

4'-O-Methylalpinumisoflavone inhibits the activation of monocytes/macrophages to an immunostimulatory phenotype induced by 27-hydroxycholesterol

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Abstract. The epidemiological, animal and cell effects of plant metabolites suggest versatile health benefits of flavonoids. However, whether flavonoids affect the deleterious biological activity of oxygenated cholesterol molecules remains to be elucidated. The present study investigated the effects of 4'-O-methylalpinumisoflavone (mAI) isolated from *Maclura tricuspidata* (*Cudrania tricuspidata*) on the 27-hydroxycholesterol (27OHChol)-induced activation of monocytes/macrophages using human THP-1 cells. mAI dose-dependently impaired the expression of C-C motif chemokine ligand (CCL)2 chemokine and the migration of monocytic cells enhanced by 27OHChol. mAI downregulated the surface and cellular levels of CD14 and inhibited the release of soluble CD14. This isoflavone significantly weakened the lipopolysaccharide responses that were enhanced in the presence of 27OHChol, and inhibited the transcription and secretion of the active gene product of matrix metalloproteinase-9. mAI also suppressed the expression of C-C motif chemokine receptor 5 ligands, including CCL3 and CCL4, and M1-phenotype markers induced by 27OHChol. Furthermore, mAI impaired phosphorylation of the nuclear factor- κ B p65 subunit without affecting the phosphorylation of Akt. These results indicate that mAI inhibits the activation of

monocytes/macrophages to the immunostimulatory phenotype in a milieu rich in 27OHChol, suggesting potential benefits of the flavonoid for the treatment of diseases in which the pathogenesis is linked to 27OHChol-induced inflammatory responses.

Introduction

The entire plant of *Maclura tricuspidata* Carr. (*Cudrania tricuspidata*) contains diverse phytochemicals, including xanthenes and flavonoids, which exert various bioactivities and have various medicinal and nutritional applications in East Asia (1). The extracts or components of *M. tricuspidata* exhibit a broad spectrum of pharmacological activities, including anti-inflammatory (2,3), antioxidant (4) and antitumor effects (5,6). Flavonoids, which represent a large subgroup of the phenolic class of specialized plant metabolites, have been confirmed to possess these beneficial effects, and >100 flavonoids have been isolated from *M. tricuspidata* (1), which are further classified into flavones, flavanones and isoflavones. Of these, certain flavonoids exhibit potential anti-inflammatory activity. The outcomes of isoflavones isolated from *M. tricuspidata* on inflammatory responses induced by endotoxins [lipopolysaccharide (LPS)] have previously been reported (2,3). However, whether flavonoids influence cell activation induced in an environment rich in oxygenated cholesterol molecules remains to be elucidated.

Oxygenated cholesterol molecules (oxysterols) are metabolites of cholesterol containing one or more hydroxyl groups (7). They possess numerous potent and diverse biological activities, of which several are implicated in the development of major chronic diseases, including atherosclerosis (8). 27-Hydroxycholesterol (27OHChol) is the major oxysterol present in advanced atherosclerotic lesions, followed by 7-oxygenated cholesterol molecules. These oxysterols comprise 75-85% of the oxysterols detected in plaques from different sites (7,9). The potent inflammatory activity of oxysterol is a major force driving the progression and complication of diseases. Among the aforementioned

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Abbreviations: mAI, 4'-O-methylalpinumisoflavone; 27OHChol, 27-hydroxycholesterol

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oxysterols, 27OHChol activates endothelial cells and monocyte/macrophage cells, which in turn enhance the expression of inflammatory mediators, including adhesion molecules, chemokines, pattern recognition receptors and matrix metalloproteinases (MMPs) (10-12). The secreted mediators then amplify inflammation by recruiting and activating monocytic cells and T cells (12-14). As the resultant chain of sequences in the presence of 27OHChol sustains and expands the inflammatory process by activating vascular cells, it is considered a promising therapeutic target for atherosclerosis (12).

Macrophages, which serve a central role in all stage of the inflammatory response, are versatile. Macrophages are activated towards an M1 phenotype upon stimulation by the Gram-negative product LPS and by inflammation-related cytokines tumor necrosis factor (TNF)- α or interferon (IFN)- γ , alone or in combination. The M1 phenotypes produce toxic effector molecules, including reactive oxygen species and nitric oxide, and inflammatory cytokines, including C-C motif chemokine ligand (CCL)2, interleukin (IL)-1 β , tumor necrosis factor (TNF)- α and IL-6. They are involved in polarized Th1 responses as inducers and effector cells, and mediate resistance against intracellular pathogens (15,16). M2-like polarized macrophages are induced in response to Th2-related cytokines IL-4 or IL-13 (M2a), to the concomitant triggering of Fc γ receptors, Toll-like receptors and immune complexes (M2b) and to anti-inflammatory molecules, including TGF- β , IL-10 and glucocorticoids (M2c) (17). The M2 phenotypes secrete anti-inflammatory mediators, IL-10 and TGF- β , have high levels of scavenger, mannose and galactose type receptors, and are involved in polarized Th2 responses, immune regulation, dampening of inflammation and tissue remodeling (15-17). M1 and M2 phenotypes are present in human atherosclerotic plaques with distinct spatial distribution. The fibrous caps of lesions exhibit no significant differences between subsets, whereas M2 macrophages outnumber the M1 phenotype in vascular adventitial tissue, and M1 macrophages dominate the rupture-prone shoulder regions over M2-type cells (18), suggesting that the balance between M1/M2 polarization is of critical importance to the homeostasis of vascular tissue and progression/regression of the vascular disease.

The present study was performed to investigate the effects of isoflavones isolated from *M. tricuspidata* on the responses of monocyte/macrophage to 27OHChol. This is the first study, to the best of our knowledge, to report that a certain type of flavonoid inhibits the activation of monocytes/macrophages to the immunostimulatory phenotype in a milieu rich in oxygenated cholesterol molecules.

Materials and methods

Cell culture. The human THP-1 monocyte/macrophage cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA; cat. no. TIB-20) and cultured in RPMI medium 1640 supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO₂. Penicillin (50 U/ml) and streptomycin (50 μ g/ml) were added to prevent bacterial contamination. A Jurkat cell line stably expressing C-C motif chemokine receptor 5 (CCR5), which was constructed by transfecting Jurkat cells (clone E6-1; ATCC; cat. no. TIB-152) with a eukaryotic expression vector

containing the CCR5 gene, was provided by Dr Y. Yun (Ewha Womans University, Seoul, Korea) (19). The CCR5-expressing Jurkat T cells were cultured in RPMI medium 1640 supplemented with 10% FBS in the presence of geneticin.

Reagents. The 4'-O-methylalpinumisoflavone (mAI) and alpinumisoflavone were isolated from the fruits of *M. tricuspidata*, as previously described (20). 27OHChol (cat. no. sc-358756) and antibodies against CD14 (cat. no. sc-9150), p65 (cat. no. c-8008), phosphorylated p65 (cat. no. sc-136548), β -actin (cat. no. sc-47778), anti-rabbit IgG-HRP (cat. no. sc-2357) and anti-mouse IgG-HRP (cat. no. sc-2005) were purchased from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA). Anti-Akt (cat. no. 4691) and anti-phosphorylated Akt (cat. no. 4060) antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). LPS prepared from *Escherichia coli* K12 (cat. no. tlr1-eklps) was purchased from InvivoGen (San Diego, CA, USA).

