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Full paper

Unique immunomodulatory effect of paeoniflorin on type I and II macrophages activities

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ABSTRACT

It has been widely accepted that macrophages are divided into M1 "pro-inflammatory" macrophages and M2 "anti-inflammatory" macrophages and an uncontrolled macrophage polarization plays an important role in the pathogenesis of different diseases. As the main substance of total glucosides of peony, paeoniflorin (PF), has been widely used to treat autoimmune and autoinflammatory diseases for years. Mechanistically, PF has been found to alter activities of many immune cells, which could further reduce inflammation and tissue damage. However, whether and how PF affects macrophages activities *in vitro* remains unknown. In current study, using M1 and M2 cells generated from mouse bone marrow precursors, we explored the role of PF in regulating M1/M2 cells activity *in vitro*. The results showed that PF inhibited LPS-induced M1 activity by reducing iNOS expression and NO production via decreasing LPS/NF-κB signaling pathway; whereas, PF enhanced IL-4-provoked M2 function by up-regulating Arg-1 production and activity via increasing IL-4/STAT6 signaling pathway. Our new finding indicates that PF can suppress M1 cells activity and enhance M2 cells function simultaneously, which could help to ameliorate autoimmune and autoinflammatory diseases in clinical treatment.

1. Introduction

Macrophages, as first identified immune cells, play critical roles in both innate and adaptive immune responses and classified into two types according to their functions (1,2). Type I macrophages (M1) are marked by producing large amounts of pro-inflammatory mediators such as TNF-α, IL-6, IL-23, IL-12 and generating reactive oxygen species such as NO via activation of inducible nitric oxide synthesis (iNOS) (3), whereas type II macrophages (M2) release high levels of anti-inflammatory molecules such as IL-10, and can be defined based on a specific genetic signature characterized by the upregulation of Ym1 (also known as *Chil3l3*) and FIZZ1 (also known as *Retnla*) genes (4). Additionally, arginase-1 (Arg-1) production is increased in M2-polarized macrophages, which in turn blocks iNOS activity by a variety of mechanisms (5). It is well known that, an uncontrolled macrophage polarization plays an important role in the pathogenesis of many autoinflammatory diseases, for example, excessive M1 polarization in adipose tissues is linked to metabolic disease (6). Moreover, the distribution of M1 and M2 polarization in human atherosclerosis shows to be related with plaque instability (7). In dextran sodium sulfate (DSS)-induced murine experimental colitis, increased M1 cells and decreased M2 cells are associated with the disease progress (8). All these evidences indicate that the balance of M1 and M2 cells is crucial for homeostasis and re-balancing the proportion of M1/M2 might be the potential therapy of autoimmune and autoinflammatory diseases.

Paeoniflorin (PF), one of the major bio-active components of Paeony root, has been widely used as an anti-inflammation and immunomodulatory agent in diverse autoimmune diseases such as rheumatoid arthritis (RA), psoriasis, sjögren's syndrome (SS) and systemic lupus erythematosus (SLE) (9–11). It was reported that, PF inhibits T and B cells proliferation in arthritis animal model (12),

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up-regulates the regulatory T cells (Treg) function in SLE patients and SS animal model (9,13). Our previous study showed that PF decreased maturation of dendritical cells (DCs) in CIA mice (14), reduced the number of $F4/80^{+}CD68^{+}$ macrophages in imiquimod (IMQ)-induced psoriasis-like mice (15). Besides, *in vitro* study, we found that PF inhibited the functions of B cells stimulated by LPS (16). Although PF has been found to alter activities of many immune cells, whether and how PF regulates M1 and M2 cells activities *in vitro* remains unknown.

In this study, we treated mouse bone marrow derived M1 and M2 cells with PF and found that PF reduced M1 cells activity stimulated by LPS through inhibiting the activation of NF-κB signaling pathway, meanwhile, PF enhanced M2 cells function provoked by IL-4 via activating STAT6 phosphorylation. Considering the critical roles of macrophages played in inflammatory and autoimmune diseases, to learn the effect of PF involved in regulating M1 and M2 cells activation will give a new insight into the therapeutic treatment. Taken together with our previous works, this study supplies that PF is benefit for not only Th1/Th17 or B cells-associated inflammation and autoimmune diseases, but also macrophage-associated diseases, providing solid evidence to the wide use of PF in clinical treatment.

2. Material and methods

2.1. Drugs

PF (molecular weight 480.05), supplied from Liwah Plant extract technology Co. Ltd. (Ningbo, China), was dissolved in double distilled water and filtrated. 1, 10, 100 μg/ml PF were used in this study.

2.2. Animals

Male 6–8w Balb/c mice were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Science (Shanghai, China). The mice were maintained under pathogen-free conditions. All of the experiments were performed according to the Animal Care and Use Committee guidelines.

