Hesperetin derivative-12 (HDND-12) regulates macrophage polarization by modulating JAK2/STAT3 signaling pathway

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[ABSTRACT] Macrophages show significant heterogeneity in function and phenotype, which could shift into different populations of cells in response to exposure to various micro-environmental signals. These changes, also termed as macrophage polarization, of which play an important role in the pathogenesis of many diseases. Numerous studies have proved that Hesperidin (HDN), a traditional Chinese medicine, extracted from fruit peels of the genus citrus, play key roles in anti-inflammation, anti-tumor, anti-oxidant and so on. However, the role of HDN in macrophage polarization has never been reported. Additional, because of its poor water solubility and bioavailability. Our laboratory had synthesized many hesperidin derivatives. Among them, hesperidin derivatives-12 (HDND-12) has better water solubility and bioavailability. So, we evaluated the role of HDND-12 in macrophage polarization in the present study. The results showed that the expression of Arginase-1 (Arg-1), interleukin-10 (IL-10), transforming growth factor β (TGF-β) were up-regulated by HDND-12, whereas the expression of inducible Nitric Oxide Synthase (iNOS) was down-regulated in LPS- and IFN-γ-treated (M1) RAW264.7 cells. Moreover, the expression of p-JAK2 and p-STAT3 were significantly decreased after stimulation with HDND-12 in M1-like macrophages. More importantly, when we taken AG490 (inhibitor of JAK2/STAT3 signaling), the protein levels of iNOS were significantly reduced in AG490 stimulation group compare with control in LPS, IFN-γ and HDND-12 stimulation cells. Taken together, these findings indicated that HDND-12 could prevent polarization toward M1-like macrophages, at least in part, through modulating JAK2/STAT3 pathway.

[KEY WORDS] Hesperetin Derivative-12; Macrophage Polarization; JAK2/STAT3; RAW264.7 cells

Introduction

Diversity and plasticity are two hallmarks of macrophages. Different stimulus induce macrophages to express distinct cytokines, surface markers, and activate arginine metabolic

pathways that eventually exert different functions seen in inflammatory and non-inflammatory, tumor and non-tumor, immunogenic and tolerogenic settings [1-2]. Two well-established polarized phenotypes are often referred to as tissue classically activated macrophages (M1) and alternatively activated macrophages (M2). M1-macrophages are induced by interferon-gamma (IFN-γ) alone or in combination with lipopolysaccharide (LPS) and produce high level of iNOS and the inflammatory cytokines such as IL-12, IL-1β, IL-6, and TNF-α. In contrast, M2-macrophages are induced by exposure to a variety of signals including the cytokines IL-4 and/or IL-13 and immune complexes and express high levels of

Arg-1, IL-10, TGF-β and so on [3-4].

Macrophage activation phenotypes have been reported to maintain treatment for several diseases probably with their powerful functions. Mona AA *et al.* [5] reported that M2 cells might work to be tumor promotive in classical Hodgkin lymphoma (CHL) and M1 cells might be tumor suppressive in mixed cellularity type. According to the reports, insulin resistance and atherosclerosis induced by obesity were associated with macrophage infiltration into adipose tissue and a phenotypic switched from M2 to M1 [6-8]. On the other hand, macrophages, as the main adjustment cells, both M1- and M2-macrophage polarization occurred during occurrence and development of liver fibrosis and played the opposite effects [9]. To the best of our knowledge, macrophage polarization played significant roles in many diseases and the polarization macrophage phenotype could turn into the novel therapeutic targets. Thus, searching the drug which could regulate the switch of M1 and M2 macrophage phenotypes has gained increasingly attention in recent years. The JAK/STAT signaling pathway affects cellular activities, such as proliferation, migration, growth, differentiation, and death [10]. Studies have shown that macrophages with the stimulation of LPS dramatically activate the Janus kinase-signal transducers2 and activators of transcription3 (JAK2-STAT3) pathway to produce inflammatory cytokines and different macrophage phenotypes [11-12]. Recent studies have revealed that IFN-γ activates the JAK and the STAT-1 phosphorylation which leads to the M1-like macrophage polarization [13].

HDN (4'-methoxy-3', 5, 7-trihydroxyflavanone), a naturally occurring flavanone found in citrus fruit peels, has been known to exhibit several key biological and pharmacological properties. Previous studies have demonstrated that HDN has plays a significant role in anti-inflammatory, anti-oxidant and anti-tumor [14-15]. However, its role in macrophage polarization remains poorly understood. In addition, its water solubility and bioavailability limited the medicinal, so we modified the structure of HDN. In previous studies, our team synthesized multiple derivatives of HDN and found that HDND-12, as shown in Fig. 1A, has better anti-inflammatory activity. The synthesis and analysis of HDND-12 had been reported in the manuscript by Wang *et al.* [16]. The objective of this study was to demonstrate whether the HDND-12 plays an important role in the transformation of M1- and M2-like macrophage and to explore the corresponding mechanism, and our research focuses on the effect role of HDND-12 in M1 polarization.