Treatment of cells. THP-1 cells (2.5 \times 10⁵ cells/ml) were serum-starved overnight by incubation in RPMI 1640 medium supplemented with 0.1% bovine serum albumin (BSA; GenDEPOT, Katy, TX, USA). After the serum-starvation, cells were treated with 27OHChol in the presence of the indicated concentrations of mAI or alpinumisoflavone. In the LPS response experiment, serum-starved THP-1 cells were cultured for 24 h with 27OHChol in the presence of mAI, followed by stimulation for 9 h with LPS (100 ng/ml).

Viability assay. The viability of the THP-1 cells was determined via the trypan blue dye exclusion method using a Vi-Cell XR cell counter (Beckman Coulter, Inc., Indianapolis, IN, USA). The viability of cells cultured in medium alone was considered 100%. Data are expressed as the mean \pm standard deviation (n=3 replicates for each group).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Reagents for RT were purchased from Promega Corporation (Madison, WI, USA) unless otherwise stated. Total RNAs isolated using TRIzol reagent (Thermo Fisher Scientific, Inc.) were reverse transcribed at 42°C for 1 h with 100 U Moloney Murine Leukemia Virus reverse transcriptase (cat. no. M1701) in a 10 μ l reaction volume, containing 50 mM Tris-HCl (pH 8.3 at 25°C), 55 mM KCl, 3 mM MgCl₂, 10 mM DTT, 1 μ g oligo (dT) 15 primers (cat. no. C110B), 0.125 mM each dNTP (cat. no. U1511), and 40 U RNase inhibitor (cat. no. N2111). Subsequent qPCR was performed following the protocol described by Kim *et al.* (21). Each 20- μ l reaction consisted of 10 μ l of SYBR-Green Master Mix, 2 μ l of forward and reverse primers (10 pM each) of genes to be analyzed, and cDNA template. The thermal cycling conditions consisted of 95°C for 10 min, followed by 45 cycles at 95°C for 10 sec, 50°C for 10 sec and 72°C for 10 sec. The relative expression of each gene was calculated as the ratio to housekeeping gene (GAPDH) using the 2^{- $\Delta\Delta$ C_q} method, as previously reported (22). The primers used were as follows: CCL2, 5'-CAGCCAGATGCAATCAATGCC-3' (forward) and 5'-TGGAAATCCTGAACCCACTTCT-3' (reverse); CCL3, 5'-AGTTCTCTGCATCACTTGCTG-3' (forward) and 5'-CGGCTTCGCTTGTTAGGAA-3' (reverse); CCL4, 5'-CTGGGT

CCAGGAGTACGTGT-3' (forward) and 5'-GCGGAGAGG AGTCCTGAGTA-3' (reverse); CD14, 5'-ACGCCAGAACCT TGTGAGC-3' (forward) and 5'-GCATGGATCTCCACC TCTACTG-3' (reverse); MMP-9, 5'-GCACGACGTCTT CCAGTACC-3' (forward) and 5'-CAGGATGTCATAGG TCACGTAGC-3' (reverse); IL-1 β , 5'-TGAGCTCGCCAGTG AAATGA-3' (forward) and 5'-AGATTTCGTAGCTGGATG CCG-3' (reverse); TNF- α , 5'-CCCAGGGACCTCTCT CTAATC-3' (forward) and 5'-ATGGGCTACAGGCTTGTC ACT-3' (reverse); C-X-C motif chemokine ligand (CXCL)10, 5'-TGTACGCTGTACCTGCATCA-3' (forward) and 5'-GGA CAAAATTGGCTTGCAAGGA-3' (reverse); CXCL11, 5'-AAG CAGTGAAAGTGGCAGAT-3' (forward) and 5'-TAAGCC TTGCTTGCTTCGAT-3' (reverse); CD80, 5'-GCAGGGA ACATCACCATCCA-3' (forward) and 5'-TCACGTGGAT AACACCTGAACA-3' (reverse); CD86, 5'-GGACTAGCAC AGACACACGGA-3' (forward) and 5'-CTTCAGAGGAGCA GCACCAGA-3' (reverse); CD163, 5'-AAAAAGCCACAAC AGGTCGC-3' (forward) and 5'-CTTGAGGAACTGCAA GCCG-3' (reverse); CD206, 5'-TGAATTGTACTGGTCTG TCCT-3' (forward) and 5'-CTGTGGTGCTGTGCATTTA TCT-3' (reverse); liver X receptor (LXR) α , 5'-AAGCCCTGC ATGCCTACGT-3' (forward) and 5'-TGCAGACGCAGTG CAAACA-3' (reverse); GAPDH, 5'-GAAGGTGAAGGTCG GAGT-3' (forward) and 5'-GAAGATGGTGATGGGATTTC-3' (reverse).

Enzyme-linked immunosorbent assay (ELISA). The protein levels of CCL2, CCL3, CCL4 and soluble CD14 (sCD14) secreted from cells were measured by using commercially available ELISA kits following the manufacturer's protocol (R&D Systems, Inc., Minneapolis, MN, USA).

Migration assay. Cell migration was investigated using Transwell permeable supports (Costar; Corning, Inc., Corning, NY, USA) as previously described (21). The top wells of the 5- μ m-pore polycarbonate Transwell inserts were loaded with THP-1 cells or Jurkat T cells expressing CCR5 (5×10^5 cells in 100 μ l of 0.1% BSA) and then inserted into a reservoir filled with supernatants containing chemoattractants. Following incubation for 2 h in a cell culture CO₂ chamber, the number of cells migrated into the reservoir were counted using the Vi-Cell XR cell counter (Beckman Coulter, Inc.).

MMP-9 gelatinolytic activity. The activity of MMP-9 was assessed by gelatin zymography following the protocol described by Kim *et al* (21). Following the removal of cell debris by centrifugation at 760 x g for 10 min at 4°C, the culture medium was concentrated 30-fold using the Vivaspin 2 centricon. An equal volume (20 μ l) of each sample was electrophoresed on 8% polyacrylamide gels containing 0.15% gelatin, and the MMPs were renatured by the removal of SDS through immersion of the gel in Triton X-100 [2.5% (v/v) in Tris-HCl, pH 7.5] for 1 h with gentle agitation. Following a rinse in water, the gels were incubated in an activation buffer (50 mM Tris-HCl pH 7.7, 0.2 M NaCl, 5 mM CaCl₂, 0.02% Brij 35 and 0.01% NaN₃) overnight at 37°C to allow proteolysis of the gelatin substrate. To visualize proteolytic bands, the gels were stained in 0.2% Coomassie Brilliant blue R-250 followed by destaining with methanol/acetic acid (20/10%) solution. The

gel was photographed by using ZoomBrowser EX 5.0 (Canon, Inc., Tokyo, Japan).

