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Full paper

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

Tianhang Zhai [a, 1], Yue Sun [a, b, 1], Huidan Li [a], Jie Zhang [a], Rongfen Huo [a], Haichuan Li [a, c], Baihua Shen [a], Ningli Li [a, *]

[a] Shanghai Institute of Immunology & Department of Immunology and Microbiology, Shanghai Jiao Tong University School of Medicine, Shanghai, China
[b] Department of Rheumatology, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, China
[c] Maternity and Child Health Hospital, Changning, Shanghai, China

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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),

* Corresponding author. Shanghai Institute of Immunology & Department of Immunology and Microbiology, Shanghai Jiao Tong University School of Medicine, No. 280, South Chong-Qing Road, Huangpu District, Shanghai 200025, China.
E-mail address: ninglixiaoxue57@163.com (N. Li).
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[1] These two authors contributed equally to this work.

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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