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Mangiferin inhibits macrophage classical activation via downregulating interferon regulatory factor 5 expression

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Abstract. Mangiferin is a natural polyphenol and the predominant effective component of *Mangifera indica* Linn. leaves. For hundreds of years, *Mangifera indica* Linn. leaf has been used as an ingredient in numerous traditional Chinese medicine preparations for the treatment of bronchitis. However, the pharmacological mechanism of mangiferin in the treatment of bronchitis remains to be elucidated. Macrophage classical activation is important role in the process of bronchial airway inflammation, and interferon regulatory factor 5 (IRF5) has been identified as a key regulatory factor for macrophage classical activation. The present study used the THP-1 human monocyte cell line to investigate whether mangiferin inhibits macrophage classical activation via suppressing IRF5 expression *in vitro*. THP-1 cells were differentiated to macrophages by phorbol 12-myristate 13-acetate. Macrophages were polarized to M1 macrophages following stimulation with lipopolysaccharide (LPS)/interferon- γ (IFN- γ). Flow cytometric analysis was conducted to detect the M1 macrophages. Reverse transcription-quantitative polymerase chain reaction was used to investigate cellular IRF5 gene expression. Levels of proinflammatory cytokines and IRF5 were assessed following cell culture and cellular homogenization using enzyme-linked immunosorbent assay. IRF5 protein and nuclei co-localization was performed in macrophages with laser scanning confocal microscope immunofluorescence analysis. The results of the present study demonstrated that mangiferin significantly

inhibits LPS/IFN- γ stimulation-induced classical activation of macrophages *in vitro* and markedly decreases proinflammatory cytokine release. In addition, cellular IRF5 expression was markedly downregulated. These results suggest that the inhibitory effect of mangiferin on classical activation of macrophages may be exerted via downregulation of cellular IRF5 expression levels.

Introduction

Mangiferin, (1,3,6,7-tetrahydroxyxanthone-C2- β -D-glucoside) is a natural polyphenol (1), and the predominant effective component of *Mangifera indica* Linn. leaf (Fig. 1) (2-4). For hundreds of years, *Mangifera indica* Linn. leaves have been used in southern China as an ingredient in various traditional Chinese medicine preparations for the treatment of bronchitis. However, the underlying pharmacological mechanism of mangiferin in the treatment of bronchitis remains to be elucidated. Previous studies have demonstrated mangiferin exerts marked anti-inflammatory properties, and the pharmacological mechanism is associated with markedly decreased release of proinflammatory cytokines (5,6). Bronchial airway inflammation is recognized as a characteristic pathological change in bronchitis (7). Peripheral blood monocytes migrate into the bronchus and surrounding lung tissue by chemotaxis. Monocytes in the lung tissue differentiates into macrophage and polarizes to M1 macrophages when activated by lipopolysaccharide (LPS) or interferon- γ (IFN- γ) (8,9). This process is defined as macrophage classical activation (10). M1 macrophages produce and release large quantities of proinflammatory cytokines, including tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6) and interleukin-8 (IL-8) (11,12). Proinflammatory cytokines are important in human infection and non-infection immunity, however, excessive proinflammatory cytokines may result in tissue and cell damage (13,14). Serious tissue and cell damage can lead to deteriorating physiological function that may be life-threatening. Thus, M1 macrophages are crucial for the pathological process of bronchitis development. It may be a potential biological target of a therapeutic agent used to treat airway inflammation treatment, as targeting it may

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appropriately regulate polarization to M1 macrophages (15). Previous studies demonstrate that IRF5 is critical in macrophage polarization to M1 macrophages, as polarization can be inhibited by suppression of IRF5 expression in macrophages (16,17). It remains to be elucidated whether mangiferin inhibits macrophage polarization to M1 macrophages via suppressing IRF5 expression, and thus decreasing proinflammatory cytokines releasing.

