with LPS or LPS and HE-chrysin for different periods and then lysed with PRO-PREP protein extraction buffer (iNtRON Biotechnology, Seongnam, Korea) for cytosolic extraction. The lysates were incubated for 20 min at 4 °C and were centrifuged for 20 min at 10,000 × *g*. at 4 °C. Nuclear extracts were prepared with a nuclear and cytoplasmic protein extraction kit (Pierce Biotechnology, Rockford, IL, USA) following the manufacturer's protocol. The protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology). The cell lysates (20 μg per sample) were separated by 10% sodium dodecyl sulfate-polyacrylamide and then electrotransferred to a polyvinylidene difluoride membrane. The membranes were blocked with 5% skim milk and incubated with each primary antibody for overnight at 4 °C. After extensive washing, the membranes were further incubated with horseradish peroxidase-conjugated secondary antibody (Calbiochem, San Diego, CA, USA) for 1 h at 25 °C. The protein was visualized using an ECL Advance kit (Bio-Rad, Hercules, CA, USA), and then exposed to X-ray medical film (Fuji-Rx, Fuji Film, Tokyo, Japan).

2.10. Mixed lymphocyte reaction (MLR) assay

To determine the T cell suppressive ability of HE-chrysin-treated BMDCs, splenocytes were isolated from spleen of BALB/c mice. The splenocytes were incubated with anti-CD4-coated magnetic microbeads (Miltenyi Biotech), and then $CD4^+$ cells were separated using an LS column (Miltenyi Biotech) following the manufacturer's protocol. The separated $CD4^+$ T cells were stained with 5 μM of CFSE (Invitrogen, Carlsbad, CA, USA) and washed with 2% FBS in PBS for 10 min. Next, BMDCs (C57BL/6 background) were treated with LPS (100 ng/mL), LPS and chrysin (10 μM), or LPS and HE-chrysin (10 μM) for 24 h and then co-cultured with CFSE-labeled $CD4^+$ T cells (BALB/c background) at a DC:T cell ratio of 0.25:1. On day 3 day of co-culture, the cells were harvested and stained with anti-CD4 antibody and then analyzed by flow cytometry. The levels of IFN-γ and IL-2 in the culture supernatant were determined by ELISA.

2.11. Induction of experimental colitis

Seven-week-old of BALB/c mice were administered either normal water or water containing 4% dextran sodium sulfate (DSS, MP Biomedical, Aurora, OH, USA) for 8 days. The mice were randomly divided into four groups ($n = 7$ per group): (1) Normal, (2) DSS only, (3) DSS + 75 mg/kg of 5-aminosalicylic acid (5-ASA; positive control), (4) DSS + 2 mg/kg of HE-chrysin. The 5-ASA and HE-chrysin were dissolved in 0.5% dimethyl sulfoxide and 0.5% Tween 20 in PBS (vehicle) and orally and daily administered to the mice during DSS treatment. The normal group and DSS only group were administered vehicle solution during DSS treatment.

2.12. Evaluation of experimental colitis

Bodyweight and the disease activity index (DAI) score were measured daily and recorded. The DAI score was calculated by summing the scores of diarrhea and bleeding. Briefly, following the parameters were used for calculation [27]; diarrhea (0 point = normal, 1 point = soft feces, 2 point = diarrhea, 3 point = no feces produced) and bleeding (0 point = no bleeding, 1 point = visual blood in rectum, 2 point = visual blood in fur). After 8 days, the mice were sacrificed by CO_2 asphyxiation and their colons were collected and photographed. Next, colon length was measured.

2.13. Measurement of colonic myeloperoxidase (MPO) activity

The colon tissues were washed with cold-PBS and homogenized in PRO-PREP protein extraction solution. Next, homogenized tissues were centrifuged twice at 13,000 rpm for 20 min at 4 °C, and the supernatant was collected. The protein concentrations of whole colon supernatants were determined with a BCA kit and MPO activity was measured with an MPO ELISA kit (Hycult Biotech, Plymouth Meeting, PA, USA) following the manufacturer's instructions.

2.14. Histological analysis

For histological observation, the colon tissues were fixed in 10% formalin, embedded in paraffin, cut into 5-mm sections, and stained with hematoxylin and eosin (H&E). Histopathological evaluation of the entire section was performed in a blinded manner by determining crypt distortion and lymphocyte infiltration (PMID: 29324769). Based on these criteria a histological store from 0 to 3 (0 = none, 1 = mild, 2 = moderate, 3 = severe). For the immunofluorescent leukocytes staining, paraffin-embedded slides were deparaffinized, and antigen retrieval was performed by microwave heating in 10 mM sodium citrate buffer (pH 6.0) for 15 min. After cooling and rinsing with PBS, slides were incubated with 5% goat serum in 1% bovine serum albumin in PBS for 1 h. After then, the sections were incubated overnight wit anti-CD45 antibody (Abcam, Cambridge, UK) at 4°C. Alexa568-conjugated secondary anti-rabbit antibody was added and incubated for 1 h, and the slides were counterstained with 4′,6-diamidino-2-phenylidole (DAPI, Invitrogen). The image was captured by confocal microscopy (LSM800, Carl Zeiss, Jena, Germany).

2.15. Isolation of lymphocytes from lymphoid organs

Mesenteric lymph nodes (mLNs) were isolated from mice in each group and kept on ice in PBS. To prepare single-cell suspension, the organs were ground and filtered through a 40-μm nylon mesh cell strainer and treated with RBC lysis buffer for 5 min. After washing with RPMI 1640 medium supplemented with 10% FBS, the cells were centrifuged at 1,800 rpm for 3 min. The cells were resuspended in RPMI 1640 containing 10% FBS and 100 U/mL penicillin/streptomycin.

2.16. T cell analysis in experimental colitis

The 48-well plates were coated with anti-CD3 (10 μg/mL, BD Biosciences) for 2 h at 37 °C, and then the cells isolated as described in Section 2.14. Next, the cells (2×10^6 cells/well) were seeded into an anti-CD3-coated 48-well plate in the presence of anti-CD28 (2 μg/mL, BD Biosciences), GolgiPlug (0.5 μg/mL) and GolgiStop (0.5 μg/mL, BD Biosciences) for 12 h at 37 °C. The cells were harvested and stained with a Live/Dead cell staining kit and anti-CD4 antibody for 30 min at 4 °C, washed in wash buffer, and incubated with the Cytofix/Cytoperm Kit. The cells were stained with anti-IFN-γ and anti-IL-17A Abs and analyzed by flow cytometry. Culture supernatants were used to measure the levels of the cytokines IFN-γ and IL-17A.