2.3. M1 and M2 macrophages generation and identification

Bone marrow precursor cells were collected from 6-8w male Balb/c mice and cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), 100 μg/ml streptomycin, 100 units/ml penicillin and 2 mM L-glutamine (Gibco, New York, USA). 20 ng/ml recombinant murine GM-CSF (PeproTech Inc., Rocky hill, USA) and 50 ng/ml recombinant murine M-CSF (PeproTech) were added to the medium respectively for M1 and M2 polarization. After 7 days, the morphology of M1/M2 cells was observed by light microscope. Generated M1/M2 cells were stained with anti-mouse F4/80, CD11b and CD11c (eBioscience Inc., California, USA) antibodies. Flow cytometry was performed using FACS Calibur cytometer (BD Biosciences) and analyzed by FlowJo7.6 software (Treestar Inc., California, USA). The adherent M1 and M2 cells were treated with 100 ng/ml lipopolysaccharide (LPS, Sigma, Missouri, USA) and 20 ng/ml murine IL-4 (PeproTech) for 24 h respectively according to previous reports (17,18), then the mRNA profiles of activated M1 and M2 cells were examined by real-time PCR.

2.4. Cell viability assay

Cell viability was examined using a Cell Counting Kit-8 (CCK-8, Dojindo, Mashikimachi, Japan) according to manufacturer's instructions as previous reports (16,19) (Supplementary material and methods 1).

2.5. RNA extraction and real-time PCR

Bone marrow precursor cells cultured with GM-CSF (20 ng/ml) or M-CSF (50 ng/ml) for 6 days to induce M1 or M2 cells, then different concentrations of PF (1, 10 and 100 μg/ml) were added for 24 h before stimulating these cells with LPS (100 ng/ml) or IL-4 (20 ng/ml) respectively for another 24 h. RNA extraction and real-time PCR were performed as previously reported (20) (Supplementary material and methods 2). Primers were designed using Primer Express 3.0 software (Applied Biosystems) and shown in Table 1.

2.6. Western blotting analysis

Bone marrow precursor cells cultured with GM-CSF (20 ng/ml) or M-CSF (50 ng/ml) for 6 days to induce M1 or M2 cells, then different concentrations of PF (1, 10 and 100 μg/ml) were added for 24 h before stimulating these cells with LPS or IL-4 respectively. Cells were harvested and lysed. Specific antibodies to iNOS, Arg-1, total STAT6, total and phosphorylated NF-κB p65 were purchased from Cell Signaling Technology Inc. (Massachusetts, USA). Antibodies to phosphorylated STAT6 were purchased from Santa Cruz Biotechnology Inc. (Dallas, USA). The Gel-Pro Analyzer 4 (Exon-Intron Inc., Loganville, USA) was used to analyze bands density. The alteration of target protein was displayed as relative fold which was derived from comparison with β-actin or their non-phosphorylated counterparts.

2.7. NO production assay

M1 cells pre-treated with PF for 24 h were subsequently stimulated with LPS. NO production in M1 cells was assessed using NO assay kit (Beyotime, China) following the manufacturer's instructions. Briefly, the supernatant of cultured cells was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2.5% phosphoric acid) and incubated at room temperature for 5 min. The concentration of nitrite was measured by reading at 570 nm absorbance wavelength. Sodium nitrite ($NaNO_2$) was used as a standard curve.

2.8. Arg-1 activity assay

M2 cells pre-treated with PF for 24 h were subsequently stimulated with IL-4. Arg-1 activity in M2 cells was assessed by an Arginase Assay Kit (Abnova, Taipeh, Taiwan), following the manufacturer's instructions (Supplementary material and methods 3).

2.9. Immunofluorescence analysis

M1/M2 cells grown on glass coverslips were treated with H_2O or PF for 24 h and then exposed to LPS/IL-4 for 0 or 15 min and fixed with 4% paraformaldehyde for 20 min. After permeabilizing with 0.3% Triton X-100 (Sigma, MO, USA) in PBS, cells were blocked with 10% normal goat serum (Cell Signaling Technology Inc.) for 1 h. Anti-NF-κB p65/anti-STAT6 antibody was applied at dilution ratio of 1:200 at 4 °C overnight. After washing, Alexa Fluor 488/594-conjugated anti-rabbit IgG (Cell Signaling Technology Inc.) were used as secondary antibody at 1:1000 dilution for 1 h at room temperature. Nuclei were counterstained with 0.25 mg/ml of 4-, 6-diamidino-2-phenylindole (DAPI; ZyMed, CA, USA) for 3 min. Coverslips with antifade fluorescent mounting medium were transferred onto glass slides. NF-κB p65/STAT6 nuclear

translocation in M1/M2 cells were studied with the confocal laser scanning fluorescence microscopy (LSM510; Zeiss, Jena, Germany) technique as described previously (20).

2.10. Statistical analysis

All experiments were performed in triplicate. The difference among groups was determined by ANOVA analysis and comparison between two groups was analyzed by the *t*-test using the GraphPad Prism 5.0 (GraphPad Software Inc., California, USA). A value of $P < 0.05$ was considered as statistical significance.