Primary human macrophages are difficult to isolate in sufficient quantities from tissue and do not proliferate in culture, and the obtained cells often exhibit notable phenotypic heterogeneity. Monocyte-derived macrophages, however, are a useful option, as human blood monocytes are readily available in large numbers and may be differentiated into macrophages *in vitro* (18). Thus, the present study used THP-1 human monocyte to investigate whether mangiferin inhibits classical activation of macrophages via suppressing IRF5 expression *in vitro*.

Materials and methods

Mangiferin preparation. Mangiferin was provided by the Key Laboratory of Traditional Chinese Medical Pharmacology, Guangxi University of Chinese Medicine (Nanning, China). It was isolated from leaves of *Mangifera indica* Linn. harvested in the Baise region of China. The preparation method was performed as described previously (19). At the end of the extraction process, a yellow powder with 97.5% purity was obtained (Fig. 1). Purity of the mangiferin powder was detected by high performance liquid chromatography (Fig. 2) (20). The HPLC measurement was performed on an Agilent 1100 LC system (Agilent, Santa Clara, CA, USA) and Elite Hypersil C18 column (5 μ m, 4.6mmIDx250mm), with a gradient system of acetonitrile-0.1% phosphoric acid solution as a mobile phase at a flow rate of 1.0 ml/min. The detective wavelength was 258 nm, the column temperature was 30°C.

Reagents and devices. Methyl thiazolyl tetrazolium (MTT), dimethyl sulfoxide (DMSO), phorbol 12-myristate 13-acetate (PMA), *E. coli* O55:B5 lipopolysaccharide (LPS) and IFN- γ were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), L-glutamine, RPMI 1640, NucBlue Fixed Cell ReadyProbes reagent, BlockAid Blocking Solution and Image-iT Fixation/Permeabilization kit were purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Mouse anti-cluster of differentiation (CD) 86-PE monoclonal human antibody (cat. no. 555658; 1:20), mouse anti-CD80-FITC monoclonal antibody (cat. no. 555683; 1:20) and LSR Fortessa flow cytometer were obtained from BD Biosciences, Franklin Lakes, NJ, USA). Mouse anti-IRF5-eFluor 660 monoclonal antibodies was bought from eBioscience, Inc. (San Diego, CA, USA; cat. no. 50-9698; 1:20) and the Membrane and Cytoplasmic Protein Extraction kit was obtained from Sangon Biotech Co., Ltd., Shanghai, China). TNF- α , IL-1 β , IL-6, IL-8 and IRF5 ELISA kits were purchased from Cusabio (College Park, MD, USA). A Multiskan Spectrum 1500 microplate reader and Applied Biosystems 7500 Fast Real-Time PCR system were obtained from Thermo Fisher Scientific, Inc. TCS SP5 II laser scanning confocal microscope was purchased from Leica Microsystems

GmbH (Wetzlar, Germany) and QIAcube nucleic acid purification device was obtained from Qiagen GmbH (Hilden, Germany).

Cell culture and treatment. The THP-1 cell line was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and maintained at 5×10^5 cells/ml in RPMI 1640 medium supplemented with 10% FBS and 2 mmol/l L-glutamine at 37°C in 5% CO₂. THP-1 cells (2×10^5 cells/ml) were differentiated to macrophages using 200 nmol/l PMA for 3 days as previously described by Daigneault *et al.* (21). Following the initial 3 days stimulus, the PMA-containing media was removed and the cells were incubated in fresh RPMI 1640 medium supplemented with 10% FBS and 2 mmol/l L-glutamine. The cytotoxicity of mangiferin was determined using the MTT assay. Macrophages (2 ml/well) were seeded in flat-bottom 24-well culture plates at a cell density of 5×10^5 cells/ml at 37°C in a humidified incubator with 5% CO₂. Cells were allowed to attach and recover for 24 h, and then the cells were treated with different concentrations of mangiferin (0, 12.5, 25, 50, 100 or 200 μ mol/l), which was dissolved in RPMI 1640 medium containing 0.1% DMSO. Cells treated with an equivalent volume of RPMI 1640 medium containing 0.1% DMSO served as a blank control (control group). Following treatment with mangiferin or DMSO for 24 h, the cells were washed twice in PBS and then incubated for 4 h with LPS (1 μ g/ml) and IFN- γ (20 ng/ml) to allow polarization to M1 macrophages according to a method previously described by Juhas *et al.* (22). Cells without mangiferin treatment were regarded as the model control group (model group).