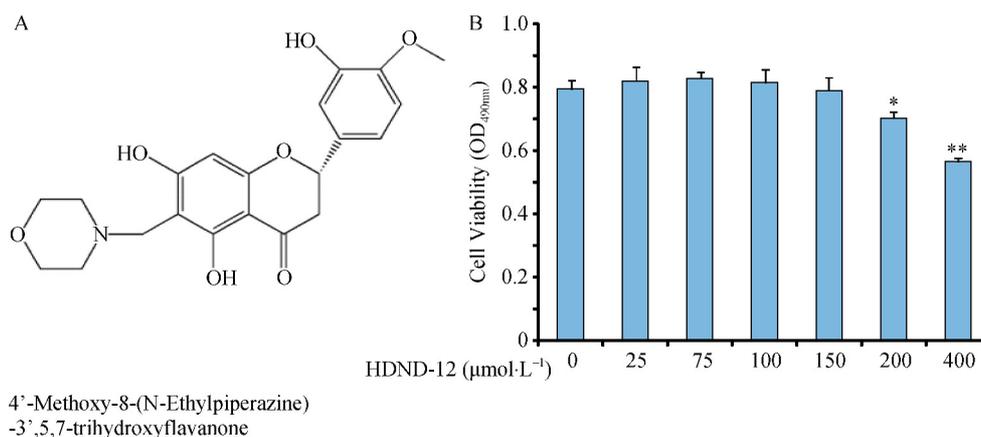4'-Methoxy-8-(N-Ethylpiperazine)
-3',5,7-trihydroxyflavanone

Fig. 1　(A) Structure of hesperetin derivative-12 (HDND-12); (B) Effect of HDND-12 on the cytotoxicity of RAW264.7 cells. Treated with HDND-12 at different concentrations (0, 25, 75, 100, 150, 200, 400 μmol·L^{-1}) caused a significant inhibition of the growth of RAW264.7 cells in 200 and 400 μmol·L^{-1} by MTT assay at 24 h

Materials and Methods

Materials and reagents

HDND-12 was provided by the pharmacy of Anhui Medical University. LPS (*Escherichia coli* 055: B5) and anddimethyl sulfoxide (DMSO) were purchased from Sigma Chemical (St.Louis, MO, USA). Recombinant Murine IFN-γ (20 μg) was purchased from PeproTech (Rocky Hill, NJ). AG490 inhibitor was purchased from Beyotime Inc (Shanghai, China). Rabbit anti-iNOS monoclonal antibodies were purchased from Cell Signaling (Beverly, MA, USA). Mouse monoclonal antibodies β-actin was purchased from Santa Cruz (California, USA) and Rabbit anti-Arg-1, JAK2, p-JAK2, STAT3, and p-STAT3 polyclonal antibody were purchased from Santa Cruz (California, USA). Arg-1, iNOS, TNF-α, IL-10, and GAPDH primers were produced by Sangon Biological and Technological Company (Shanghai, China). Enzyme Linked Immunosorbent Assay (ELISA) kit was acquired from Hushang Biotechnology Co., Ltd. (Shanghai, China). APC anti-mouse CD206 (MMR) and APC Rat IgG2a, κIsotype Ctrl were purchased from Biolegend (USA).

Cell culture and treatment

RAW264.7, a cell line of mouse peritoneal macrophages, was obtained from Cell Bank of Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco' smodified Eagle's medium (DMEM; HyClone, USA) supplemented
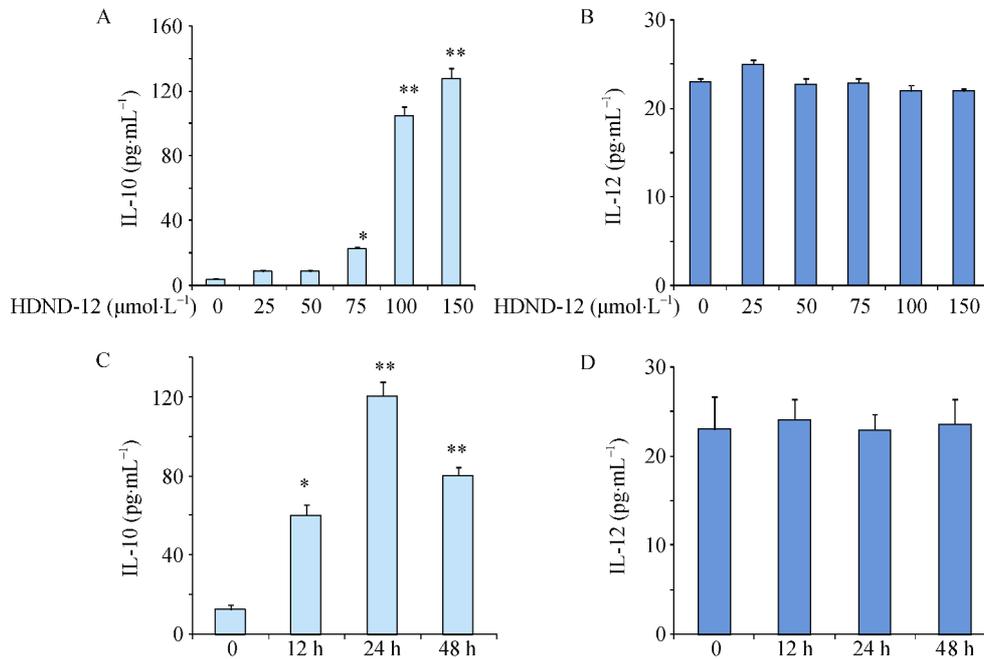
Fig. 2   The expression of cytokines (IL-10 and IL-12) in RAW264.7 stimulating by different concentrations and times of HDND-12. (A, B) Cells were incubated for 24 h in fresh medium without or with HDND-12 (25, 50, 75, 100, 150 µmol·L^{-1}). (C, D) Macrophages were incubated with 100 µmol·L^{-1} HDND-12 for 0, 12, 24, 48 h. The expressions of IL-10 and IL-12 in the supernatants of macrophages are detected by Elisa analysis. Statistical analysis was performed by One-way ANOVA. $^{*}P < 0.05$; $^{**}P < 0.01$ *vs* normal