2.17. Statistical analysis

Statistical significance was analyzed using one-way analysis of variance followed by Tukey's multiple comparison or Kruskal-Wallis test using the GraphPad Prism 5.0 software (GraphPad, Inc., San Diego, CA, USA). The results are expressed as the mean along with standard deviation. The significance of differences was defined as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

3. Results

3.1. HE-chrysin inhibits cytokine production in LPS-stimulated BMDCs

The structures of HE-chrysin and chrysin are shown in Fig. 1A. Prior to investigating the anti-inflammatory effects of HE-chrysin, cell

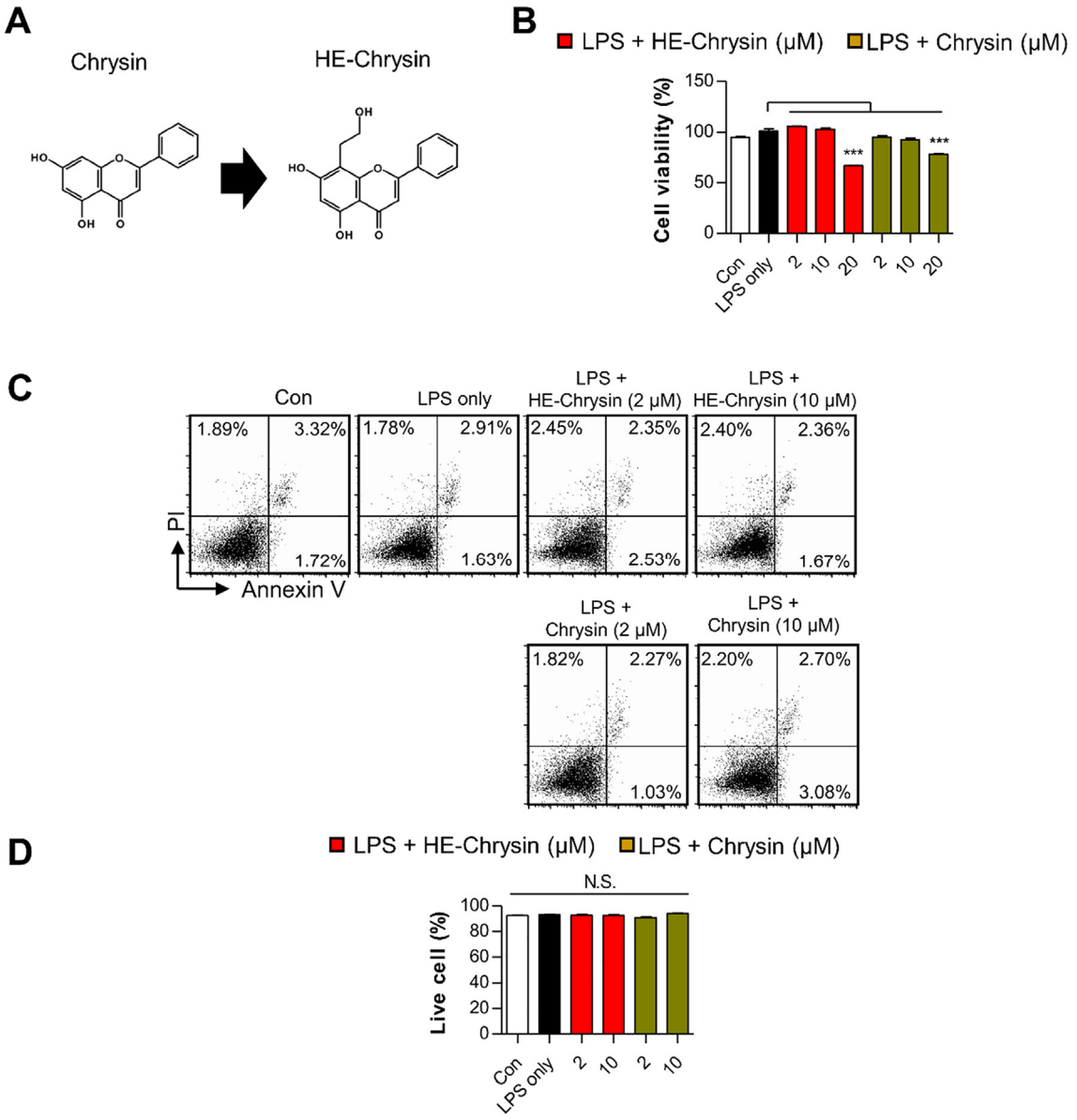

Fig. 1. Effects of HE-chrysin and chrysin on viability of BMDCs. (A) Chemical structure of HE-chrysin and chrysin. Cell viability effects of HE-chrysin and chrysin determined using Ez-cytox cell viability kit (B) and annexin V/PI (C) assay. (D) Average percentage of live cells means negative populations of annexin V/PI. Statistical analysis was performed by Tukey's multiple test. The data represent the mean ± SD ($n = 3$). ***$p < 0.001$ represent significant differences.

viability was determined. We confirmed that both HE-chrysin and chrysin were not cytotoxic towards BMDCs at doses of 2–10 μM (Fig. 1B–D). Activated DCs induce inflammation by producing large amounts of pro-inflammatory cytokines [28]. Thus, we next examined the inhibitory effects of HE-chrysin on the cell culture supernatant level of these cytokines in LPS-stimulated BMDCs. Treatment of HE-chrysin significantly inhibited LPS-induced overproduction of TNF-α, IL-12p70, and IL-1β in BMDCs, which had a stronger inhibitory effect on cytokine production than chrysin treatment (Fig. 2A). The inhibitory effect of HE-chrysin on LPS-induced cytokine production was confirmed at the intracellular level. The HE-chrysin-treated group showed lower expression of intracellular TNF-α, IL-12p70, and IL-1β compared to the chrysin-treated group (Fig. 2B and C). In summary, HE-chrysin more strongly inhibits LPS-induced cytokine production in BMDCs than intact-chrysin.