Flow cytometric analysis. Flow cytometric measurements were performed using an 11 color LSR Fortessa flow cytometer. Forward and side scatter light was used to identify cell population and measure size and granularity of the cells. Auto-fluorescence was recorded by analyzing unstained cells. Fc receptors were blocked by incubating cells with 100 μ g recombinant human IgG (from the Image-iT Fixation/Permeabilization kit; Invitrogen, Thermo Fisher Scientific, Inc.) for 15 min at 4°C prior to antibody staining. For detection of cell surface markers, 1 μ g CD86-PE and CD80-FITC antibodies were incubated with samples containing 3×10^5 THP-1 cells for 30 min at 4°C. Following incubation, all cells were washed in PBS and fluorescence was compared to unstained controls with 10,000 events recorded. A gate with CD86-PE⁺ and CD80-FITC⁺ was configured to select M1 macrophages. The FlowJo for Windows of version 7.1 (Emerald Biotech Co. Ltd, Hangzhou, China) was used for analysis.

Confocal microscopy analysis. Following washing twice in PBS, cells in the culture dishes were incubated for fixation and permeabilization using Image-iT Fixation/Permeabilization kit. Blocking solution was added following removal of the permeabilization solution and washing twice with PBS. Subsequently, 1 μ g of monoclonal anti-human IRF5-eFluor 660 antibodies and 2 drops of DAPI NucBlue Fixed Cell ReadyProbes reagent were added, and incubated with cells for 30 min at 4°C in the dark. Images of whole cell



Figure 1. *Mangifera indica* Linn. leaves, mangiferin powder and the chemical structure.

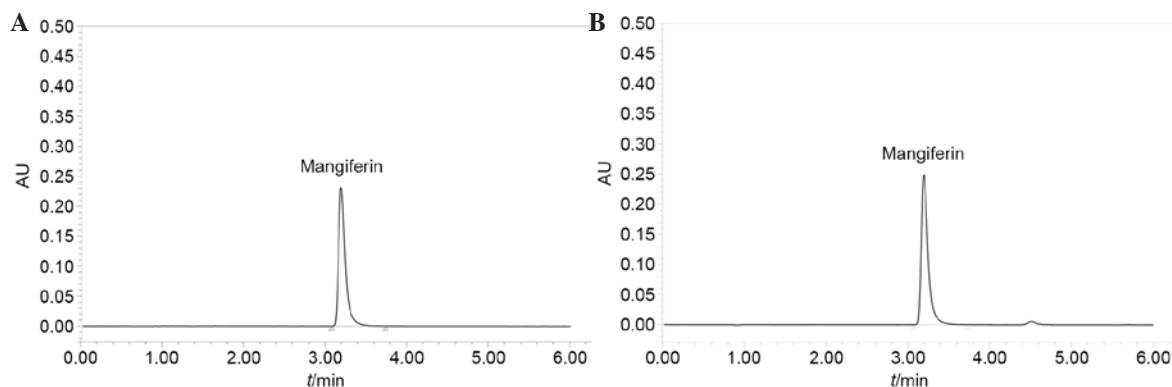


Figure 2. High performance liquid chromatograms of (A) mangiferin standard substance and (B) sample. AU, arbitrary units.