with 10% (*V/V*) heat-inactivated fetal bovine serum (Bovine, China), 100 U·mL^{-1} of penicillin (Gibco), and 100 µg·mL^{-1} of streptomycin (Gibco). Cells were grown in a humidified 5% CO_2 and 37 °C atmosphere. Cells were cultured by adding different concentrations for 24 h, or adding 1000 ng·mL^{-1} LPS and 10 ng·mL^{-1} IFN-*γ* for 12 h and then adding different concentrations for 24 h in the suitable conditions.

Total RNA isolation and real-time quantitative PCR analysis

Total RNA was extracted from RAW264.7 cells by using TRIzol (Invitrogen) according to the manufacture's instruction. The first-strand cDNA was synthesized from total RNA using Thermoscript RT-PCR synthesis kit (Fermentas). The reaction mixture was prepared according to the manufacture's instruction using SYBRGreen qPCR Master Mix (QIAGEN). Real-time quantitative PCR was carried out under standard protocol using the following primers: GAPDH (forward: 5'-GGACCTCATGGCCTACATGG-3'; reverse: 5'-TAGGGC CTCTCTTGCTCAGT-3'), iNOS (forward: 5'-CTGCAGCAC TTGGATCAGGAACCTG-3; reverse: 5'-GGAGTAGCCTGT GTGCACCTGGAA-3'), Arg-1 (forward: 5'-TGACATCAAC ACTCCCCTGACAAC-3'; reverse: 5'-GCCTTTTCTTCCTT CCCAGCAG-3'), TNF-*α* (forward: 5'-TGTCCCTTTCACTC ACTGGC-3'; reverse: 5'-CATCTTTTGGGGGAGTGCCT-3'), IL-10 (forward: 5'-ACCTCCAGGACGACTTTGAT-3'; reverse: 5'- GCTGCCTGCTCTTACTGACT-3'). PCR was performed 95 °C for 10 min followed by 40 cycles at 95 °C for 15 sec and at 60 °C about 1 min by using Thermo Step One. Reactions were conducted three times, and threshold cycle values were normalized to GAPDH gene expression. The specificity of the products was determined by melting curve analysis. The ratio of the relative expression of target genes to GAPDH was calculated by the formula $2^{-\Delta\Delta Ct}$.

Western blot analysis

Cells were harvested and homogenized using RIPA lysis buffer (Beyotime, China) and quantified with a Bicin Choninic Acid (BCA) protein assay kit (Boster, China). Total proteins were separated by denaturing 10% or 12% SDS-PAGE and subsequently electrophoretically transferred to a PVDF membrane (Millipore Corp, Billerica, MA, USA). The membranes were then incubated in TBST containing 5% skim milk at 37 °C for 3 h, and with specific primary antibodies at 4 °C overnight. The next day, the membranes were washed 3 times with TBS/Tween 20 (0.075%), followed by incubation with HRP-conjugated secondary antibodies (1 : 8000–1 : 10000) at 37 °C for 1 h. Proteins were visualized with ECL-chemi-luminescent kit (ECL-plus, Thermo Scientific, USA). The bands were visualized by chemiluminescence reagents (Model No. ChemiQ 4600, Bioshine)

Elisa assay

The levels of IL-12, IL-10, TGF-*β* and TNF-*α* in the supernatants of macrophages were determined using an enzyme-linked immunosorbent assay (ELISA) kit (R&D USA) according to the manufacturer's instruction.

Flow cytometry analysis

The expression of CD206 on RAW264.7 cells treatment with HDND-12 was studied by flow cytometry. Purified cells

following specific treatments were washed with normal saline twice and labeled with fluorescence-labelled antibody (APC-conjugated CD206 anti mouse antibody and isotype control) 1 μL for 15 min as manfacturer's recommendation. Then the mean fluorescence intensities were determined using quadrant statistics.

AG490 treatment

RAW264.7 cells were seeded overnight in six-well plates and pretreated with AG490 (10 μmol·L^{-1}) for 14 h, then treat it with or without M1 inducers (LPS and IFN-γ) for 12 h and HDND-12 until the processing time finished. The medium containing DMEM only was regarded as a control.