3.2. HE-chrysin modulates surface molecule expression and antigen-presenting ability in BMDCs

Activated DCs highly express MHC class molecules and co-stimulatory molecules (e.g., CD80 and CD86), which are important for inducing T cell-mediated immune responses [29]. To evaluate the effect of HE-chrysin on surface molecules, BMDCs were treated with HE-chrysin or chrysin with LPS for 24 h, and then surface molecules were analyzed by flow cytometry. Treatment of HE-chrysin significantly reduced overexpression of LPS-induced surface molecule expression, including that of CD80, CD86, and MHC-II, while the chrysin-treated group did not show reduced overexpression of surface molecules (Fig. 3A and B). We subsequently examined the effects of HE-chrysin on the antigen-presenting ability in BMDCs using anti-Y-Ae mAb, which recognizes the $E\alpha_{52\text{-}68}$ peptide-MHC-II complex. As shown in Fig. 3C and D, the HE-chrysin-treated group showed a lower percentage of $E\alpha_{52\text{-}68}$/MHC-II complexes (Y-Ae-positive cells), while chrysin-treated group did not show a reduction. These results indicate that HE-chrysin effectively

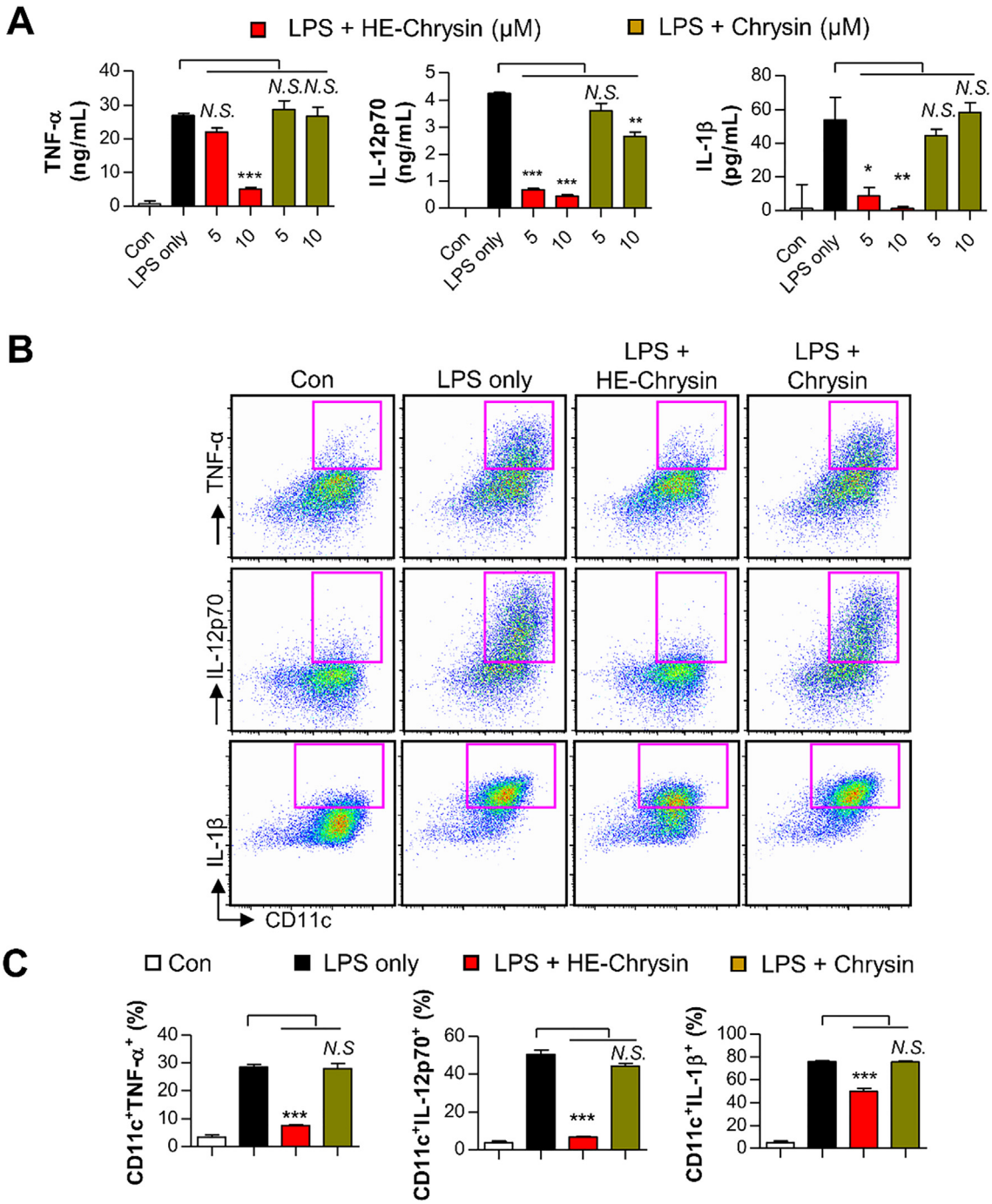

Fig. 2. Effects of HE-chrysin and chrysin on cytokine production in LPS-stimulated BMDCs. (A) BMDCs were treated with HE-chrysin or chrysin in the presence of LPS (100 ng/mL) for 24 h, and then TNF-α, IL-12p70, and IL-1β levels in the culture medium were analyzed by ELISA. (B) Intracellular levels of TNF-α and IL-12p70 in BMDCs were determined by flow cytometry after 12 h in the presence of GolgiPlug (1 μg/mL), and the intracellular level of IL-1β in BMDCs was determined after 18 h in the absence of GolgiPlug. (C) Bar graph shows the mean ± SD ($n = 3$) of percentage of intracellular cytokine levels in BMDCs. Statistical analysis was performed by Tukey's multiple test. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ represent significant differences.

prevents DC activation by inhibiting overexpression of surface molecules and exogenous antigen-presenting ability.