Flow cytometric analysis. The THP-1 cells were harvested by centrifugation at 200 x g for 5 min at room temperature, which was followed by incubation for 40 min with anti-CD14 antibody conjugated with fluorescent dye (Santa Cruz Biotechnology, Inc.) diluted to 1:100 in FACS buffer (2 mM EDTA and 0.2% BSA in PBS) at 4°C. The cells were washed twice with PBS and resuspended in 1% paraformaldehyde in phosphate-buffered saline (PBS). The fluorescence was analyzed using a flow cytometer.

Western blot analysis. Western blotting was performed as described previously (21). The cells were lysed with lysis buffer (1% SDS, 1 mM NaVO₃ and 10 mM Tris-HCl, pH 7.4) containing protease inhibitors, and the supernatants were isolated following centrifugation (15,000 x g) for 5 min at 4°C. Following determination of protein concentration using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.), an equal quantity of protein (20 μ g) was separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. Following blocking for 1 h in 5% skim milk in 0.1% Tween-20/TBS at room temperature, the membranes were incubated with primary antibodies diluted to 1:1,000 (v/v) in the blocking solution overnight at 4°C. The membranes were then washed three times with 0.1% Tween-20/TBS for 10 min each and incubated with horseradish peroxidase-conjugated secondary antibodies diluted in the blocking solution (1:8,000) for 1 h at room temperature. Following three washes with washing buffer for 10 min each, the bands were detected using ECL western blotting substrate.

Statistical analysis. Statistical analysis was performed via one-way analysis of variance, followed by Dunnett's multiple comparison test, using PRISM 5.0 (GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

mAI exerts minimal effects on the viability of monocytic cells. The present study determined the effects of alpinumisoflavone and mAI, the two most abundant isoflavones isolated from the fruits of *M. tricuspidata*, on the viability of THP-1 cells (Fig. 1A). Cell viabilities were 92.4, 89.7 and 86.5% following exposure for 48 h to 2.5, 5 and 10 μ g/ml of mAI, respectively. However, significant cytotoxicity was observed with identical concentrations of alpinumisoflavone. When the monocytic cells were treated with 1 μ g/ml of mAI in the presence of 27OHChol at the concentration used of 2 μ g/ml, viability was not decreased (Fig. 1B). Therefore, subsequent experiments were performed with mAI at sub-cytotoxic concentrations.

mAI inhibits the expression of CCL2 and monocytic cell migration. Whether mAI influences the activation of monocytes/macrophages was investigated by measuring the expression of CCL2 and monocytic cell migration. 27OHChol activated the THP-1 monocyte/macrophage cells as indicated

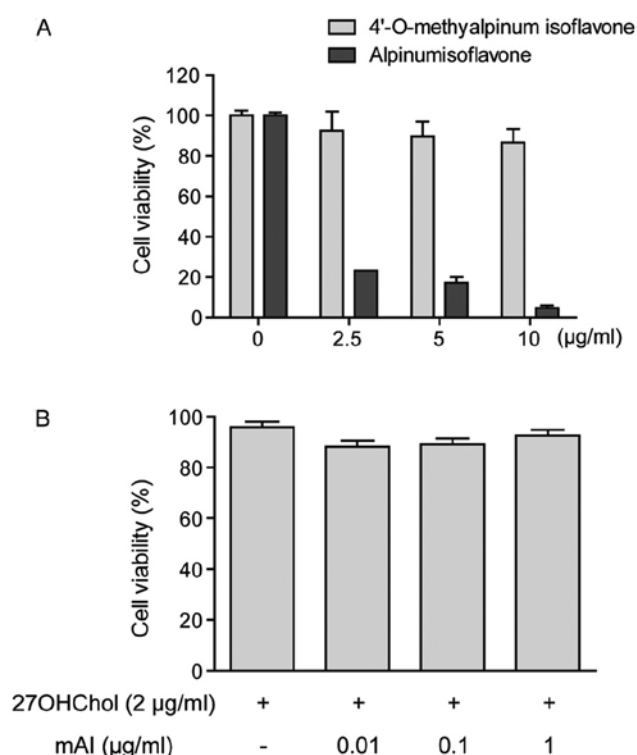


Figure 1. Effects of mAI or alpinumisoflavone on the viability of THP-1 cells. (A) THP-1 cells (2.5×10^5 cells/ml) were serum-starved overnight in RPMI medium containing 0.1% endotoxin-free BSA, followed by treatment for 48 h with the indicated concentrations of mAI or alpinumisoflavone, and cell viability was determined. (B) Serum-starved THP-1 cells were treated for 48 h with the indicated concentrations of mAI in the presence of 27OHChol, and cell viability was determined. mAI, 4'-O-methylalpinumisoflavone; 27OHChol, 27-hydroxycholesterol.

by enhanced expression of the CCL2 chemokine, however, the expression was inhibited by mAI in a dose-dependent manner. The levels of CCL2 transcript increased 26.4-fold following stimulation with 27OHChol, whereas this increase was suppressed to 19.3- 11.3- and 3.7-fold in the presence of 0.01, 0.1 and 1 µg/ml of isoflavone, respectively (Fig. 2A). However, the basal expression level of CCL2 was not altered by treatment with mAI (Fig. S1A). mAI affected the secretion of CCL2 in a pattern similar to that observed with transcription of the gene. The level of secreted CCL2 significantly increased following stimulation with 27OHChol compared with that of unstimulated THP-1 cells, however, the levels of secreted CCL2 were dose-dependently reduced in the presence of mAI (Fig. 2B). Whether the decreased secretion of CCL2 altered the migration of monocytic cells was also investigated. An increase in monocytic cell migration was observed in response to the supernatant isolated from cells stimulated with 27OHChol, however, the migration was significantly reduced in response to the media isolated from cells treated with 0.1 and 1 µg/ml of the isoflavone (Fig. 2C). Collectively, these results suggested that mAI inhibited the production of CCL2 induced by 27OHChol and thereby monocytic cell migration.

mAI downregulates the expression of CD14. The present study also investigated whether mAI has any effects on the expression of CD14 pattern recognition receptor. Stimulation