3. Results

3.1. Generation and identification of M1 and M2 cells in vitro

To explore whether PF affects the activities of M1 and M2 cells, we first generated and identified these two type macrophages. The results showed that adherent cells exhibited typical morphology after 7 days culture in accordance with previous reports (21): M1 cells displayed a rounded morphology, while M2 cells appeared elongated and spindle-shaped morphology (data not shown). Next, we examined the surface markers by flow cytometry. The results showed that more than 90% of generated cells were F4/80$^+$CD11b$^+$

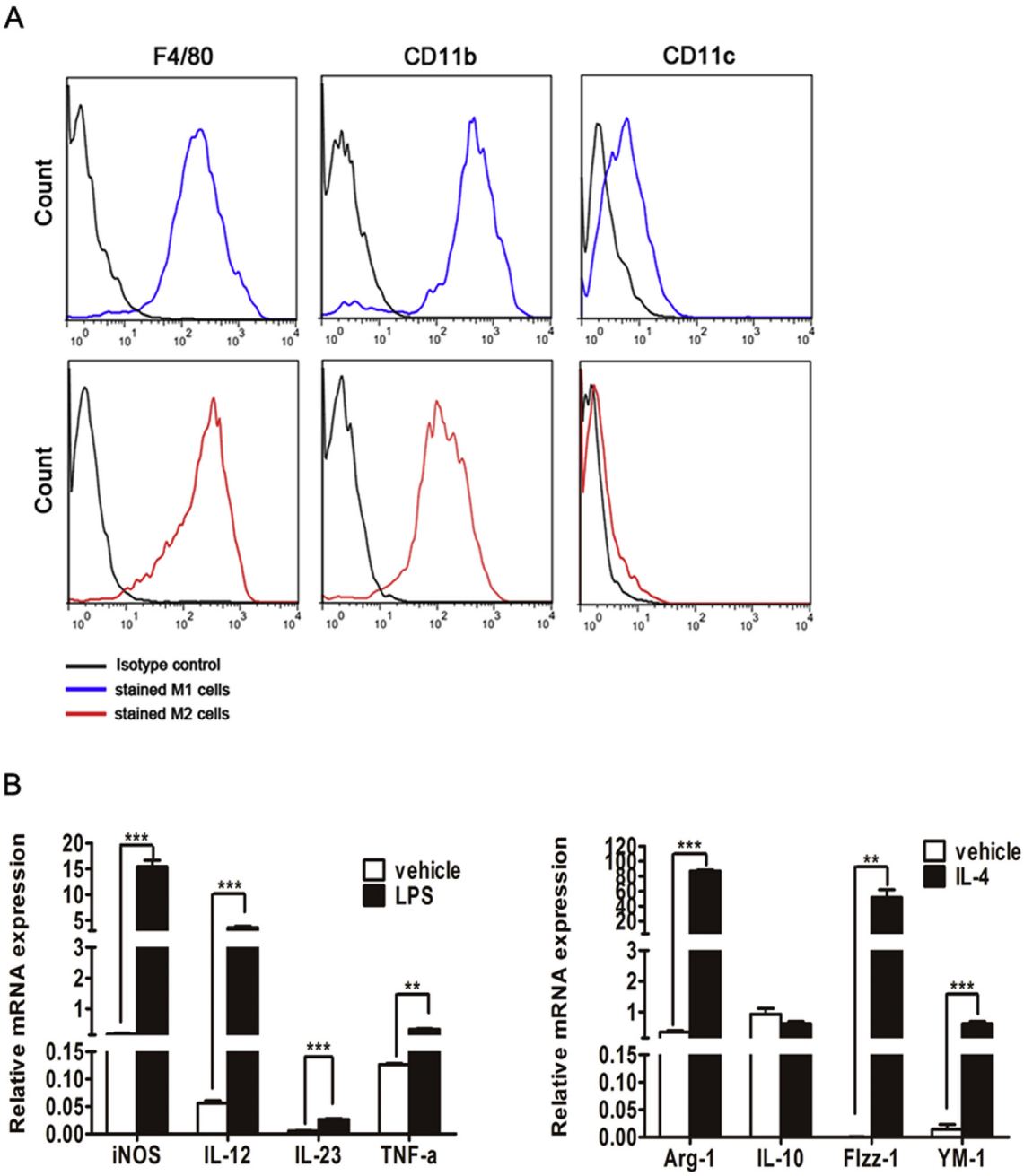

Fig. 1. Identification of M1 and M2 cells generated from mouse bone marrow precursor cells. (**A**) F4/80$^+$CD11b$^+$CD11c$^+$ M1 cells (blue line) and F4/80$^+$CD11b$^+$CD11c$^-$ M2 cells (red line) were estimated by flow cytometry. (**B**) mRNA profiles of M1 and M2 cells stimulated by LPS and IL-4 respectively were detected by real-time PCR. The results are expressed as mean ± SEM of at least three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$.