3.3. HE-chrysin inhibits mitogen-activated protein kinases (MAPKs) and NF-κB signals in LPS-stimulated BMDCs

The MAPK and NF-κB pathways are important regulations of the activation of DCs, which promote the expression of cytokine genes and certain surface molecules [30]. Thus, we investigated whether the inhibitory effects of HE-chrysin on LPS-induced overexpression of inflammatory mediators occurs through regulation of MAPK and NF-κB signaling. As shown in Fig. 4, treatment with LPS induced phosphorylation of ERK, JNK, p38, and phosphorylation/degradation of IκB-α as well as nuclear translocation of NF-κB, while treatment with HE-chrysin significantly inhibited phosphorylation of ERK (15 min, 30 min; $p < 0.05$), JNK (60 min; $p < 0.05$), p38 (15 min, 30 min; $p < 0.05$), and phosphorylation/degradation of IκB-α (30 min, 60 min; $p < 0.05$). However, chrysin-treated group did not inhibited phosphorylation of these molecules. Furthermore, treatment with HE-chrysin inhibited translocation of NF-κB at 60 min ($p < 0.05$), but chrysin-treated group

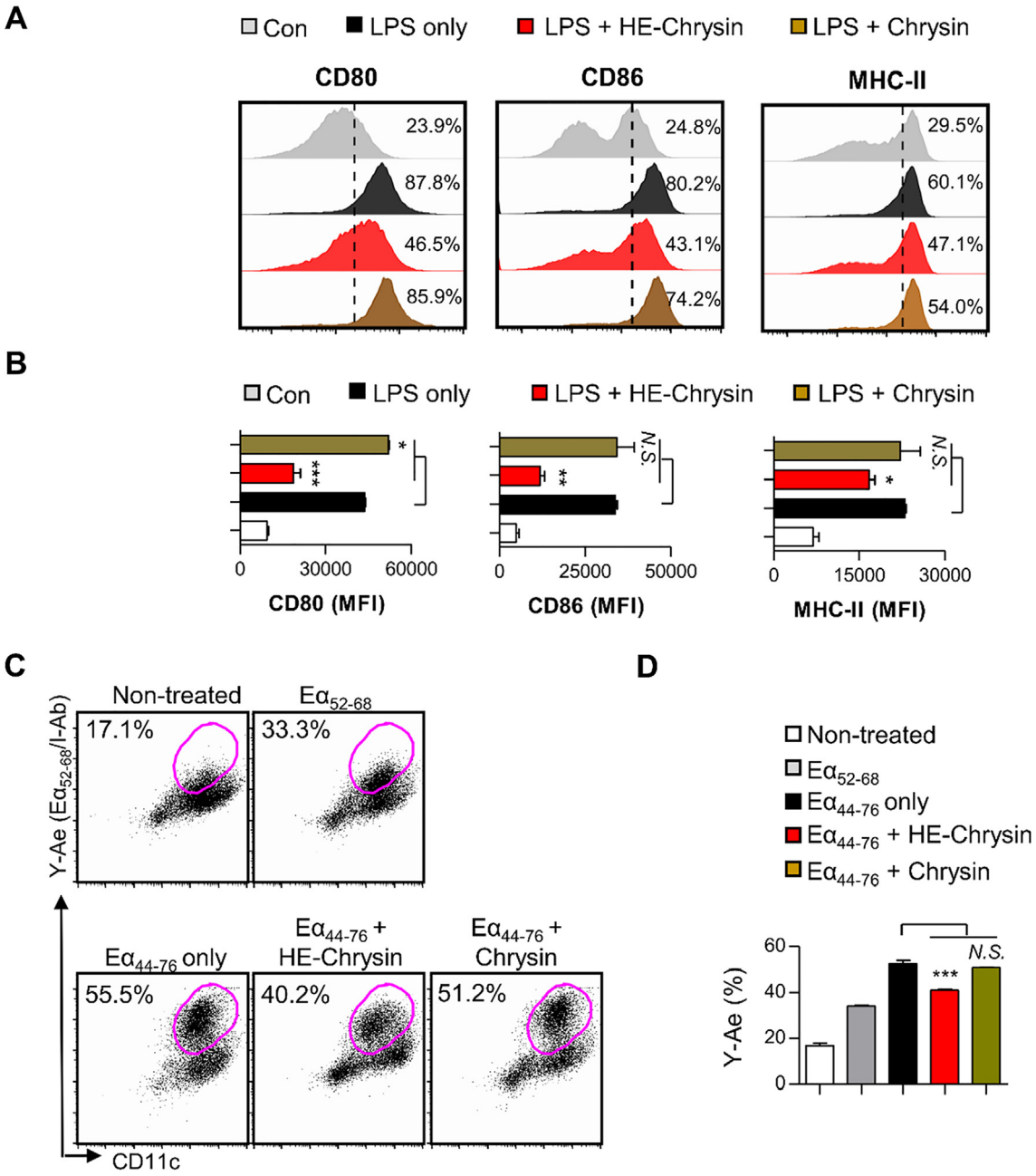

Fig. 3. Effects of HE-chrysin and chrysin on surface molecules and antigen-presenting ability in BMDCs. (A and B) BMDCs were treated with HE-chrysin (10 μM) or chrysin (10 μM) in the presence of LPS (100 ng/mL) for 24 h, and then the cells were stained with anti-CD80, anti-CD86, anti-MHC-II, and anti-CD11c and analyzed by flow cytometry. (B) Bar graphs show the mean ± SD of median fluorescence intensity (MFI) of each surface molecule expressed by $CD11c^+$ cells. (C) Antigen-presenting ability in HE-chrysin (10 μM)- or chrysin (10 μM)-treated BMDCs in the presence of $E\alpha_{44\text{-}76}$ peptide. After 24 h, the cells were stained with anti-CD11c and anti-Y-Ae Abs for 30 min at 4 °C and analyzed by flow cytometry. $E\alpha_{52\text{-}68}$ peptide (1 μg/mL)-treated group used as positive control. (D) The bar graphs show the mean ± SD of $Y\text{-}Ae^+$ (%) in expressed by $CD11c^+$ cells. Statistical analysis was performed by Tukey's multiple test. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ represent significant differences.

did not inhibited translocation of NF-κB. These results indicate that HE-chrysin has anti-inflammatory activity in BMDCs by regulating the MAPK and NF-κB signaling pathways.