Statistical analysis

Statistical analysis was completed with the Statistical package for Social Sciences (SPSS Inc., Chicago, IL, USA, version 13.0). The data were expressed as mean ± standard error (SE). The Bartlett's test was used to determine whether the data were heterogeneous or homogeneous. Statistical significance was determined by either the Student's t test for comparison between means or one-way analysis of variance with a post hoc Dunnett's test. Differences were considered significant at *P* < 0.05.

Results

HDND-12 affects macrophage polarization in RAW264.7 cells

MTT assay was used to screen the influences of HDND-12 on the viability of RAW264.7 cells. We treated the RAW264.7 cells with different concentrations of HDND-12 (0, 25, 50, 75, 100, 200, 400 μmol·L^{-1}) in the 24 h. As shown in Fig. 1B, we found HDND-12 (0–150 μmol·L^{-1}) had no effects on RAW264.7 cells, but concentrations in the 200–400 μmol·L^{-1} range significantly reduced cell viability. This result revealed that HDND-12 in the concentrations of 0–150 μmol·L^{-1} had no toxicity to RAW264.7 cells. To elucidate the functional role of HDND-12 during macrophage polarization, RAW264.7 cells were stimulated by HDND-12 with different concentrations (0, 25, 50, 75, 100, 150 μmol·L^{-1}) for 24 h, then we detected the markers of M1 and M2 (IL-12 and IL-10) in normal group and HDND-12 group (Fig. 2A), the expression of IL-10 significantly up-regulated compared to the control group, and were much higher at 100 μmol·L^{-1}. Then we chose 100 μmol·L^{-1} for 12, 24, 48 h (Fig. 2B), which peaked at 24 h after HDND-12 treatment. Ultimately we chose the appropriate concentrations (50, 100, 150 μmol·L^{-1}) and time point (24 h) for the next studies. As illustrated in Fig. 3A, it is observed that the expression of IL-10 and TGF-β in HDND-12 concentration groups was increased strikingly compared with the control. In conjunction with RT-QPCR (Fig. 3B) and Western Blot (Fig. 3C) analysis, the expression of Arg-1, IL-10 mRNA and Arg-1 protein was also up-regualted significantly compared with the control. In addition, FACS analyses (Fig. 4) showed that HDND-12 with different concentrations increased the mean fluorescence intensity (MFI) and percentage of CD206 cells in RAW264.7 and the changes of HDND-12 (100 μmol·L^{-1}) were apparent especially. In light of these evidences, we suggested that HDND-12 might be a regulator in the transformation of M1- and M2-like macrophage.

HDND-12 inhibits the switch of M1-type macrophage

To further elucidate the regulation role of HDND-12 in polarization macrophages, we chose the classic model in RAW264.7 cells, M1-like macrophage was induced by LPS (1000 ng·mL^{-1}) and IFN-γ (10 ng·mL^{-1}) for 12 h [17], and then stimulated with different concentrations of HDND-12 (0, 50, 100, 150 μmol·L^{-1}) for 24 h. Interestingly, we found that the marker of M1-type was down-regulated in HDND-12 groups, suggesting that HDND-12 could inhibit the switch of M1-like macrophage. Western blot analysis showed that (Fig. 5) iNOS, the hallmark of M1 macrophage, was increased remarkably by LPS and IFN-γ treatment when compared with the normal. On the other hand, the expression of iNOS in the HDND-12 concentrations groups was decreased compared with the LPS and IFN-γ-induced cells. In light of these evidences, we suggested that HDND-12 could inhibit the shift of M1-like macrophages in RAW264.7 cells.

HDND-12 regulates macrophage polarization through the JAK2/STAT3 pathway

We have proved that HDND-12 could up-regulate the expressions of M2 markers and inhibit M1 polarization of macrophage, however, the possible regulatory mechanism of HDND-12 in macrophage polarization is still unknown to date. It is well known that the JAK2/STAT3 pathway play an important role in macrophage inflammatory response and occupies a central role among the regulatory factors of macrophage polarization [18-19]. Thus, we paid more attention to p-JAK2 and p-STAT3.