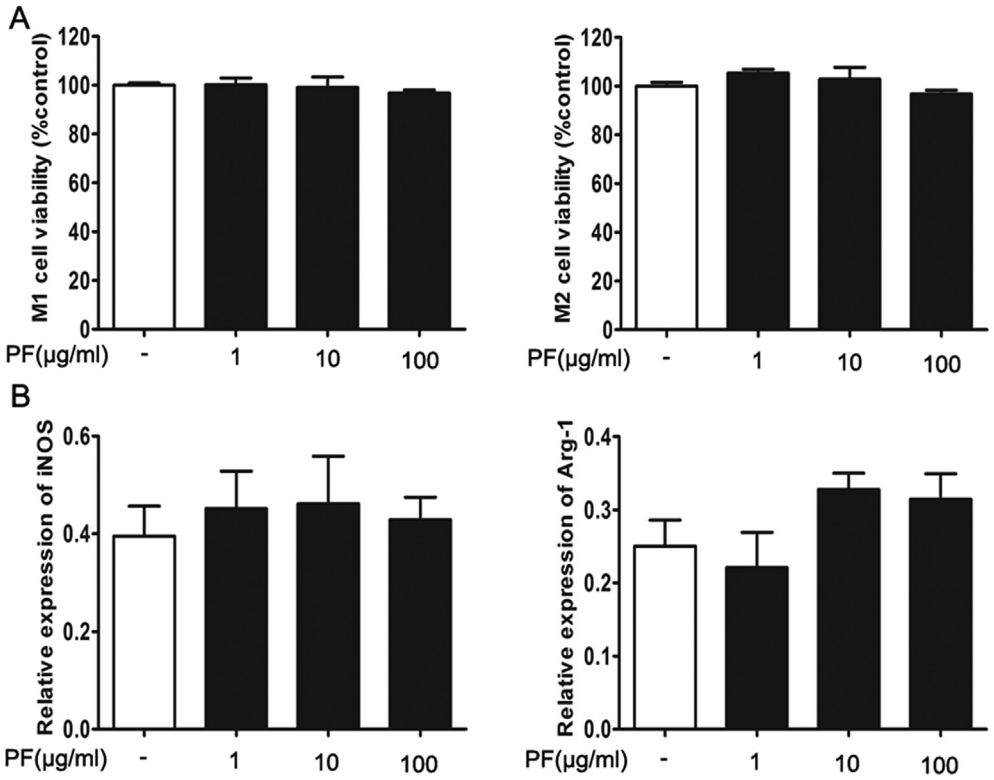

Fig. 2. PF had no effect on the viability and activity of un-stimulated M1 and M2 cells *in vitro*. M1 or M2 cells were treated with (black bar) or not (open bar) different concentrations of PF. (**A**) Cell viability was measured by the CCK-8 assay. (**B**) The mRNA expression of iNOS in M1 cells and Arg-1 in M2 cells was examined by real-time PCR. The results are expressed as mean ± SEM of at least three independent experiments.

macrophages. Further, we identified M1 cells using CD11c, which is uniquely expressed on M1 cells. The results showed that GM-CSF induced cells are F4/80^{+}CD11b^{+}CD11c^{+} M1 cells (Fig. 1A, upper panel), and M-CSF induced cells are F4/80^{+}CD11b^{+}CD11c^{-} M2 cells (Fig. 1A, lower panel). Further, we examined the activity of generated M1 and M2 cells stimulated by LPS and IL-4 respectively. The results showed that LPS stimulated M1 cells expressed high levels of iNOS, IL-12, IL-23 and TNF-a (Fig. 1B, left panel), and IL-4 activated M2 cells expressed high levels of Arg-1, Fizz-1 and YM-1 (Fig. 1B, right panel). These data indicated that we generated typical M1 and M2 cells successfully *in vitro*.

3.2. PF has no effect on un-activated M1 and M2 cells in vitro

Next, we studied the effect of PF on the viability and activity of un-stimulated M1 and M2 cells. The results showed that PF did not affect either the M1 or M2 cells viability (Fig. 2A) or the expression of the iNOS mRNA in M1 cells and the Arg-1 mRNA in M2 cells (Fig. 2B), indicating that PF did not alter the viability and activity of un-stimulated M1 and M2 cells.

3.3. PF impaired the activity of M1 cells stimulated by LPS

It is well known that LPS-induced M1 cells produce high levels of pro-inflammatory cytokines (22), we future explored the effects of PF on LPS-stimulated M1 cells. The results showed 1 μg/ml of PF did not show inhibitory effect, but 10 and 100 μg/ml of PF reduced the iNOS mRNA expression (Fig. 3A). Subsequently, we examined the NO production and iNOS protein expression in LPS-stimulated M1 cells upon PF treatment. The results showed both 10 and 100 μg/ml of PF inhibited NO (Fig. 3B) and iNOS protein production (Fig. 3C). These data suggested that PF indeed decreased LPS-stimulated M1 cells activity by inhibiting iNOS expression at both mRNA and protein levels in a dose dependent manner.

3.4. PF enhanced activity of IL-4-inducing M2 cells

M2 cells show the opposite function of M1 cells in inflammatory and autoimmune diseases (1,2). As we had found that PF could reduce M1 activity, we next tested the potential effect of PF on M2 activity. The results showed that 10 μg/ml and 100 μg/ml of PF enhanced Arg-1 mRNA expression (Fig. 4A). Consistently, we found that 10 μg/ml and 100 μg/ml of PF up-regulated the Arg-1 activity (Fig. 4B) and protein expression (Fig. 4C), indicating that PF indeed increased IL-4-activated M2 cells function by promoting Arg-1 production in a dose dependent manner.