3.4. HE-chrysin-treated BMDCs inhibits proliferation and activation of T cells

To characterize the effect of HE-chrysin on alloactivation ability of BMDCs, the BMDCs treated with the LPS or LPS and HE-chrysin were co-cultured with CFSE-labeled $CD4^+$ T cells for 3 days. The $CD4^+$ T cells co-cultured with LPS-treated BMDCs showed significantly increased proliferation, while LPS/HE-chrysin-treated BMDCs did not increased T cell proliferation (Fig. 5A and B). Furthermore, the $CD4^+$ T cells co-cultured with LPS/HE-chrysin-treated BMDCs did not increase level of IFN-γ and IL-2 compared to $CD4^+$ T cells co-cultured with LPS-treated BMDCs (Fig. 5C). These findings indicate that HE-chrysin-treated BMDCs did not increase the proliferation and cytokine production of T cells.

3.5. HE-chrysin alleviates DSS-induced colitis

To investigate whether the anti-inflammatory properties of HE-

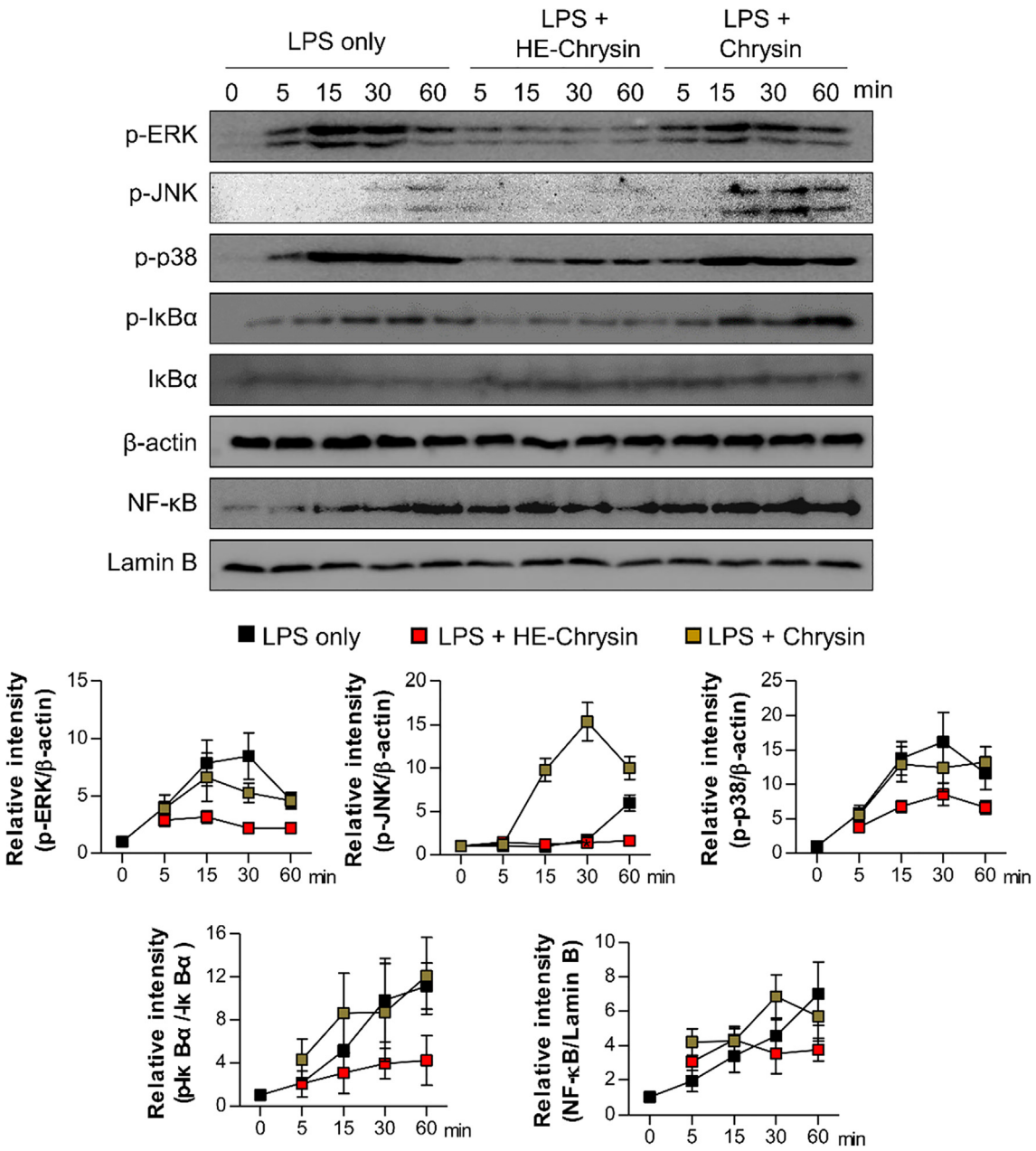

Fig. 4. Effects of HE-chrysin and chrysin on MAPKs and NF-κB signaling pathway in BMDCs. The BMDCs were treated with HE-chrysin (10 μM) or chrysin (10 μM) with LPS (100 ng/mL) for the indicated times. The protein levels of cytosolic p-ERK, p-JNK, p-p38, p-IκBα, IκBα, and nuclear NF-κB were analyzed by western blotting. Western blotting data are shown by a representative image of three independent experiments. The graphs showed the mean ± SD of relative intensity of each protein expression level. Statistical analysis was performed by Tukey's multiple test.

chrysin can protect against DSS-induced colitis, HE-chrysin was orally administered to DSS-treated mice for 8 days. In the disease model, the 5-ASA treatment (8 days; $p < 0.05$) and HE-chrysin treatment (8 days; $p < 0.05$) protected against DSS-induced bodyweight reduction (Fig. 6A). The 5-ASA-treated (6 days, 7 days, 8 days; $p < 0.05$) and HE-chrysin-treated (6 days, 7 days; $p < 0.05$ and 8 days; $p < 0.05$) groups showed lower DAIs than the group treated with DSS alone (Fig. 6B). Furthermore, both the 5-ASA-treated and HE-chrysin-treated groups showed lower levels of colonic MPO (Fig. 6C) and longer colon lengths (Fig. 6D and E) compared to the group treated with DSS alone. For histological changes, treatment of HE-chrysin effectively attenuated DSS-induced lymphocytes infiltration and induced crypt regeneration (Fig. 6F and G). The number of $CD45^+$ leukocytes was also reduced in HE-Chrysin-treated group (Supplemental Fig. 1)

3.6. HE-chrysin reduces Th1- and Th17-cytokines in MLN

In the progression of IBD, a homeostasis imbalance in T lymphocytes is an important hallmark that tends to exacerbate the symptoms of disease progression [31,32]. Therefore, to determine whether treatment with HE-chrysin can reduce Th1- and Th17-type cytokines in DSS-induced colitis mice, cytokine levels in the culture supernatant were measured by ELISA. As shown in Fig. 7A, treatment with DSS induced overproduction of IFN-γ and IL-17A, while HE-chrysin effectively reduced IFN-γ and IL-17A in MLN. The inhibitory effects of HE-chrysin on DSS-induced IFN-γ and IL-17A in MLN were confirmed at the intracellular level (Fig. 7B and C). HE-chrysin did not affect splenic IFN-γ and IL-17A (data not shown). These results indicate that HE-chrysin exhibits protective efficacy against DSS-induced colitis by regulating Th1- and Th17-type cytokines.