In order to examine whether JAK2 and STAT3 were keys in the process of the shift of RAW264.7 macrophage cells by HDND-12, JAK2, p-JAK2, STAT3 and p-STAT3 protein levels were analyzed by western blot. As shown in Figs. 6A, 6B and 6C, the expression of p-JAK2 was significantly down-regulated after stimulation with HDND-12 (50, 100, 150 μmol·L^{-1}). Moreover, not only macrophage induced by LPS and IFN-γ significantly increased p-JAK2 protein expression, but also inhibited HDND-12-treated M1-type macrophage p-JAK2 protein level up-regulation. Furthermore, the expression of p-STAT3 was in accordance with the above results of p-JAK2. As indicated by Western Blot assay (Fig. 7), when we stimulated cells with AG490 , the protein levels of iNOS and TNF-α were significantly reduced in LPS, IFN-γ, HDND-12 and AG490 stimulation group compare control group. Therefore, the above data indicated that the regulate role of HDND-12 in shift of M1- and M2-type macrophages may be, at least in part, mediated by JAK2/STAT3 signaling pathway.
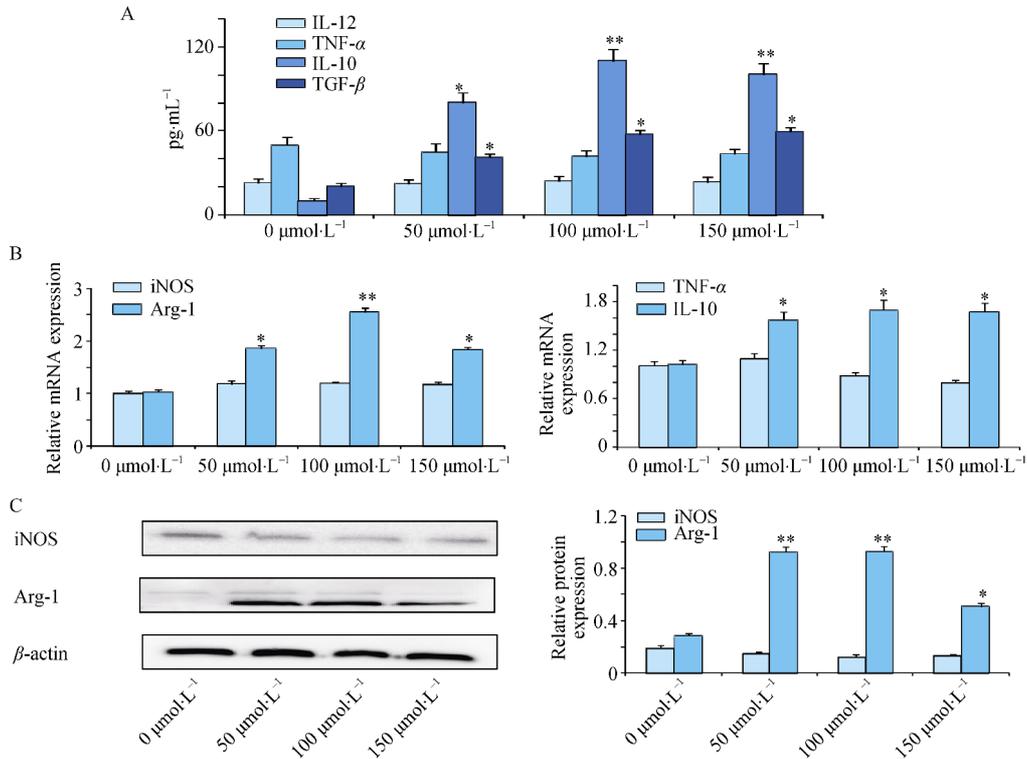
Fig. 3   HDND-12 with different concentrations (50, 100, 150 μmol·L^{-1}) induces the secretion of cytokines and the mRNA and protein expressions of markers of polarization macrophages in RAW264.7. (A) Elisa analysis shows increased expression of IL-10 and TGF-β by HDND-12; (B) Gene expression of pro-inflammatory cytokines TNF-α, anti-inflammatory cytokine IL-10 and the markers of polarization macrophages (iNOS and Arg-1) were measured by quantitative PCR; (C) The protein expressions of iNOS and Arg-1were tested by Western Blot analysis. Values are the mean ± SEM of three independent experiments. ANOVA was used to analyze the differences. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* control
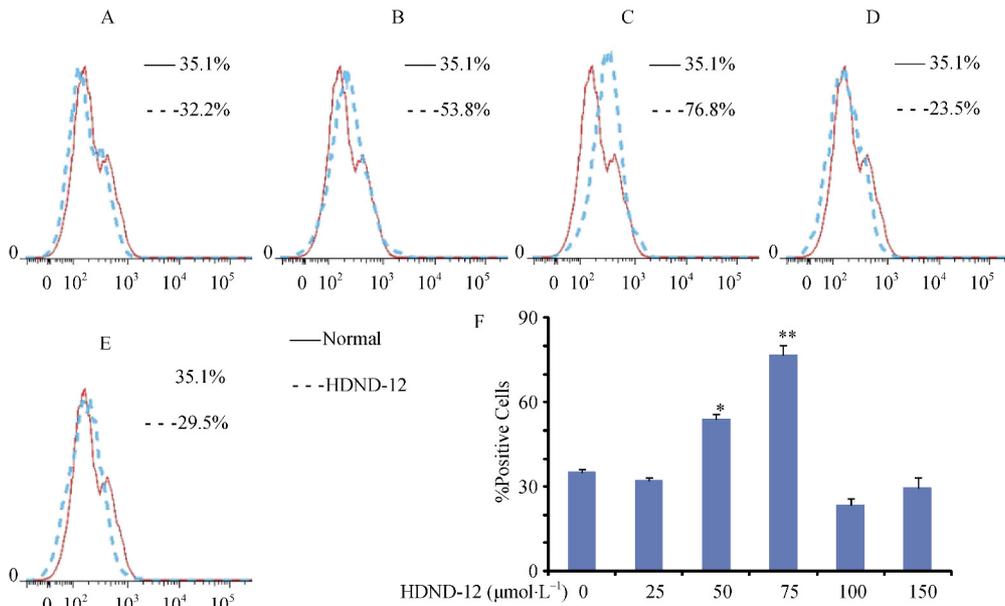