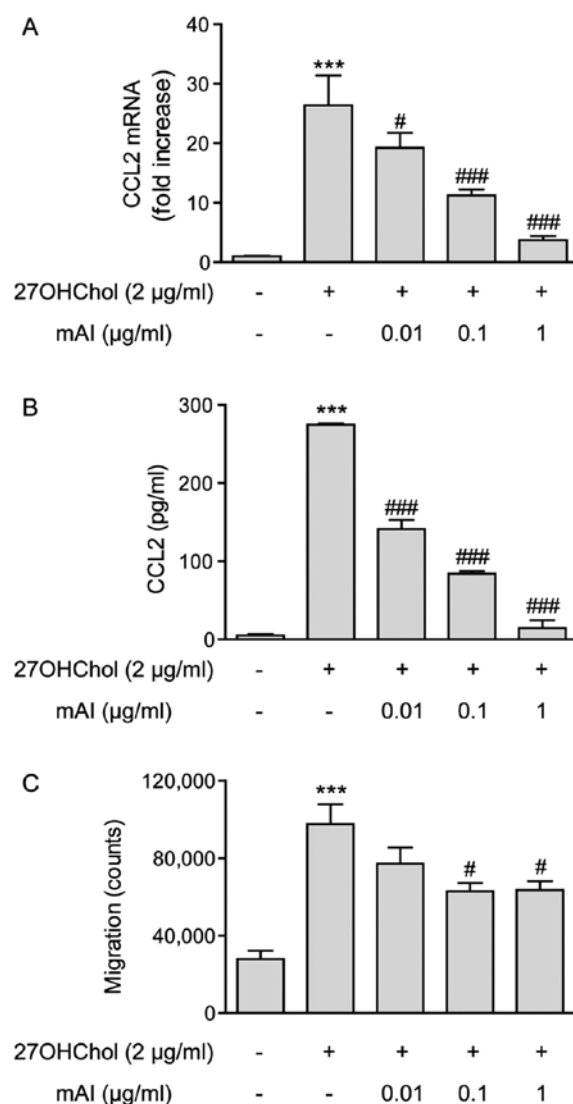


Figure 2. mAI decreases the expression of CCL2 and monocytic cell migration. THP-1 cells (2.5×10^5 cells/ml) were serum-starved by incubating overnight in RPMI medium supplemented with 0.1% BSA (endotoxin-free) and cultured for 48 h with 27OHChol in the presence of the indicated concentrations of mAI. (A) CCL2 transcript levels were assessed by reverse transcription-quantitative polymerase chain reaction analysis. The y-axis values represent the increases in mRNA levels of CCL2 normalized to GAPDH levels, relative to that of the untreated THP-1 cells (control). *** $P < 0.001$, vs. control; ### $P < 0.001$, vs. 27OHChol; # $P < 0.05$, vs. 27OHChol. (B) Culture media were isolated, and the protein levels of CCL2 in the media were measured by enzyme-linked immunosorbent assay. *** $P < 0.001$, vs. control; ### $P < 0.001$, vs. 27OHChol. (C) Monocytic cells were exposed to the conditioned media isolated above, and migration of monocytic cells was measured using a chemotaxis assay. *** $P < 0.001$, vs. control; # $P < 0.05$, vs. 27OHChol. Data in all graphs are expressed as the mean \pm standard deviation ($n = 3$ replicates for each group). CCL2, C-C motif chemokine ligand 2; mAI, 4'-O-methylalpinumisoflavone; 27OHChol, 27-hydroxycholesterol.

of the THP-1 cells with 27OHChol resulted in an increased percentage of cells expressing membrane CD14 (mCD14), from 11.2 to 27.9%, compared with that in untreated cells. However, the increase was suppressed to 25.9, 18.0 and 8.4%, following treatment with 0.01, 0.1 and 1 µg/ml of mAI, respectively, as determined by flow cytometry (Fig. 3A). Whether mAI affected the cellular level of CD14 was determined by western blotting. The protein level of CD14 in the THP-1 cells increased following stimulation with 27OHChol, however,

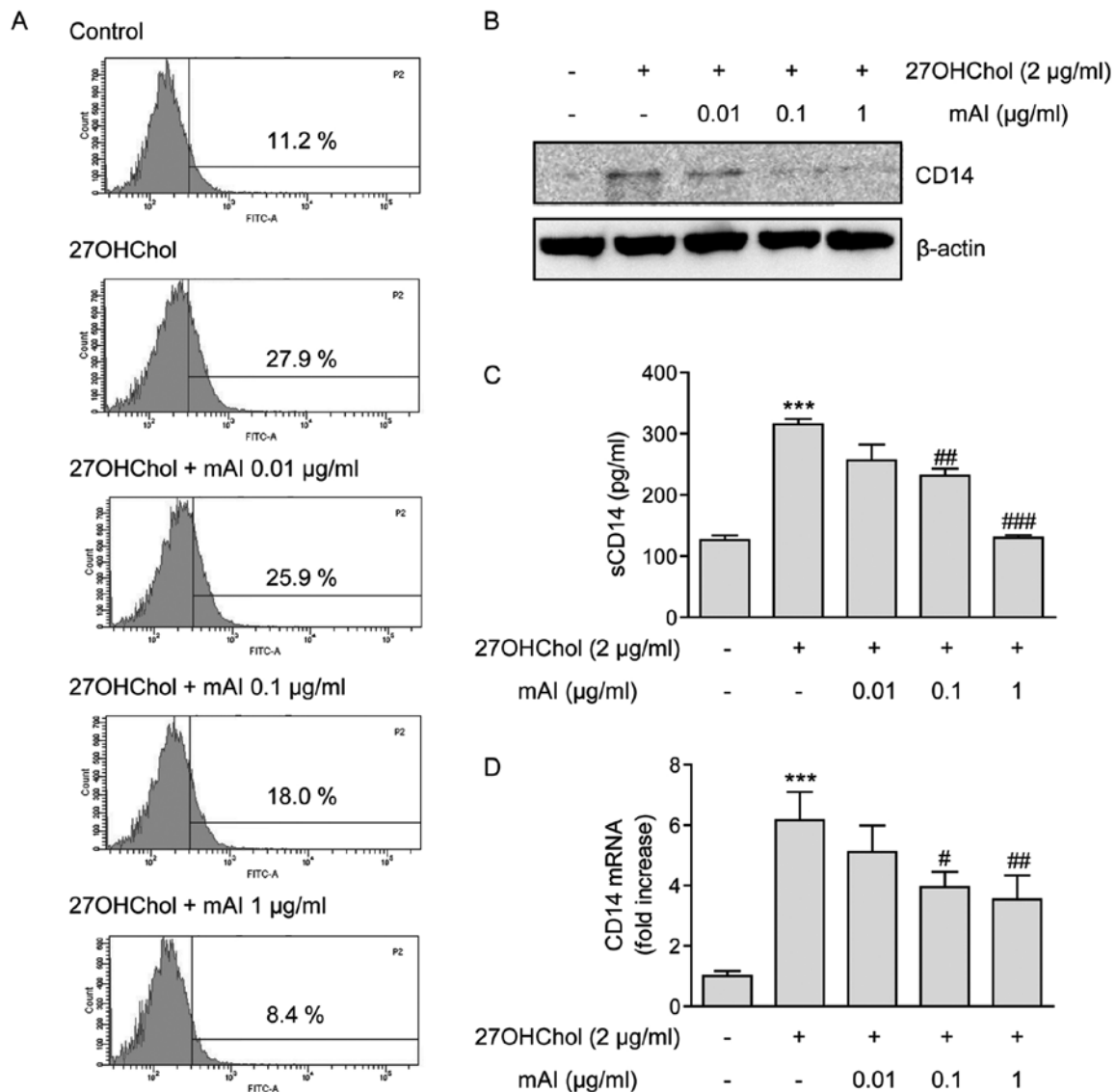


Figure 3. Downregulation of mCD14 on monocytic cells and the inhibition of sCD14 release by treatment with mAI. Serum-starved THP-1 cells (2.5×10^5 cells/ml) were cultured for 48 h with 27OHChol (2 $\mu\text{g/ml}$) in the presence of the indicated concentrations of mAI. (A) THP-1 cells immunostained with surface CD14 were analyzed by flow cytometry. (B) CD14 protein was detected by western blot analysis. (C) Secretion of sCD14 proteins into medium was measured with an enzyme-linked immunosorbent assay. *** $P < 0.001$, vs. control; ### $P < 0.001$, vs. 27OHChol; ## $P < 0.01$, vs. 27OHChol. (D) CD14 transcript levels were assessed by reverse transcription-quantitative polymerase chain reaction analysis. Data are expressed as the mean \pm standard deviation ($n = 3$ replicates for each group). *** $P < 0.001$, vs. control; # $P < 0.01$, vs. 27OHChol; # $P < 0.05$, vs. 27OHChol. mAI, 4'-*O*-methylalpinumisoflavone; 27OHChol, 27-hydroxycholesterol; sCD14, soluble CD14.