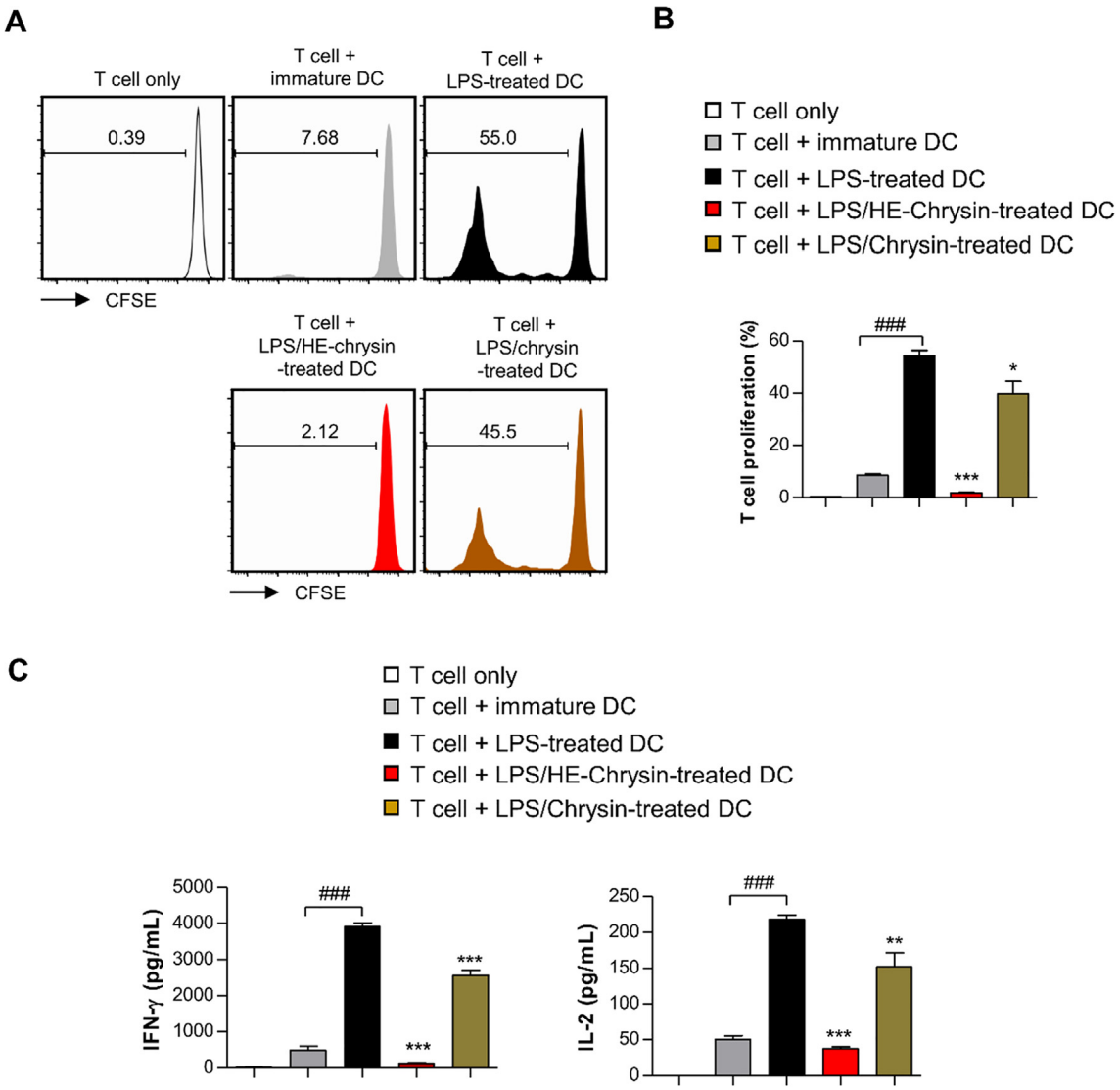

Fig. 5. Effects of HE-chrysin-treated DCs on proliferation and activation of $CD4^+$ T cells. The DCs were treated with HE-chrysin (10 μM) or chrysin (10 μM) in the presence of LPS (100 ng/mL) for 24 h, and then CFSE-labeled $CD4^+$ T cells were co-cultured with each group of DCs for 3 days. (A) The proliferation of $CD4^+$ T cells was analyzed by flow cytometry. (B) Bar graph shows the mean ± SD ($n = 3$) of proliferation percentage of $CD4^+$ T cells. (C) Levels of IFN-γ and IL-2 were analyzed by ELISA using co-culture supernatant. Statistical analysis was performed by Tukey's multiple test. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ represent significant differences.

4. Discussion

In the present study, to estimate the therapeutic potential of our new flavonoid derivative, HE-chrysin, we examined its anti-inflammatory effects *in vitro* and *in vivo*.