Fig. 4   Phenotypic analysis of RAW264.7 cells treatment with HDND-12 by FACS. The expression of M2-type macrophage marker on CD206^{+}-enriched cells from cells treatment with HDND-12 was assessed. The solid line stand for the normal group and the dotted line show the HDND-12 groups. The median fluorescence intensity and percentage of CD206 in Figs. 4A, B, C, D, Ewere on behalf of different concentrations of HDND-12 (25, 50, 100, 150, 200 μmol·L^{-1}). Bar graphs present the mean and SEM of the percentage of positive cells (n = 3 for each genotype). $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* control
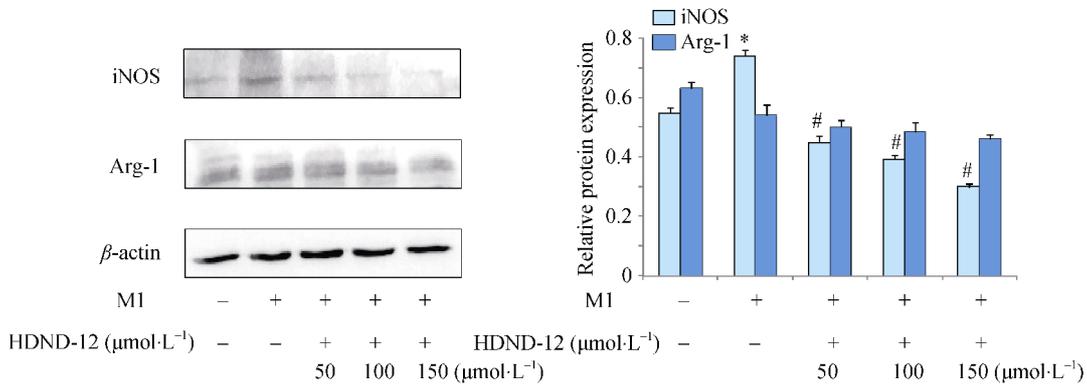
Fig. 5   HDND-12 could inhibit the expression of the hallmarks of M1-like macrophages and could not affect the marker of M2-like macrophages. RAW264.7 cells were treated with the different concentrations (50, 100, 150 μmol·L^{-1}) HDND-12 followed by LPS and IFN-γ for 12 h. Western blotting analysis was assessed to determine the levels of iNOS and Arg-1. Results are representative of three separate experiments. $^*P$ < 0.05 *vs* control condition, $^\#P$ < 0.05 *vs* model condition
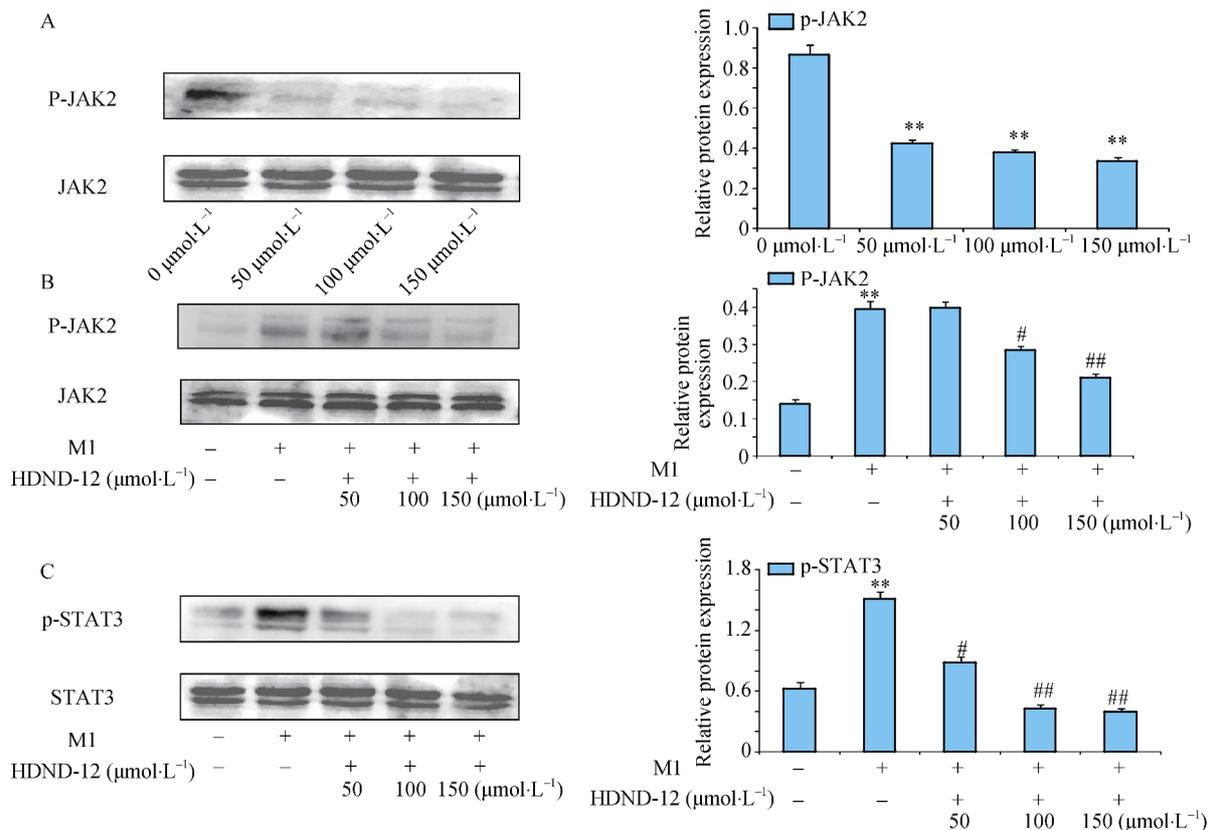