treatment with mAI resulted in decreased levels of cellular CD14 (Fig. 3B). The effects of mAI on sCD14 release were also determined. The THP-1 cells released a basal level of sCD14 (129.9 ± 7.6 pg/ml) into the media, and this was significantly increased to 315.3 ± 15.6 pg/ml following stimulation with 27OHChol (Fig. 3C). However, the release of sCD14 was significantly reduced in a dose-dependent manner by treatment with varying concentrations of the isoflavone. The level of sCD14 released from cells was reduced to the basal level following treatment with 1 $\mu\text{g/ml}$ of mAI.

As mAI inhibited the expression of CD14 at the protein level, its effects on the transcription of the gene were investigated. The levels of CD14 transcript increased 6.2-fold following stimulation with 27OHChol, compared with control cells cultured in medium, however, this was reduced to 3.9- and 3.5-fold in the presence of 0.1 and 1 $\mu\text{g/ml}$ of mAI, respec-

tively (Fig. 3D). However, the basal expression of CD14 was not influenced by treatment with mAI (Fig. S1B). Collectively, these results indicated that mAI suppressed the expression of CD14 induced by 27OHChol at the transcriptional and protein levels.

mAI impairs the secretion of active MMP-9. Activated monocyte/macrophage cells secrete active MMP-9 (22). Therefore, the present study investigated whether mAI influenced the expression of MMP-9. The stimulation of THP-1 cells with 27OHChol resulted in a 4.5-fold increase in the transcript levels of MMP-9, however, the level decreased to 2.7-, 1.8- and 0.9-fold in the presence of 0.01, 0.1 and 1 $\mu\text{g/ml}$ of the isoflavone, respectively (Fig. 4A). However, treatment with mAI alone did not cause a change in the basal level of the MMP-9 transcript (Fig. S1B). The activity of secreted MMP-9 was also

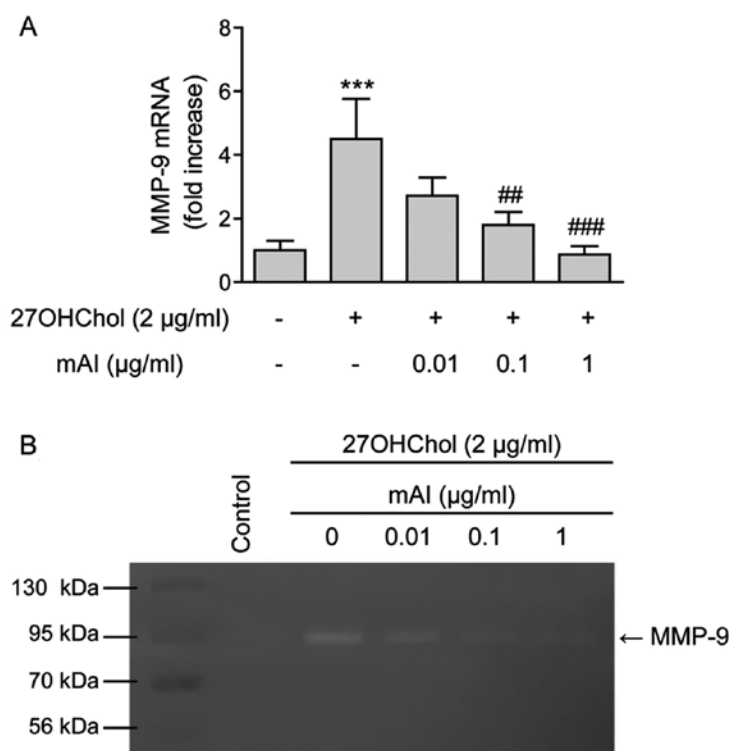


Figure 4. Effects of mAI on the expression of MMP-9. Serum-starved THP-1 cells were cultured for 48 h with 27OHChol in the presence of mAI. (A) MMP-9 transcript levels were assessed by reverse transcription-quantitative polymerase chain reaction analysis. Data are expressed as the mean \pm standard deviation (n=3 replicates for each group). ***P<0.001, vs. control; ###P<0.001, vs. 27OHChol; ##P<0.01, vs. 27OHChol. (B) Activity of MMP-9 secreted by cells was assessed by gelatin zymography. Control THP-1 cells were cultured for 48 h in medium alone. mAI, 4'-O-methylalpinumisoflavone; 27OHChol, 27-hydroxycholesterol; MMP-9, matrix metalloproteinase-9.