3.5. PF inhibited NF-κB signaling pathway in LPS-stimulated M1 cells

Previous studies have identified that NF-κB pathway links to the LPS-induced M1 activation (23). We thus examined the alteration of NF-κB signaling pathways in PF-treated M1 cells. Using western blotting assay, we found that PF inhibited NF-κB p65 phosphorylation in M1 cells dramatically compared with PF-untreated cells after incubation with LPS for 15 min (Fig. 5A). Consistently, the immunofluorescence analysis showed that PF reduced LPS stimulated NF-κB p65 translocation into the nucleus of M1 cells (Fig. 5B), suggesting that NF-κB is the major signal in LPS-provoked M1 cells affected by PF, which is consistent with our previous reports (14,16).

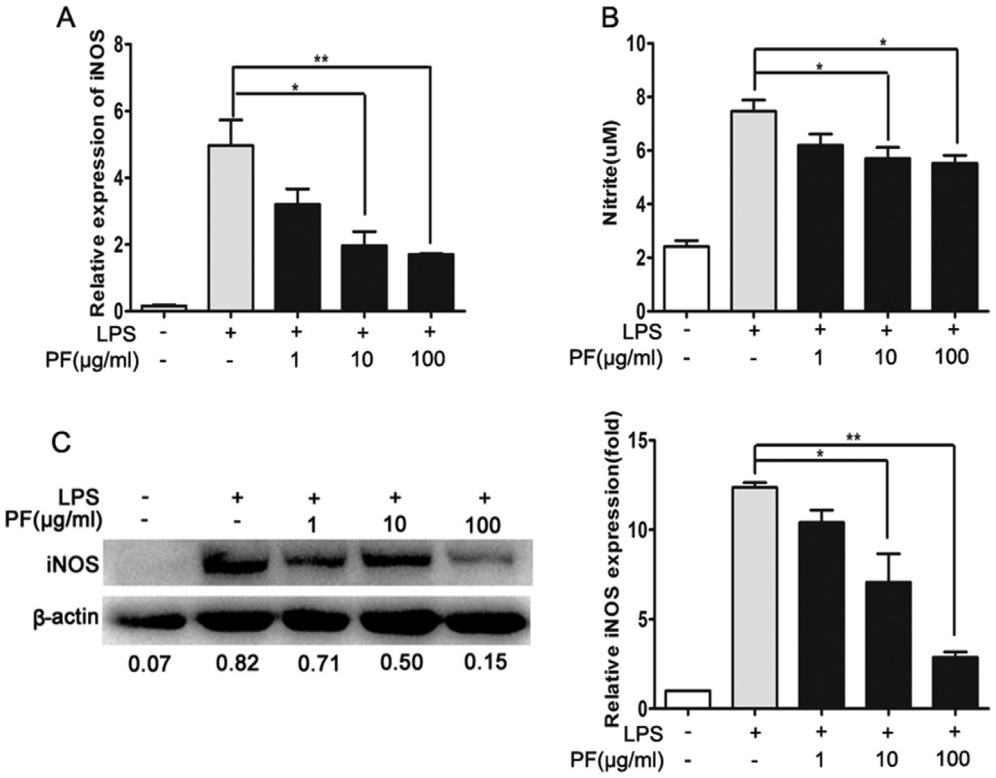

Fig. 3. PF inhibited iNOS and NO production in LPS-induced M1 cells. M1 cells were cultured in the absence or presence of PF and subsequently stimulated with LPS (100 ng/ml). Open bar represented cells without LPS and PF treatment. Grey bar meaned that cells were only treated with LPS. Black bar represented that cells were treated with LPS and PF. (**A**) mRNA expression of iNOS was detected by real time PCR. (**B**) NO levels in the cell culture supernatant were measured by Griess reagent kit. (**C**) iNOS protein expression was evaluated by western blotting (left panel). The relative fold of alterated iNOS was calculated and displayed (right panel). Data are expressed as mean ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