Fig. 6   Effects of JAK2/STAT3 pathway in HDND-12-induced macrophage polarization. Western blotting analysis was assessed to determine the levels of phosphorylated and non-phosphorylated JAK2 and STAT3. (A) Cells were only treated with HDND-12 (50, 100, 150 μmol·L^{-1}) for 24 h; (B) RAW264.7 cells were treated with HDND-12 followed by LPS and IFN-γ for 12 h. n = 3, $^*P$ < 0.01, $^{**}P$ < 0.05 vs control; $^\#P$ <0.05, $^{\#\#}P$ <0.01, significantly decrease *vs* model condition

Discussion

Macrophages, which manifest great plasticity, are important components of the innate immune defense system and constitute an essential element of inflamed environment [20]. Macrophages show significant heterogeneity in function and phenotype, which can shift into different populations of cells in response to exposure to various micro-environmental sig-nals. These changes, also termed as macrophage polarization, can regulate the initiation, development, and cessation of some diseases, such as inflammatory diseases, cancers, metabolic diseases and so on [21-22]. In recent years, macrophage polarization has gained increasing attention and looking for an operative medicine, which could target macrophage polarization and skew their phenotypes to adapt to the micro-environment, might hold great promise for the development of diseases.
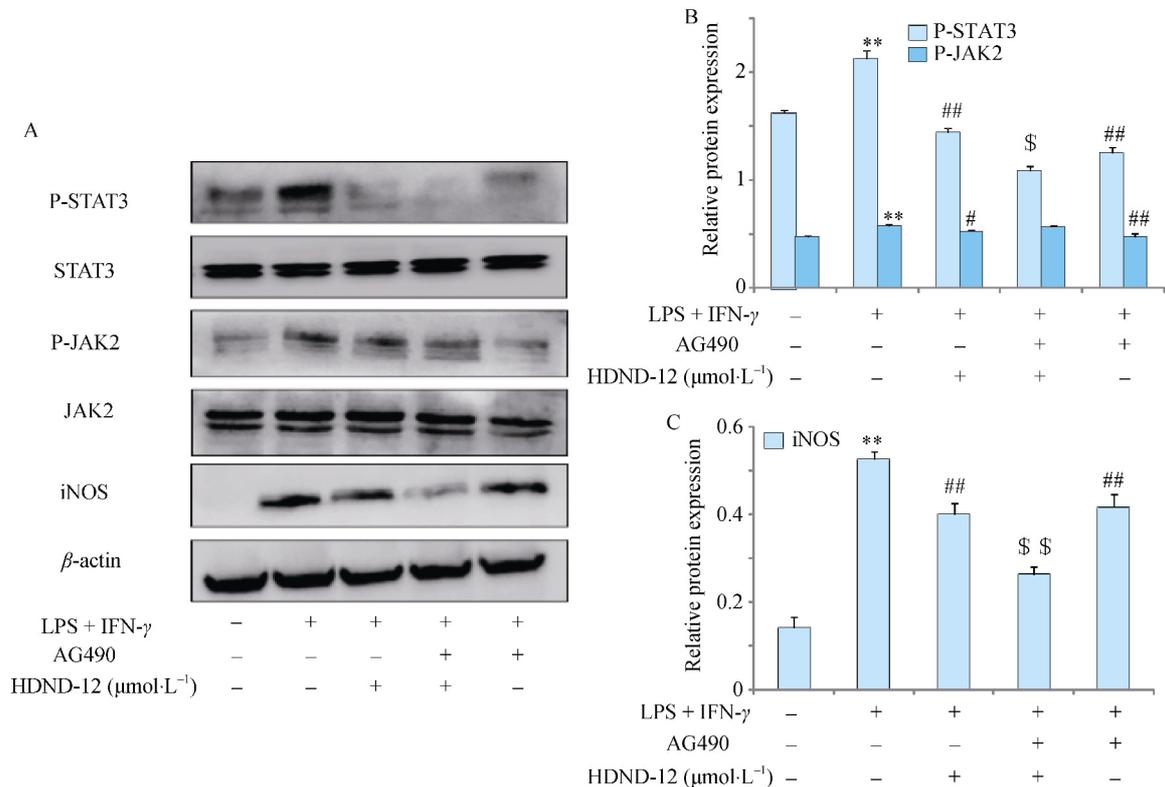
Fig. 7 The effect of AG490 on the level of iNOS in M1-type macrophage. (A) Treatment with AG490 and HDND-12 separately on RAW264.7 cells produced a significant decrease in the levels of p-JAK2 and p-STAT3. And both stimulus can decrease more compared with the two groups of single stimulus. (B) We detected the protein level of iNOS in RAW264.7 cells treated with AG490 and or HDND-12. The results are expressed as the mean ± SEM of three different experiments. $^{**}P < 0.01$, $^{*}P < 0.05$ *vs* normal, $^{##}P < 0.01$, $^{#}P < 0.05$ *vs* M1 group, $^{\$}P < 0.05$, $^{\$\$}P < 0.01$*vs* M1 plus HDND-12 group