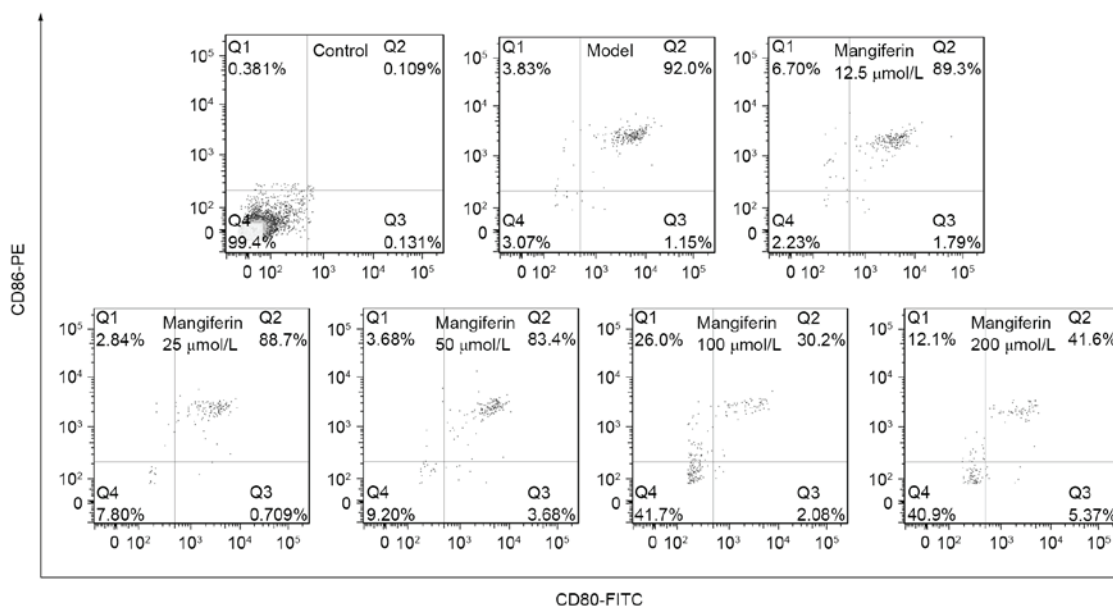


Figure 3. Flow cytometry dot plot of CD80⁺/CD86⁺ M1 macrophages in each group. Macrophages polarized to M1 macrophages following lipopolysaccharide/interferon- γ stimulation. Flow cytometric analysis was conducted to detect the M1 macrophages stained with anti-human CD86-PE and anti-human CD80-FITC. M1 macrophages were calculated and shown in second quadrant. CD, cluster of differentiation; PE, phycoerythrin; FITC, fluorescein isothiocyanate.

morphology and IRF5 staining were acquired using a Leica TCS SP5 II laser scanning confocal microscope.

Reverse transcription-quantitative polymerase chain reaction (RT-PCR). RNA was isolated and purified from cell homogenates using RNeasy Plus Mini kit on a QIAcube nucleic acid purification device. RNA was reverse transcribed with QuantScript RT kit according to the manufacturer's protocol. The primer and MGB-probe used to analyze IRF-5 gene expression were

designed and supplied by Invitrogen (Thermo Fisher Scientific, Inc.), the sequences are as follows: Forward, 5'-GTTGTTAAAGAGCCTGGCACCTA-3' and reverse, 5'-CTGGAGTGTGCA GAGATGACACA-3' for the primer; and 5'-CCGCTCTCACTTCAT-3' for the MGB-probe. RT-PCR was conducted on an ABI 7500 system. Samples were run in triplicate. The thermocycling conditions were as follows: 95°C for 5 min; 40 cycles of 95°C for 30 sec and 60°C for 90 sec. Data were collected at the end of each cycle. Gene copies were calculated from a standard