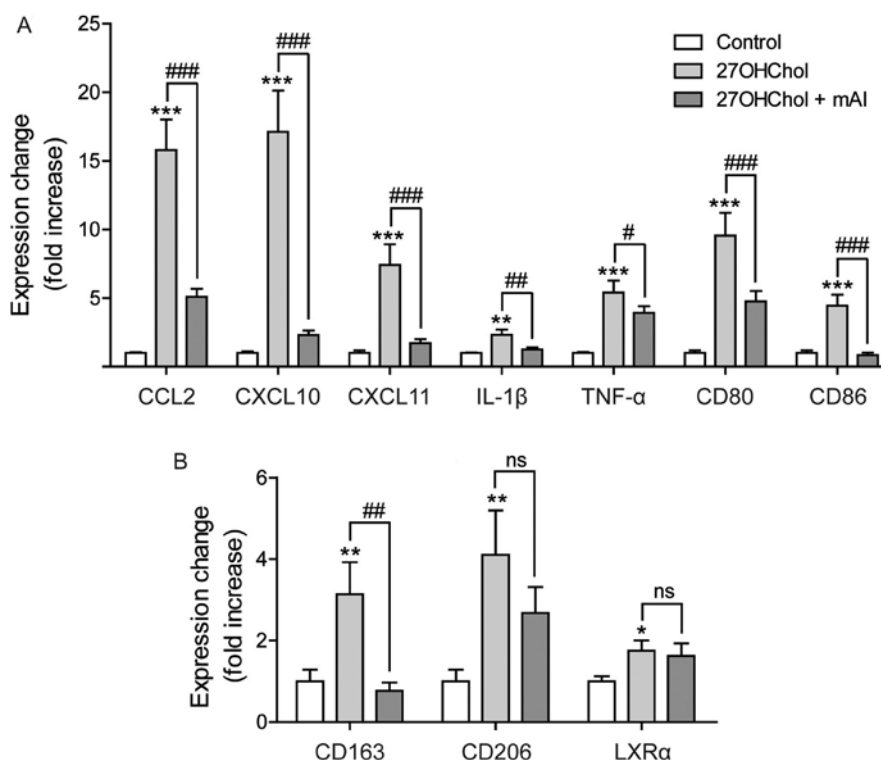


Figure 5. Effects of mAI on M1/M2 polarization markers. Serum-starved THP-1 cells (2.5×10^5 cells/ml) were cultured for 48 h with 27OHChol (2 μ g/ml) in the presence of mAI (1 μ g/ml). The transcript levels of markers for (A) M1 and (B) M2 polarization were assessed by reverse transcription-quantitative polymerase chain reaction analysis. Data are expressed as the mean \pm standard deviation (n=3 replicates for each group). ***P<0.001, vs. control; **P<0.01, vs. control; *P<0.05, vs. control; ###P<0.001, vs. 27OHChol; ##P<0.01, vs. 27OHChol; #P<0.05, vs. 27OHChol; ns, not significant; mAI, 4'-O-methylalpinumisoflavone; 27OHChol, 27-hydroxycholesterol; CCL2, C-C motif chemokine ligand 2; CXCL, C-X-C motif chemokine ligand; IL-1 β , interleukin-1 β ; TNF- α , tumor necrosis factor- α ; LXR α , liver X receptor α .

analyzed by gelatin zymography (Fig. 4B). The stimulation of THP-1 cells with 27OHChol resulted in increased MMP-9 activity in the supernatant. However, the gelatinolytic activity elevated by 27OHChol was attenuated by treatment with mAI. These results indicated that mAI inhibited the secretion of active MMP-9.

mAI suppresses the expression of M1 markers. M1 phenotype macrophages enhance the secretion of inflammatory and immunostimulatory mediators, whereas M2-type macrophages release immunoregulatory cytokines (18). Therefore, the present study investigated whether mAI affected macrophage polarization by examining the expression of M1 markers. The transcript levels of M1 markers CCL2, IL-1 β , TNF- α , CXCL10, CXCL11, CD80 and CD86 increased following stimulation with 27OHChol, and the levels of all seven markers were significantly reduced by treatment with mAI (Fig. 5A). The effects of mAI on the M2 marker were also investigated. 27OHChol increased the transcript levels of three M2 markers of CD163, CD206 and LXR- α . The IL-10 transcript was not detected by RT-qPCR analysis (data not shown). The levels of CD163 were significantly reduced by treatment with mAI, whereas the isoflavone did not significantly affect the expression of CD206 or LXR- α (Fig. 5B). Overall, these results suggested that mAI mainly regulates the expression of M1 markers.

mAI inhibits the production of CCR5 ligands and influences the migration of CCR5-expressing cells. Monocytic cells activated by 27OHChol secrete chemokines in addition to CCL2, which enhance the migration of CCR5-positive cells (13). Therefore, the present study investigated the effects of mAI on the expression of CCR5 ligands (CCL3 and CCL4) involved in the migration of the cell. The transcript levels of CCL3 and CCL4 were significantly elevated following stimulation with 27OHChol, and this elevation was significantly suppressed in a dose-dependent manner in the presence of 0.1 and 1 μ g/ml mAI (Fig. 6A). However, mAI did not alter the basal expression of CCL3 or CCL4 (Fig. S1A). mAI affected the secretion of CCL3 and CCL4 in a similar pattern to that observed for their transcription. Stimulation with 27OHChol resulted in enhanced secretion of CCL3 and CCL4, and their secretion was significantly suppressed by treatment with mAI (Fig. 6B). A migration assay was performed using CCR5-expressing Jurkat T cells. The supernatant isolated from cells exposed to 27OHChol significantly enhanced the migration of the CCR5-expressing cells (Fig. 6C). However, the migration was significantly decreased when the cells were exposed to supernatant isolated following treatment with 1 μ g/ml mAI. Overall, these results suggested that mAI inhibited the production of CCR5 ligands induced by 27OHChol and affected the migration of CCR5-expressing cells.

mAI impairs the phosphorylation of the nuclear factor (NF)- κ B p65 subunit, but not of Akt. The transcription factor NF- κ B serves a central role in the inducible expression of inflammatory genes (23). Therefore, whether mAI had any effects on the expression or phosphorylation of the NF- κ B p65 subunit was investigated (Fig. 7). Increased phosphorylation of p65 was observed following stimulation with 27OHChol,