HDN, a naturally occurring flavanone compound, was previously proved that HDN has several functions such as anti-oxidant, anti-tumor, anti-inflammatory, immune regulation and so on [23-24]. In the present study, we explored the regulation role of HDND-12 in the polarization of macrophage. HDND-12 is one of the derivatives of hesperetin by structural modifications in order to improving its water solubility and bioavailability. In the current study, we tested the hypothesis that HDND-12 may be regulator in a prominent M2 macrophage phenotype and inhibitor in M1 macrophage switch. In our study, we showed that HDND-12 led to a time- and dose-dependent increase in the expression of M2-type markers, such as Arg-1, IL-10 and so on. Additionally, we demonstrated that HDND-12 suppressed the expression levels of the markers of LPS and IFN-*γ*-induced M1-type macrophage including iNOS, IL-12. In addition, in order to explore the underlying mechanisms in RAW264.7 cells. We investigated HDND-12 was related to macrophage polarization and then observed the up-regulation of M2 markers at both the mRNA and protein levels after treatment with HDND-12 compared with normal RAW264.7 cells. These results proved that HDND-12 may be used as a useful drug for switching to M2-type. Moreover, LPS from Gram-negative bacteria and IFN-*γ* are the most potent innate immune activating and M1- type polarization

stimuli known [4]. LPS and IFN-*γ* exert their toxic effects by potently activating macrophage cells and activating TLR4 signaling, which could up-regulate the expression of M1 markers. On the contrary, we found HDND-12 down-regulated M1 related markers, such as iNOS, IL-12 and TNF-*α*. Finally, we study the possible mechanism about HDND-12 in regulating macrophage polarization. Various signaling pathways, including JAK/STAT, SOCS2/SOCS3, TLR4, and IRF5/IRF4, were reported to regulate macrophage activation and polarization [25-27]. Specifically, JAK/STAT signaling plays a central role in macrophage polarization and a growing body of evidences suggests that IFN-*γ* was one of the most potent endogenous macrophage-activating factors [28]. Thus, we explored whether HDND-12 regulate the JAK/STAT signaling pathway Intriguingly, HDN could attenuate the phosphorylation level of JNK in lipopolysaccharide-induced H9C2 cardiomyocytes [29]. So we wonder if HDND-12 may function in JAK/STAT signaling pathway through the expression of phosphorylation level of JAK2 and STAT3, respectively. We found the expressions of p-JAK2 and p-STAT3 were up-regulated in M1-like macrophage and down-regulated in HDND-12-induced groups, which suggested the inhibitor role of HDND-12 to M1-like macrophage in RAW264.7 cells may be correlated with the JAK2/STAT3 pathway. Which puzzleed us was that some publications had

already reported that JAK2/STAT3 signaling were activated in M2-like macrophage. But in our study the JAK2/STAT3 signaling were activated in M1-like macrophage, so we need more research to explain this problem. Additional, we pre-treated the cells with AG490 (10 μmol·L^{-1}) for 14 h, then treat it with or without M1 inducers (LPS and IFN-γ) for 12 h and HDND-12 until the processing time finished. It was noteworthy that the protein levels of iNOS and TNF-α were significantly reduced in LPS, IFN-γ, HDND-12 and AG490 stimulation group compare with LPS, IFN-γ and HDND-12 stimulation group. Taken together, HDND-12 could regulate the switch the ratio of M1-type and M2-type macrophages and the underlining mechanisms might be contributed to its beneficial effects on JAK2/STAT3 signaling.

In this study, we explored the potential role of HDND-12 in macrophage polarization and how did it work in RAW264.7 cells. However, macrophage polarization as related diseases' therapeutic target is in infancy, and there are little effective drugs against it. Traditional Chinese medicine is an attractive therapeutic direction, and this study demonstrated HDND-12 is a good beginning but also need further research. In future work, the important areas should focus on exploring the full spectrum of macrophage polarization, searching new drugs with regard to the transformation of polarization, and ultimately helping treat related diseases.

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