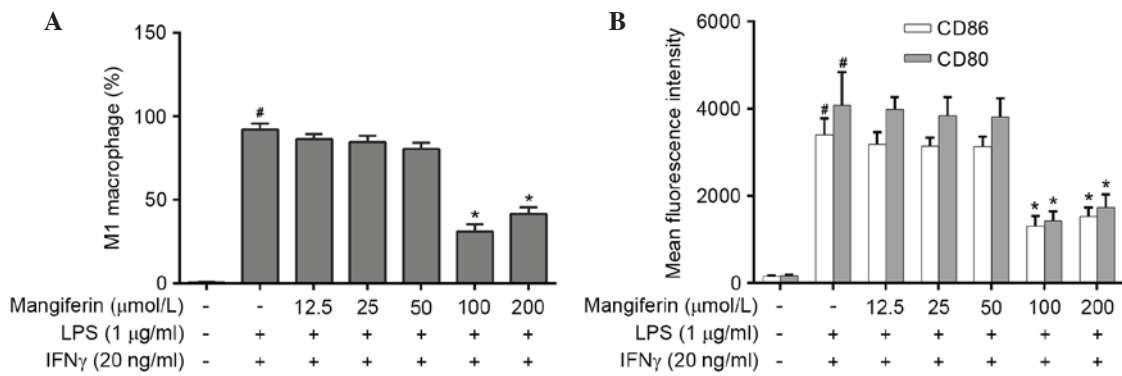


Figure 4. Bar plots of (A) mean percentage of M1 macrophages and (B) mean fluorescence intensity of CD80⁺/CD86⁺ in each group. Data are expressed as the mean \pm standard deviation. [#]P<0.01 vs. the control; ^{*}P<0.01 vs. the model. CD, cluster of differentiation; LPS, lipopolysaccharide; IFN, interferon.

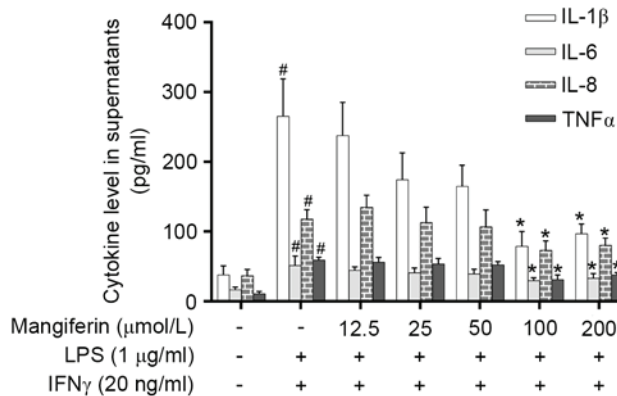


Figure 5. Levels of TNF- α , IL-1 β , IL-6 and IL-8 in the supernatant of each group. LPS/IFN- γ stimulation upregulated the levels of TNF- α , IL-1 β , IL-6 and IL-8 levels in the model group. Mangiferin at doses of 100 and 200 μ mol/l markedly decreased the levels of TNF- α , IL-1 β , IL-6 and IL-8. Data are expressed as the mean \pm standard deviation. [#]P<0.01 vs. the control. ^{*}P<0.01 vs. the model. TNF- α , tumor necrosis factor- α ; IL, interleukin; LPS, lipopolysaccharide; IFN- γ , interferon- γ .

curve. The PCR results were analyzed with the 7500 software 2.0 included with the in ABI 7500 real-time quantitative PCR system (Thermo Fisher Scientific, Inc.) (23).

Proinflammatory cytokine and IRF5 protein ELISA. Cells were washed twice in PBS. The cell protein was extracted with Membrane and Cytoplasmic Protein Extraction kit, the supernatants were collected (300 x g for 5 min at 4°C) and cryopreserved at -20°C for the ELISA assays. ELISA kits were used to assess the concentrations of TNF- α , IL-1 β , IL-6, IL-8 and IRF5 according to the manufacturer's protocols.