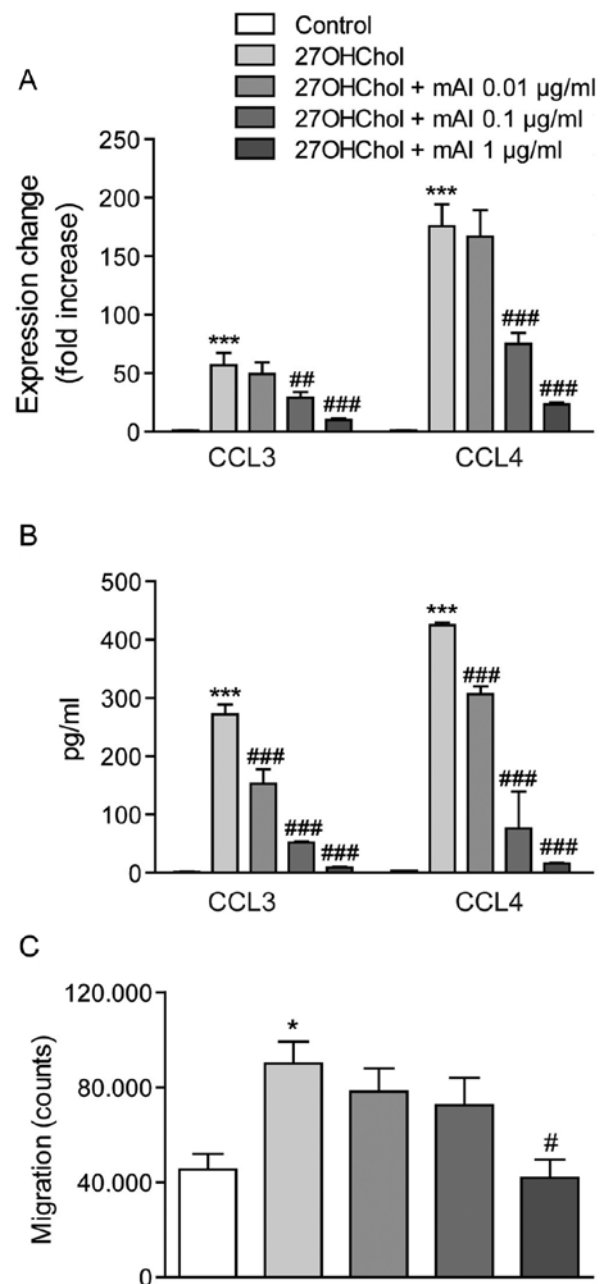


Figure 6. mAI inhibits the expression of CCL3 and CCL4 and reduces migration of CCR5-positive T cells. Serum-starved THP-1 cells (2.5×10^5 cells/ml) were cultured for 48 h with 27OHChol (2 μ g/ml) in the presence of the indicated concentrations of mAI. (A) Levels of CCL3 and CCL4 transcripts were assessed by reverse transcription-quantitative polymerase chain reaction analysis. *** $P < 0.001$, vs. control; *** $P < 0.001$, vs. 27OHChol; ** $P < 0.01$, vs. 27OHChol. (B) Secretion of CCL3 and CCL4 proteins into the media was measured by enzyme-linked immunosorbent assay following isolation of culture media. *** $P < 0.001$, vs. control; *** $P < 0.001$, vs. 27OHChol. (C) CCR5-expressing Jurkat T cells were exposed to the conditioned media, and migration of the cells was measured by chemotaxis assay. * $P < 0.05$, vs. control; # $P < 0.05$, vs. 27OHChol. In all graphs, data are expressed as the mean \pm standard deviation ($n = 3$ replicates for each group). mAI, 4'-O-methylalpinumisoflavone; 27OHChol, 27-hydroxycholesterol; CCR5, C-C motif chemokine receptor 5; CCL, C-C motif chemokine ligand.

which was almost completely inhibited by treatment with 1 μ g/ml mAI. However, this flavonoid did not influence the level of cellular p65 protein. The effects of mAI on Akt were also investigated. The phosphorylation of Akt was elevated upon stimulation with 27OHChol, but no alterations were

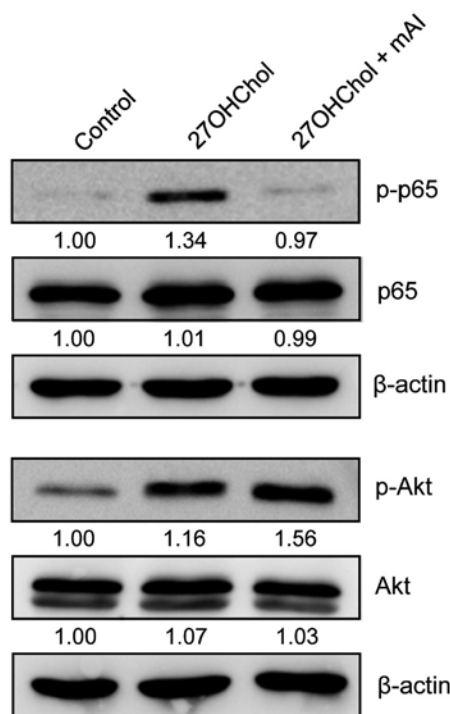


Figure 7. Effects of mAI on phosphorylation of p65 subunit. Serum-starved THP-1 cells (2.5×10^5 cells/ml) were exposed to 27OHChol (2 μ g/ml) for 4 h in the absence or presence of mAI (1 μ g/ml). Isolated cell extracts were subjected to immunoblotting for phosphorylated and unphosphorylated forms of p65 and Akt. mAI, 4'-O-methylalpinumisoflavone; 27OHChol, 27-hydroxycholesterol; p-, phosphorylated.

observed in phosphorylation or cellular protein levels of Akt following mAI treatment. These results indicated that mAI regulated the phosphorylation of the NF- κ B p65 subunit.

Discussion

The present study demonstrated inhibition of the 27OHChol-induced expression of multiple chemokines, including CCL2, CCL3 and CCL4, by treatment with mAI. CCL2 recruits monocytes to the sites of inflammation, thereby amplifying inflammation (24). CCL3 and CCL4 interact with CCR5 and enhance the migration of Th1 lymphocytes, as this receptor is characteristic of Th1 cells (25). Th1 cells produce IFN- γ , IL-2 and TNF- α , and evoke cell-mediated immunity and phagocyte-dependent inflammation (26). The decreased levels of chemokines result in the reduced migration of the corresponding aforementioned cell types. mAI also inhibits the secretion of active MMP-9, which is required for the migration of macrophages during inflammatory responses (27). The results of the present study suggest that these inhibitory effects are not due to cytotoxicity, but are specific; treatment with mAI and 27OHChol did not decrease cell viability, and treatment with mAI alone had no effects on the basal expression of the multiple genes, including CCL2, CCL3, CCL4 and MMP-9. The secretion of high levels of chemokines and active MMP-9 indicated the activation of monocyte/macrophage cells. Therefore, the results of the present study suggest that mAI is likely to inhibit activation of monocytic cells and thereby regulate inflammation and Th1 responses in an environment rich in 27OHChol.

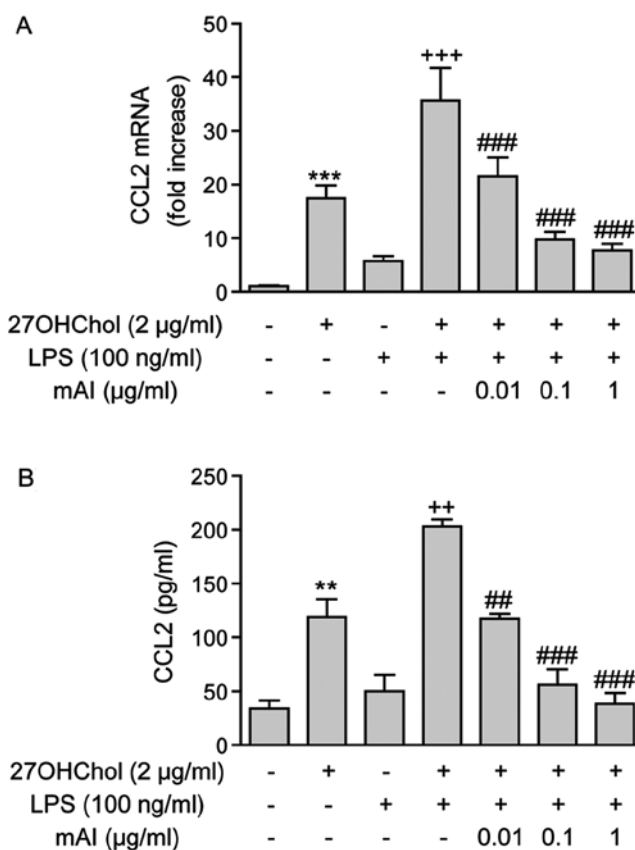


Figure 8. Effects of mAI on enhanced expression of CCL2 induced by 27OHChol + LPS. Serum-starved THP-1 cells (2.5×10^5 cells/ml) were cultured for 24 h with 27OHChol (2 μ g/ml) in the presence of the indicated concentrations of mAI, followed by stimulation for 9 h with or without LPS (100 ng/ml) from *Escherichia coli* K12. (A) Levels of the CCL2 transcript were assessed by reverse transcription-quantitative polymerase chain reaction analysis. *** $P < 0.001$, vs. control; *** $P < 0.001$, vs. 27OHChol; ### $P < 0.001$, vs. 27OHChol + LPS. (B) Culture media were isolated, and the level of CCL2 protein secreted into the media was measured by enzyme-linked immunosorbent assay. ** $P < 0.01$, vs. control; ++ $P < 0.01$, vs. 27OHChol; ### $P < 0.001$, vs. 27OHChol + LPS; ## $P < 0.01$, vs. 27OHChol + LPS. In both graphs, data are expressed as the mean \pm standard deviation ($n = 3$ replicates for each group). mAI, 4'-O-methylalpinumisoflavone; 27OHChol, 27-hydroxycholesterol; LPS, lipopolysaccharide; CCL2, C-C motif chemokine ligand 2.