DCs, as professional antigen-presenting cells, are a key link between the innate and adaptive immune systems by presenting pathogen-derived antigens to naïve-T cells [33]. When pathogen invasion occurs, mature DCs undergo endocytosis to migrate to draining lymph nodes and activate naïve-T cells by up-regulating inflammatory mediators [34]. Numerous DCs are present in the intestinal mucosa and protect against infectious molecules and innocuous antigens; thus, these cells play key role in maintaining immune homeostasis and tolerance in the gut [35]. Increased levels of pro-inflammatory cytokines, including IL-1, IL-12, TNF, and IL-6, produced by activated APCs are a major immunological feature in IBD [36]. Particularly, elevated IL-1β level is observed in the initial process of colonic inflammation, which subsequently leads to the recruitment of IL-17A-producing innate lymphoid cells 3 and Th17 cells [37,38]. Furthermore, activated DCs in the mucosal tissue highly express CD40, CD86, CD83, and CD80, triggering consecutive inflammation, including increased production IL-12, overexpression of chemokine receptor, and elongation of DCs retention in inflamed tissue [39–41]. These inflammatory responses effectively return to normal when overproduction of TNF-α is inhibited [42]. In the case of IBD patients, DCs are accumulated at sites of inflammation, and they express higher level of co-stimulator molecules and pro-inflammatory cytokines than healthy control tissue. These activated mediators prime abnormal T cell responses to the enteric flora, which increase reactivity of effector T cells with inflamed mucosa [35]. Therefore, phenotypical and functional control of DCs is considered as a valuable therapeutic target for IBD.

During pathogen-induced DC activation, Toll-like receptors recognize microbial structural elements and consecutively lead to nuclear transcription of NF-κB and activation of MAPK family members [43]. Activation of these signals is important in mediating up-regulation of MHC class and costimulatory molecules as well as pro-inflammatory cytokines [44]. Furthermore, NF-κB induces production of IL-1β by providing the signal which activates the NLRP3 inflammasome [45].

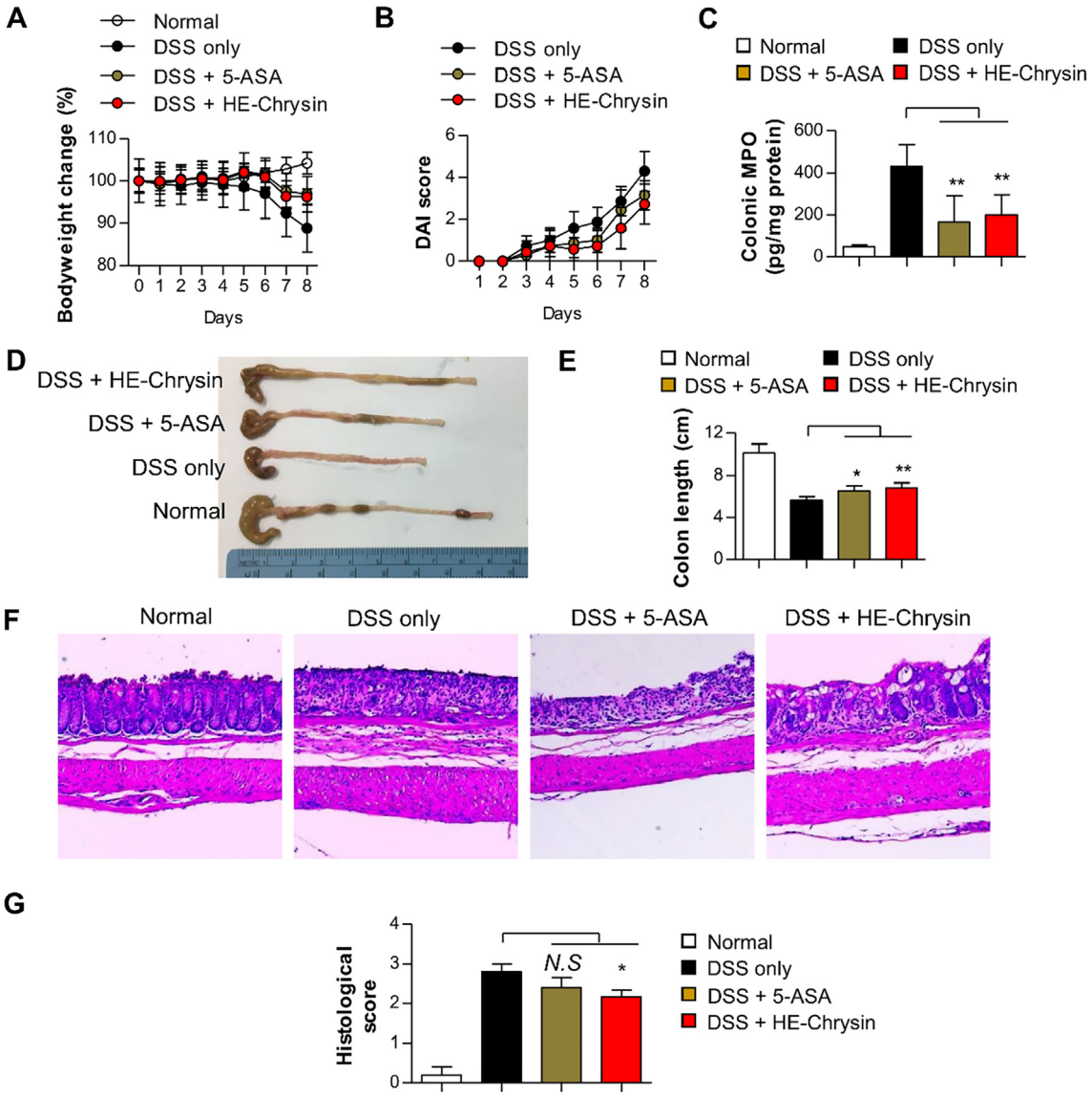

Fig. 6. Effects of HE-chrysin on DSS-induced colitis in mice. Mice were treated with 4% DSS in drinking water for 8 days. (A) Body weight changes and (B) Disease activity index (DAI) scores were monitored daily. Statistical analysis was performed by Kruskal-Wallis test. (C) Colonic MPO was measured by ELISA using colon tissue lysate. (D) Representative image of colon. (E) Changes in colon length. (F) Representative histological images (H&E staining) of mouse colon, 100 × magnification. (G) Histological score in H&E staining. Statistical analysis was performed by Tukey's multiple test. All data are shown as the mean ± SD ($n = 7$) of mice per group. *$p < 0.05$, and **$p < 0.01$ represent significant differences.

Our results showed HE-chrysin effectively inhibits LPS-induced overexpression of surface molecules and cytokines in BMDCs by inhibiting MAPK and NF-κB signaling, which inhibits the proliferation and activation of T cells.