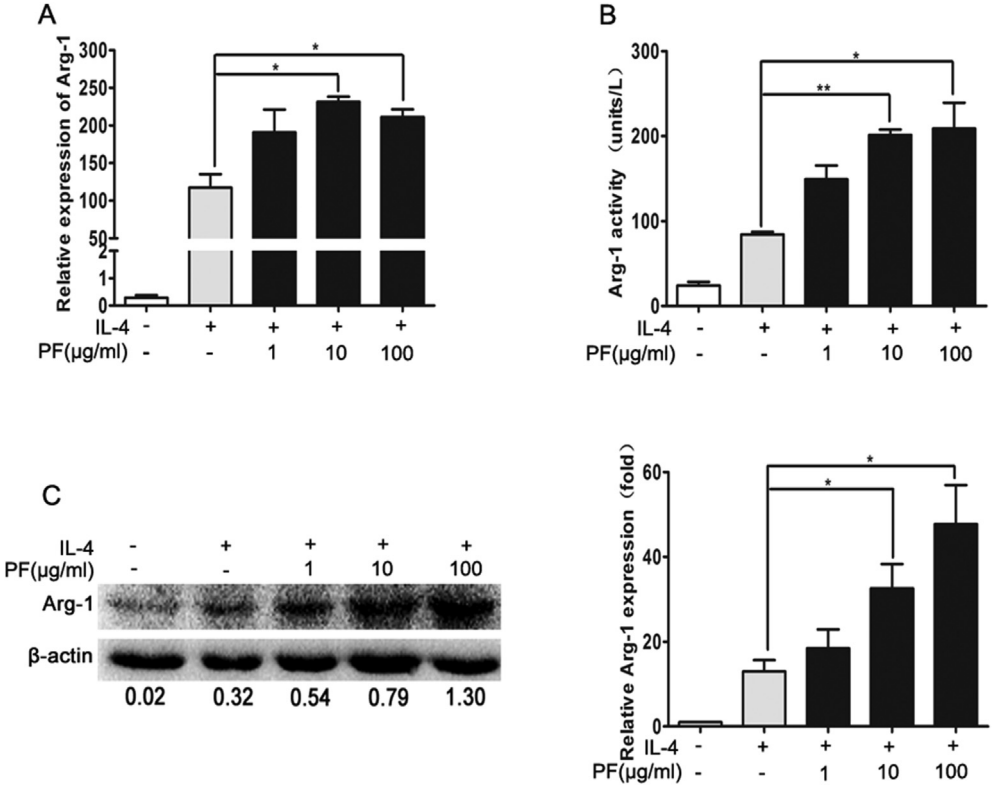

Fig. 4. PF enhanced IL-4-induced Arg-1 activity and expression in M2 cells. M2 cells were stimulated with IL-4 (20 ng/ml) in the absence or presence of PF. Open bar represented cells without IL-4 and PF treatment. Grey bar represented that cells were only treated with IL-4. Black bar meaned that cells were treated with IL-4 and PF. (**A**) Arg-1 mRNA expression was examined by real-time PCR. (**B**)Arginase activity in cell lysate was determined by Arginase activity assay kit. (**C**) Arg-1 protein was evaluated by western blotting (left panel). The relative fold of alterated Arg-1 was calculated and displayed (right panel). Data are expressed as mean ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