Statistical analysis. All data were analyzed for statistical significance using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Data are presented as the mean \pm standard deviation. Statistical analysis was conducted using one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of mangiferin on macrophage polarization towards M1 macrophages. A commonly accepted marker profile for M1

macrophages is CD80⁺/CD86⁺. Cells with CD80⁺ and CD86⁺ surface markers can be identified as M1 macrophage (24). The present study observed few cells with CD80⁺/CD86⁺ surface markers prior to LPS/IFN- γ stimulation, while large quantities of M1 macrophages with high fluorescence intensity of CD80⁺/CD86⁺ surface markers were detected following LPS/IFN- γ stimulation (Figs. 3 and 4). Following treatment with different concentrations of mangiferin, the percentage of M1 macrophages in each mangiferin-treated group was reduced to various degrees. However, there was a statistically significant decrease in M1 macrophage percentage and cell CD80⁺/CD86⁺ surface markers mean fluorescence intensity in the 100 and 200 μ mol/l mangiferin groups (P<0.01). Furthermore, the decreases in the 100 μ mol/l mangiferin group was lower than in the 200 μ mol/l group, but there was no significant difference between the two groups (Figs. 3 and 4).

Effect of mangiferin on cytokine levels in the supernatant. With LPS/IFN- γ stimulation, macrophages polarized to M1 macrophages and released large quantities of proinflammatory cytokines, including TNF- α , IL-1 β , IL-6 and IL-8. Thus, these proinflammatory cytokines in the supernatant can indicate the classical activation of macrophages. In the present study, the levels of TNF- α , IL-1 β , IL-6 and IL-8 levels in the cell culture supernatants were increased by LPS/IFN- γ stimulation, and were significantly higher in the model group compared with the control group (P<0.01; Fig. 5). Following treatment with various concentrations of mangiferin, the TNF- α , IL-1 β , IL-6 and IL-8 were significantly decreased by mangiferin at doses of 100 and 200 μ mol/l (P<0.01), but not at other doses. Furthermore, the decrease in the 100 μ mol/l mangiferin group was lower than in 200 μ mol/l, however, there was no significant difference between the two groups (Fig. 5).

Effect of mangiferin on macrophage IRF5 expression. An important role for IRF5 in macrophage classical activation has been previously recognized (16,22). IRF5 is expressed at the highest levels in M1 macrophages (17,25). In the present study, macrophages in the control group expressed low gene and protein levels of IRF5, but significant increases in IRF5 gene and protein expression was observed in the model group and higher than in the control group (P<0.01; Fig. 6). Following treatment with various concentrations of mangiferin, IRF5 expression levels were significantly decreased by mangiferin

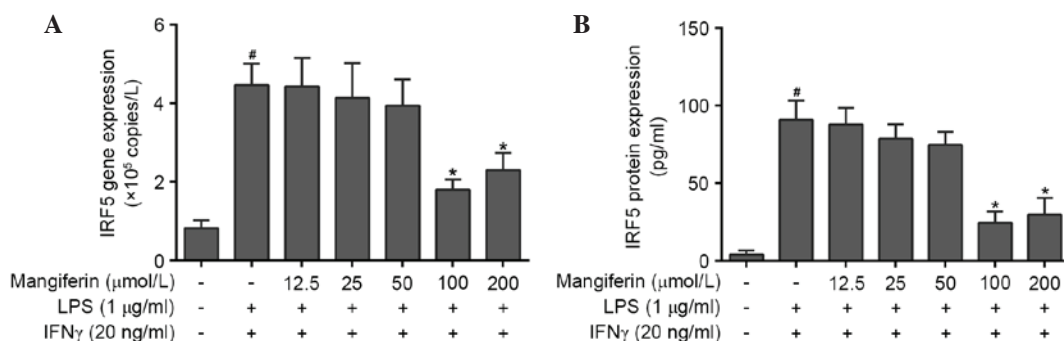


Figure 6. Bar plots of IRF5 (A) gene and (B) protein expression in each group. Data are expressed as the mean \pm standard deviation. [#]P<0.01 vs. the control; ^{*}P<0.01 vs. the model. IRF5, interferon regulatory factor 5; LPS, lipopolysaccharide; IFN- γ , interferon- γ .