Because of the similarities with human IBD and the convenience of using mouse models, the DSS-induced colitis model is widely used as an experimental murine model [46]. Exposure to DSS induced bodyweight loss, diarrhea, bleeding, increased MPO as an index of neutrophil infiltration, and histological changes, including infiltration of mononuclear cells and mucosal damage. Treatment with HE-chrysin alleviated these symptoms and pathological changes in the experimental colitis model. Many studies have shown that aberrant $CD4^+$ T cell responses cause colitis-associated pathology [47]. Generally, the DSS-induced colitis model is associated with the Th1- and Th17-type immune response caused by overproduction of cytokines, including IFN-γ, IL-1β, TNF-α, and IL-12 [32,48]. Our data showed that treatment with HE-chrysin reduced IFN-γ and IL-17A levels in MLN. These results indicate that HE-chrysin protects against DSS-induced colitis by attenuating the Th1- and Th17-type immune response.

5. Conclusion

In this study, we observed that HE-chrysin exhibited stronger inhibitory effects on LPS-induced pro-inflammatory cytokine production, overexpression of surface molecules, and antigen-presenting ability than chrysin in DCs. Treatment of DCs with HE-chrysin effectively impaired T cell proliferation and activation, and these anti-inflammatory activities were mediated by the regulation of MAPK and NF-κB signaling. Importantly, oral administration of HE-chrysin attenuated DSS-induced colitis symptoms and reduced Th1- and Th17-type cytokine levels. Further studies regarding toxicity and pharmacokinetics studies of HE-chrysin are recommended to utilize as a candidate for colitis.

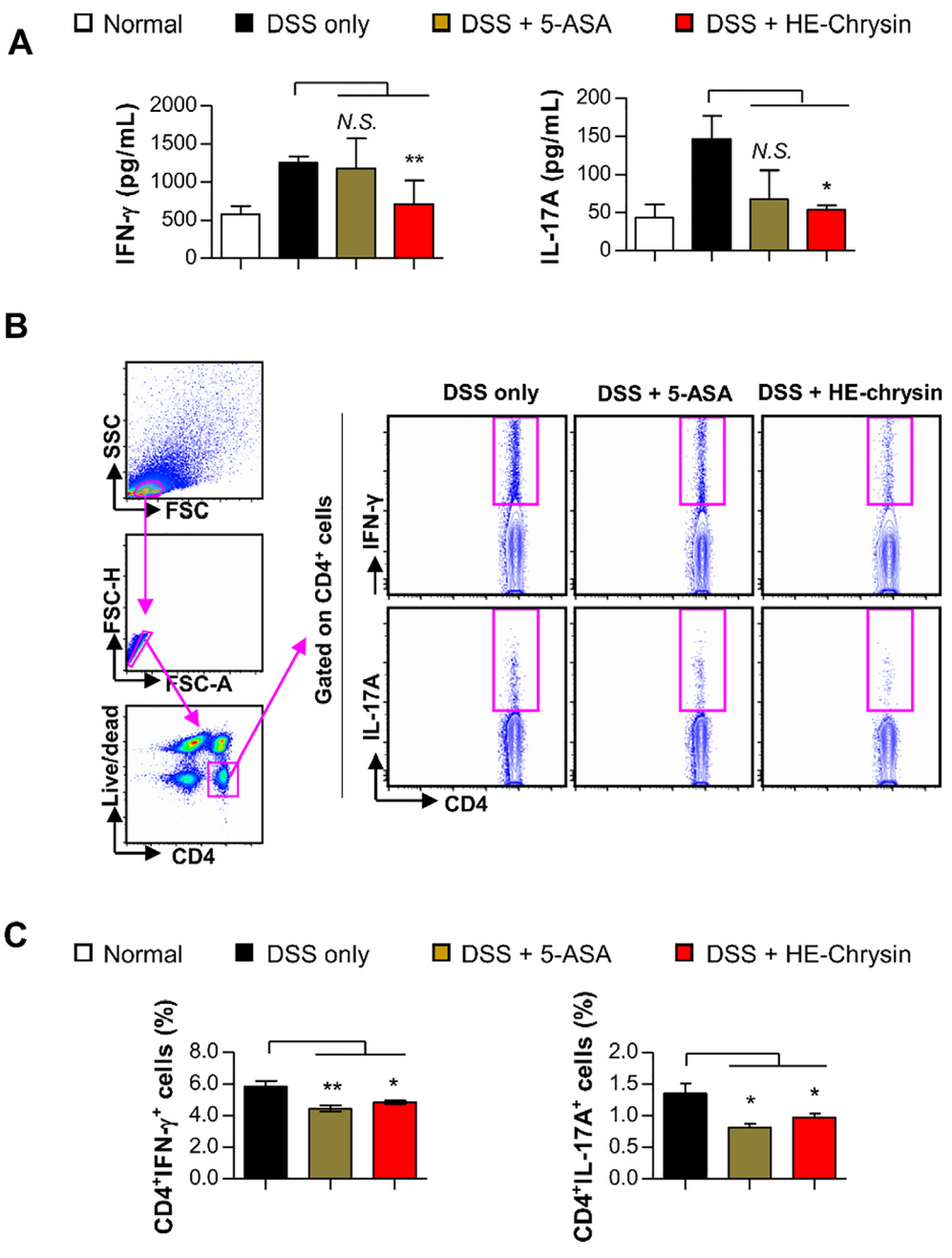

Fig. 7. Effects of HE-chrysin on Th1- and Th17- type cytokines in DSS-induced colitis. (A) Single cells isolated from MLN of mice in each group were stimulated with anti-CD3/CD28 Abs for 24 h, and the levels of IFN-γ and IL-17A in the culture supernatant were measured by ELISA. (B) Single cells isolated from MLN were stimulated with anti-CD3/CD28 in the presence of GolgiPlug and GolgiStop. The cells were stained with Live/Dead, anti-CD4, anti-IFN-γ, and anti-IL-17A Abs, and the intracellular levels of IFN-γ and IL-17A were analyzed by flow cytometry. (C) Bar graphs show the percentage of $CD4^+IFN\text{-}\gamma^+$ or $CD4^+IL\text{-}17A^+$ T cells. The value shows the mean ± SD ($n = 7$) of mice per group. Statistical analysis was performed by Tukey's multiple test. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ represent significant differences.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.105958.

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