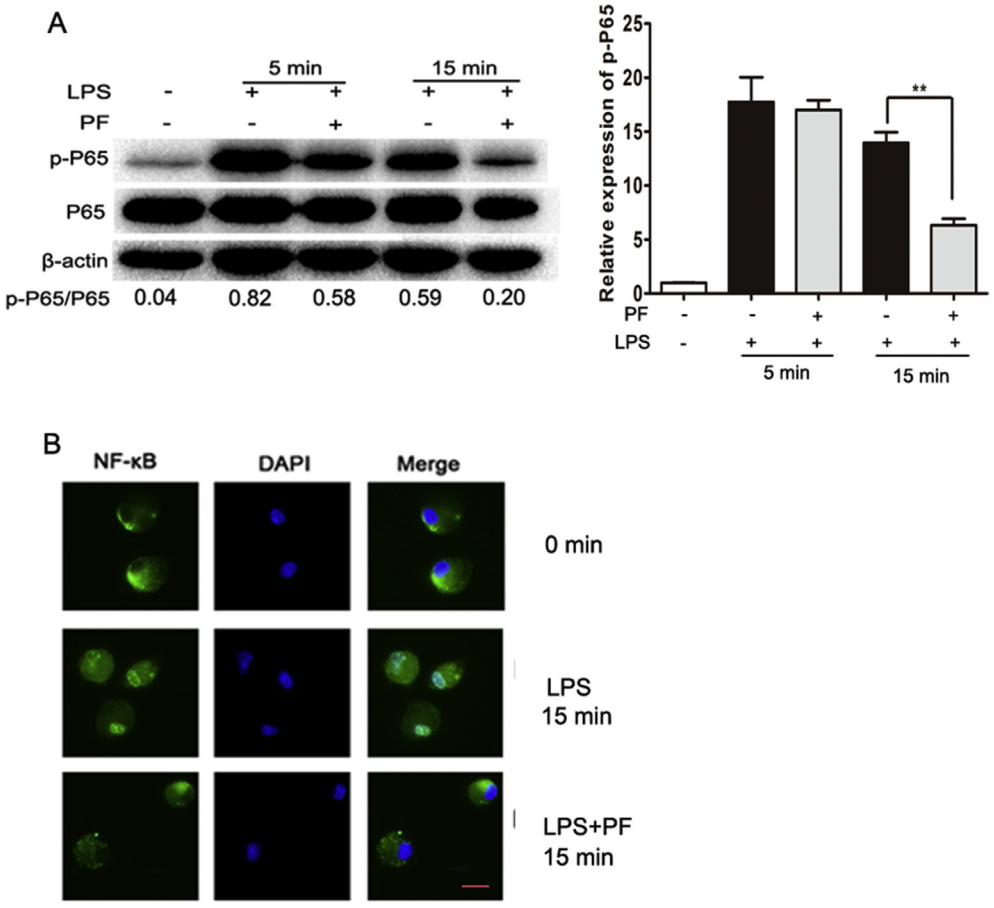

Fig. 5. Signaling pathway involved in PF alleviated M1 cell activity stimulated by LPS. (**A**) M1 cells were stimulated with LPS in the presence or absence of PF for 5 min and 15 min. NF-κB phosphorylation was analyzed by western blotting and the altered phosphorylation of NF-κB was displayed as relative fold which was derived from comparison with their non-phosphorylated counterparts. Data are expressed as mean ± SEM of three independent experiments. $^{**}P < 0.01$. (**B**) Nuclear translocation of NF-κB in M1 cells was monitored by confocal fluorescence microscopy. Top panels, Unstimulated M1 cells; Center panels, M1 cells stimulated with LPS for 15 min; Bottom panels, PF-pretreated M1 cells stimulated with LPS for 15 min. NF-κB p65 was detected by Alexa Fluor 488-anti-p65 (green); Nuclei were stained with DAPI (blue). Merged pictures show NF−κB translocation into the nucleus. Original magnification ×400. Bar, 10 μm. Data represent one of three independent experiments.

3.6. PF activated STAT6 signaling pathway in IL-4-activated M2 cells

Given that STAT6 pathway is related with IL-4-induced M2 activation (24), we examined whether STAT6 was involved in the function of PF-treated M2 cells. First, we performed the western blotting experiments and the data showed that PF increased STAT6 phosphorylation dramatically in M2 cells after stimulation with IL-4 for 5 min (Fig. 6A). To further corroborate the result, we examined the nuclear translocation of STAT6 in PF-treated M2 cells by laser scanning confocal immunofluorescence microcopy. The result showed that PF enhanced STAT6 nuclear translocation in activated M2 cells (Fig. 6B).

4. Discussion

Macrophages, mainly classified into M1 and M2 phenotypes, as first identified immune cells, play critical roles in both auto-inflammatory and autoimmune diseases. It is believed that a switch in the M1/M2 balance is involved in maintaining tissue homeostasis, and once the balance is broken, immune-mediated inflammation and tissue damage occur (6–8). As an extract from the root of *Paeonia lactiflora*, PF has been widely used to treat autoimmune diseases, including macrophage associated diseases (9,12). In this study, using M1 and M2 cells generated from mouse bone marrow precursors, we found that PF inhibited the activity of LPS-induced M1 cells by reducing iNOS/NO production but enhanced IL-4-provoked M2 cells function via upregulating Arg-1 expression and activity. Further analysis showed that PF impaired LPS/NF-κB signaling pathway in activated M1 cells but enhanced IL-4/STAT6 signaling pathway in M2 cells.

It is reported that M1 macrophages drive the recruitment of Th1 cells through producing cytokines, thereby amplifying the pro-inflammatory response in autoinflammatory and autoimmune diseases (25). For example, in rheumatoid arthritis patients, over-active M1 cells produce abundant M1 cytokines (TNF-α, IL-1β and IL-12) and accelerate the development of the disease (26). Besides, excessive M1 polarization in adipose tissues is linked to metabolic disease (27). Furthermore, macrophages accumulate in lesional skin in psoriasis (28) and attenuating macrophage function has been proposed as an effective strategy for psoriasis treatment (29). We reported that PF decreased the number and activities of F4/80^{+}CD68^{+} macrophages, inhibited production of IL-12 and iNOS in IMQ-induced psoriasis-like mice (15). In this study, we further identified that PF decreased LPS-stimulated M1 cells activity by inhibiting iNOS expression in a dose dependent manner.

As we know, M1 subset does not produce pro-inflammatory factors in homeostatic state. We found that PF has no effect on the viability and activity of un-stimulated M1 cells *in vitro*, which is consistent with our pervious study: PF has no effect on un-activated B cells (16). These data revealed that PF selectively inhibited activated immune cells, which is benefit for the clinical treatment.