CD14, a pattern recognition receptor, recognizes and binds to pathogen-associated molecular patterns, including LPS, and enhances responses to them (28). As mAI downregulated the expression of mCD14 and sCD14, whether the isoflavone altered responses to LPS was investigated. The transcript levels of the CCL2 gene increased following stimulation with 27OHChol or LPS alone (Fig. 8A). The addition of LPS to 27OHChol-stimulated cells resulted in a significant elevation of the levels of CCL2. However, the enhanced transcription of CCL2 was significantly attenuated by treatment with mAI. The isoflavone also influenced the secretion of CCL2 in a pattern similar to that of transcription of the gene (Fig. 8B). These results suggest that mAI prevents the overproduction of CCL2 induced by LPS, which is in agreement with a previous study that reported the inhibition of LPS-induced expression of inflammatory molecules in murine microglial cells, following treatment with this isoflavone (3). Collectively, these findings suggest that mAI downregulates the expression of CD14, thereby weakening responses to LPS.

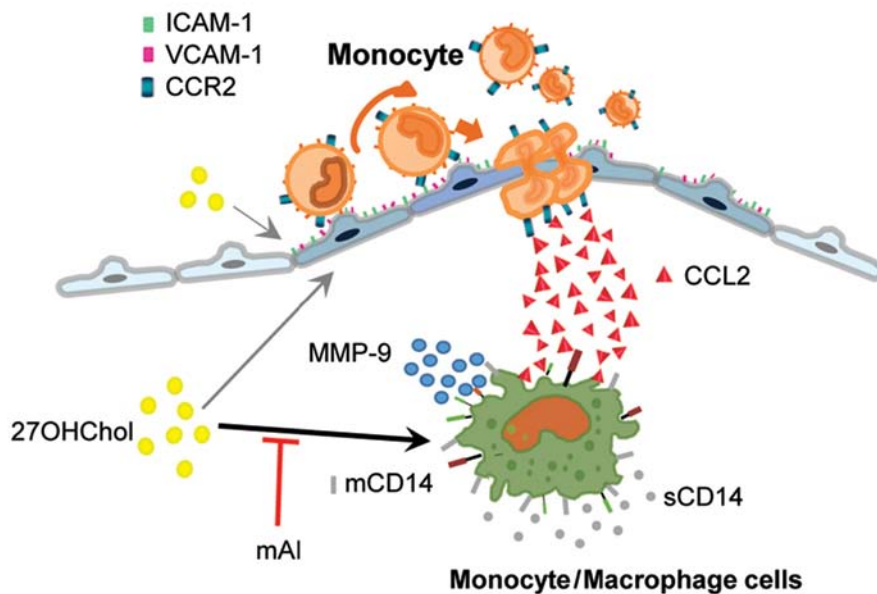


Figure 9. Beneficial effects of mAI in a milieu rich in 27OHChol. 27OHChol induced the expression of adhesion molecules on endothelial cells, enhanced the migration of monocytic cells and Th1 cells, and activates monocytic cells to produce pro-inflammatory molecules. mAI inhibits the activation of monocytes/macrophages induced by 27OHChol, and decreases the production of MMP-9 and chemokines, including CCL2, CCL3 and CCL4. The decreased concentration of chemokines results in impaired migration of the inflammatory cells to the sites of inflammation. mAI also attenuates responses to lipopolysaccharide via the downregulation of CD14. The anti-inflammatory effects may slow progression and reduce complications of diseases in which 27OHChol is involved in the pathogenesis. mAI, 4'-O-methylalpinumisoflavone; 27OHChol, 27-hydroxycholesterol; MMP-9, matrix metalloproteinase-9; CCL, C-C motif chemokine ligand; mCD14, membrane CD14; sCD14, soluble CD14; ICAM-1, intercellular adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1; CCR-2, C-C chemokine receptor type 2.

The present study attempted to determine the molecular mechanisms underlying the inhibitory effects of mAI. NF- κ B, of which the most common and well-characterized form is the p65/p50 heterodimer, induces the expression of genes involved in inflammation following phosphorylation and translocation into the nucleus (29). Akt induces the expression of chemokines and pattern recognition receptors in response to 27OHChol (13,14,30). Therefore, the effects of mAI on Akt and the p65 subunit, which is responsible for the high transcription activating potential of NF- κ B, were investigated. mAI inhibited the phosphorylation of p65, whereas the phosphorylation of Akt was unchanged, indicating specific inhibition of the phosphorylation of p65. In addition, the decreased levels of the phosphorylated p65 subunit coincided with decreased inflammatory mediators following treatment with mAI, which is consistent with a previous study that reported the inhibited phosphorylation of p65 and p50 subunits following LPS stimulation in the presence of this isoflavone (3). Collectively, these findings suggest that mAI is likely to exert its effects via the post-translational modification of NF- κ B.

Macrophages polarize to different functional phenotypes, M1 and M2, in response to microenvironmental stimuli. The present study demonstrated that mAI primarily suppresses the expression of M1 markers, suggesting the regulation of polarization to the M1 macrophage phenotype. The M1-type macrophages produce high levels of pro-inflammatory cytokines and promote Th1 responses, whereas the M2-type macrophages promote tissue and functional recovery (15). The inhibitory effects suggest that the isoflavone can suppress Th1 responses and subsequent inflammatory responses, as demonstrated by reduced chemokine expression and immune

cell migration. Taken together, these findings suggest that mAI regulates inflammation by inhibiting the expression of chemokines and affecting the polarization to M1-type macrophages. The negative effects of mAI on macrophage M1 polarization may also be linked to attenuated responses to LPS (2,3).

The present study demonstrated that mAI impairs the inflammatory and immunostimulatory effects of 27OHChol on monocytes/macrophages at the cellular and molecular levels. As 27OHChol is the most abundant cholesterol oxidation product in atherosclerotic lesions (9), the results of the present study suggest that mAI can reduce inflammation in the lesions (Fig. 9). Further investigation is required to evaluate the beneficial effects of mAI using animal models.

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Availability of data and materials

The datasets used and/or analyzed in the present study are available from the corresponding author on reasonable request.

Authors' contributions

KK, DL, and SKE designed the study and analyzed the data; JL, BYK, YS, and DHG performed the experiments and analyzed the data; JL, BYK, and KK wrote the manuscript. All authors approved the final version of the manuscript for publication.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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