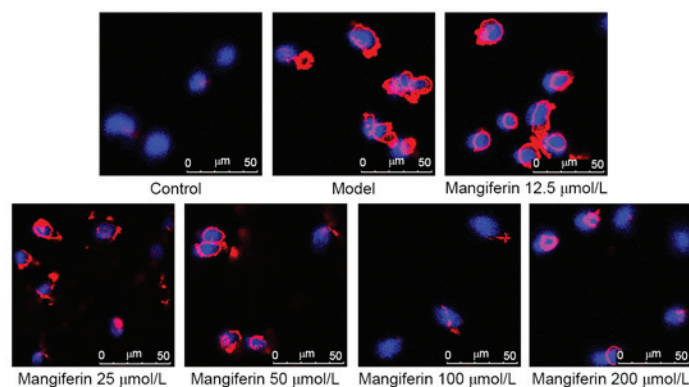


Figure 7. Laser scanning confocal microscope immunofluorescence analysis of IRF5 protein expression in macrophages and nucleus co-localization. Cells were stained using monoclonal anti-human IRF5-eFluor 660 antibody (red) and the blue pseudo color is a result of DAPI staining. IRF5, interferon regulatory factor 5.

at doses of 100 and 200 $\mu\text{mol/l}$ ($P<0.01$; Fig. 6), but not at other doses. Furthermore, the decrease in 100 $\mu\text{mol/l}$ mangiferin group was lower than in the 200 $\mu\text{mol/l}$ group, however, there was no significant difference between the two groups (Fig. 6).

Localization of IRF5 protein expression as observed by confocal microscopy. IRF5 has been reported to be important during macrophage polarization to M1 macrophages (26,27). IRF5 protein was detected and nucleus co-localization was observed using fluorescence confocal microscopy to evaluate the effect of mangiferin on of IRF5 protein expression during macrophage polarization to M1 macrophages. Without LPS/IFN- γ stimulation, IRF5 protein expression level was low. However, following stimulation by LPS/IFN- γ , IRF5 protein expression was significantly increased (Fig. 7). Following treatment with various concentrations of mangiferin, IRF5 protein expression levels were significantly decreased by mangiferin at doses of 100 and 200 $\mu\text{mol/l}$ (Fig. 7). Furthermore, 100 $\mu\text{mol/l}$ mangiferin resulted in a greater decrease in IRF5 protein expression levels than 200 $\mu\text{mol/l}$ (Fig. 7).

Discussion

Previous studies have demonstrated that mangiferin exerts a number of beneficial biological effects on inflammation (28), oxidative injury (29), tumor cell growth (30), microorganism infections (31), metabolic regulations (32), immune regulations (33) and radioprotection (34). Among these

pharmacological activities of mangiferin, its anti-inflammatory activity appears particularly prominently due to its beneficial effects against acute or chronic inflammation with various causes (35-39). Previous studies have also reported mangiferin exerts an anti-inflammatory effect via altering the biological behavior of macrophages to exert its anti-inflammatory activity (40,41), however its sub-cellular target and molecular mechanism remain to be elucidated. Similarly, further research is required to understand the effect of mangiferin on classical activation of macrophages, which is vital for inflammation. IRF5 has been recognized as a key regulatory factor in macrophage classical activation (27,42). However, little is known about the association between IRF5 and the effects of mangiferin on macrophage classical activation and, thus, further research is required.

As a member of the interferon regulatory factor family of transcription factors, IRF5 is closely associated with the inflammatory reaction and autoimmune response (43,44). Previous studies demonstrate inflammatory and autoimmune diseases may be associated with increased IRF5 expression levels (45,46). IRF5 is also an important regulatory factor in cell signal transduction pathway from Toll-like receptor (TLR) 7 to TLR9 (47,48). IRF5 can regulate the release of multiple cytokines, including TNF- α , IL-1 β , IL-6 and a number of chemokines, suggesting the inflammatory reaction is adjusted and controlled by IRF5 (49).