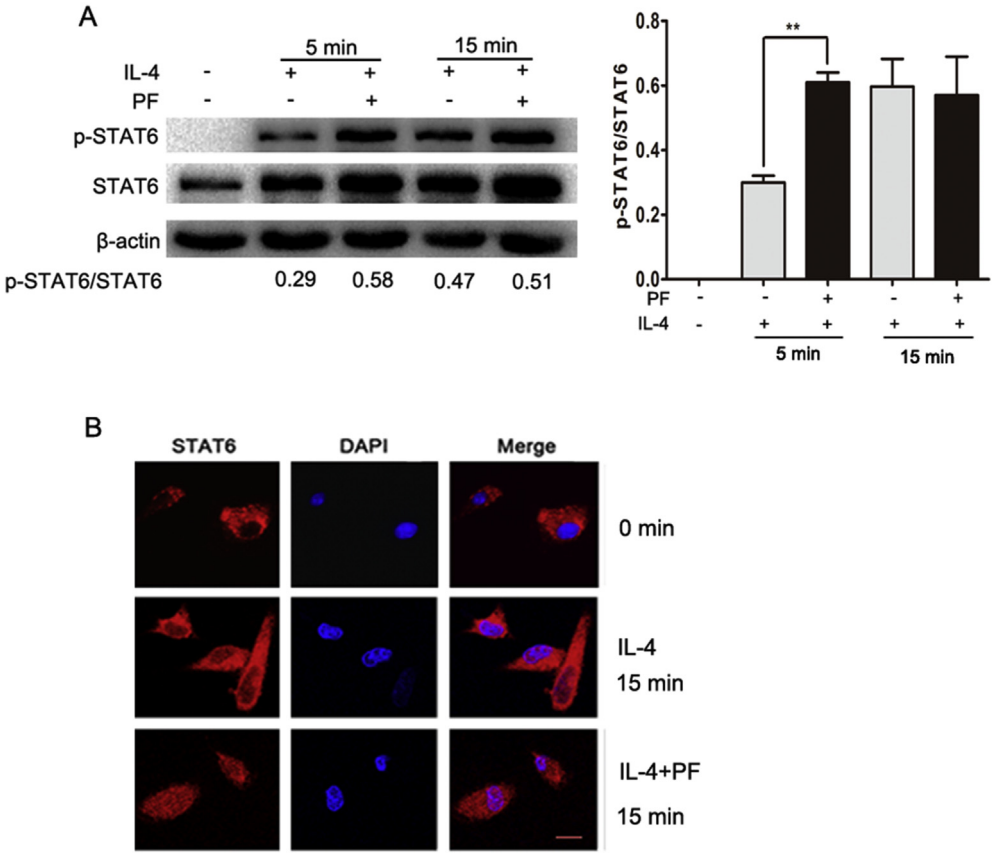

Fig. 6. Signaling pathway involved in PF enhanced M2 function provoked by IL-4. (**A**) M2 cells were stimulated with IL-4 in the presence or absence of PF for 5 min and 15 min. STAT6 phosphorylation was analyzed by western blotting and the altered phosphorylation of STAT6 was displayed as relative fold which was derived from comparison with their non-phosphorylated counterparts. Data are expressed as mean ± SEM of three independent experiments. $^{**}P < 0.01$. (**B**) Nuclear translocation of STAT6 in M2 cells was monitored by confocal fluorescence microscopy. Top panels, Unstimulated M2 cells; Center panels, M2 cells stimulated with IL-4 for 15 min; Bottom panels, PF-pretreated M2 cells stimulated with IL-4 for 15 min. STAT6 was detected by Alexa Fluor 594-anti-STAT6 (red); Nuclei were stained with DAPI (blue). Merged pictures show STAT6 translocation into the nucleus. Original magnification ×400. Bar, 10 μm. Data represent one of three independent experiments.

Considering the opposite roles of M1 and M2 cells during immune response and tissue damage (30), we deduced that PF would be in favor of M2 activation. Indeed, in this study, we found that PF could increase IL-4-provoked M2 cells activities by enhancing Arg-1 production and activity. It is well known that in immune system, the regulatory subsets play very important role in maintaining homeostasis and attenuating inflammation by producing cytokines (IL-10, TGF-β) or integrating with target cells directly (31). For example, regulatory T cells (Treg) are found to inhibit the inflammation and immune response in many diseases (32). Accordingly, increasing the number and activity of these regulatory cells could be developed as an effective strategy in the treatment of immune and autoinflammatory diseases (33,34). PF has been shown to promote Treg differentiation and improve Th2 cells function *in vivo* (35). It is reported that IL-4-activated macrophages (considered as M2 cells) were involved in magnifying Treg cells and Th2 cells responses (25,36). Together with our current study that PF enhanced the function of IL-4-activated M2 cells, we suggested that PF could improve the activity of immune regulatory cells widely *in vivo* and *in vitro*. These data showed that PF could suppress M1 cells and enhance M2 cells activity simultaneously.

What might be the mechanism responsible for the activation of M1/M2 regulated by PF?

It is believed that a predominance of NF-κB activation contributes to LPS-induced M1 response (23). LPS, an agonist of TLR4, cooperating with CD14, initiates innate immunity response and inflammation (37). In our previous studies, PF was found to selectively arrest LPS-induced DC maturation (14), inhibit B cells activation provoked by LPS but not anti-CD40 or IL-4 (16). In this study, we firmly established that PF negatively regulated M1 cells activity by targeting LPS-induced NF-κB pathway, indicating that PF could selectively inhibit LPS/TLR4/NF-κB signaling pathway. Moreover, STAT6 is an important transcriptional factor that plays a key role in M2 macrophages polarization, and IL-4 can bind to the complex of the type 1 IL-4 receptors (IL-4Ra) and the common γ-chain (γ-c) in M2 macrophages, then the complex receptor system induces phosphorylation of JAK2, which leads to selective activation of STAT6, and the phosphorylated STAT6 can translocate into the nucleus where it binds to the promoter region of target genes, thereby regulating expression of M2 cells associated genes (38). In this study, we first proposed that PF enhanced M2 cells activity via promoting IL-4-mediated STAT6 signaling pathway.

In conclusion, our data investigated that PF could suppress M1 and enhance M2 cells activity simultaneously. Given that numerous studies have shown that PF shows unique bidirectional function by bringing dysfunctional immune system back into balance when treat autoimmune diseases (39), our results provide new insight into the bidirectional regulatory function of PF, which could be benefit for autoinflammatory and autoimmune disease treatment.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jphs.2015.12.007.

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