IRF5 is highly expressed in monocytes, macrophages and plasmacytoid dendritic cells, however, it is also expressed in

B cells and T cells (50,51). An important role for IRF5 in the macrophage classical activation has been identified. In M1 macrophages, IRF5 directly upregulates expression of multiple proinflammatory cytokines, but suppresses IL-10 expression, an anti-inflammatory cytokine (25,52). Furthermore, exogenous expression of IRF5 in M1 or M2-polarized (alternatively activated) macrophages induces expression of M1-associated cytokines and chemokines (16). Thus, high expression levels of IRF5 is characteristic of M1 macrophages, in which it directly activates transcription of the genes encoding various proinflammatory cytokines. Thus, the present study hypothesizes that macrophage classical activation may be inhibited via suppressing IRF5 expression in M1 macrophages using a therapeutic agent.

The cell surface markers of M1 macrophages, remain disputed, however, previous studies have reported that classically activated macrophages (M1 macrophages) exhibit high levels of CD80 and CD86 markers (53), and their upregulated expression is associated with the release of a large quantity of inflammatory cytokines (54,55). Thus, CD80⁺/CD86⁺ cytomembrane markers have been widely accepted as cell surface markers for M1 macrophages. Consistent with a previous study (24), cell with CD80⁺/CD86⁺ surface markers were identified as M1 macrophage in the present study. Macrophages exhibited few CD80⁺/CD86⁺ surface markers prior to LPS/IFN- γ stimulation, and reduced proinflammatory cytokine levels in cell culture supernatant and lower cellular IRF5 expression were also observed. However, CD80⁺/CD86⁺ cells and levels of proinflammatory cytokines were markedly increased following LPS/IFN- γ stimulation, and cellular IRF5 expression was markedly upregulated. These results indicated LPS/IFN- γ stimulation results in macrophage M1 polarization, and was consistent with previous studies (56,57).

Subsequent experiment results demonstrated that 100 and 200 μ mol/l of mangiferin significantly inhibited LPS/IFN- γ stimulation-induced macrophage polarization *in vitro* ($P < 0.01$), and the inhibition of 100 μ mol/l of mangiferin was more marked than 200 μ mol/l. Similarly, mangiferin results in the most notable inhibitory effect on cellular IRF5 expression at 100 μ mol/l rather than 200 μ mol/l. These results suggest the effect of mangiferin was not improved at the highest dose when mangiferin was used to inhibit macrophage classical activation, however, the reason remains to be elucidated. Notably, the results of the present study also indicate a possible association between the inhibitory effect of mangiferin on macrophage classical activation and decreasing cellular IRF5 expression. Mangiferin may downregulate cellular IRF5 expression, which then affects macrophage classical activation. The results of the present study may provide further experimental support for research into the anti-inflammatory properties of mangiferin and its underlying mechanism.

Macrophage classical activation is required in the normal protective immune response (58), particularly, in the early stage of the inflammatory reaction. However, chronic inflammatory diseases or excessive inflammation injury are not part of the normal protective response and immoderate macrophage polarization to M1 macrophages has been considered to be an important factor in chronic bronchitis or other inflammatory diseases (59,60). Mangiferin may inhibit macrophage classical activation via suppressing IRF5 expression

levels. Thus, mangiferin results in beneficial effects against diseases with marked macrophage classical activation. This pharmacological effect suggest mangiferin may be a potential anti-inflammatory therapeutic agent.

In conclusion, mangiferin can inhibit classical macrophage activation *in vitro*. The depression of cellular IRF5 expression was shown to be closely associated with this effect. However, more research is required to fully elucidate the mechanism of action of mangiferin.

Acknowledgements

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