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Full paper

Paeoniflorin selectively inhibits LPS-provoked B-cell function

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ABSTRACT

B cells are important in the development of autoimmune disorders through mechanisms involving dysregulated polyclonal B-cell activation, production of pathogenic antibodies, and targeting which reduces inflammation and tissue damage effectively but often leads to patients suffering from secondary infection. Paeoniflorin (PF) is the main substance of the Total glucosides of peony and has been widely used to treat autoimmune diseases for years. However, whether PF affects B cell activity remains unknown. In this study, using purified murine spleen B cells, we analyzed the effects of PF on B-cell function *in vitro*. We found that PF inhibited the expression of CD69/CD86 and the proliferation of B cells stimulated by LPS. In addition, PF reduced the B-cell differentiation and immunoglobulin production that was stimulated by LPS. Interestingly, PF did not alter B-cell activation and proliferation provoked by anti-CD40 or IL-4. These results indicated for the first time that PF inhibits B-cell activation, proliferation and differentiation by selectively blocking the LPS/TLR4 signaling pathway. Furthermore, our data suggest that PF selectively inhibits inflammation and tissue damage mediated by LPS-activated B cells but does not alter CD40/CD40L- or IL-4-provoked B-cell function in autoimmune diseases treatment, which might aid in protecting patients from secondary infection.

1. Introduction

As a unique cell type in the immune system, B lymphocytes are the principal components of the adaptive immune system, but also serve various innate immune functions that play an important role in protective immunity as producers of both antibodies and cytokines (1, 2). It is well known that B-cell receptor (BCR), CD40/CD40 ligand (CD40L), IL-4 and lipopolysaccharide (LPS) signaling are involved in B-cell activation (3, 4). The CD40/CD40L signaling promotes B cell proliferation and cytokines production (5, 6). IL-4 mediates B-cell activation and proliferation by enhancing the antigen presentation of B cells (7). Thus, CD40/CD40L and IL-4 signaling are indispensible in T-cell-dependent B cell activation and play a critical role in adaptive immunity. LPS is ligand of TLR4 and is expressed on the membrane of many cells, including B cells (8). LPS/TLR4 can stimulates B cell activation, leading to pro-inflammatory cytokine production (9), induction of class switch recombination in innate and adaptive immune responses (10).

Through presenting auto-antigens and producing auto-antibodies, B cells are involved in the pathogenesis of autoimmune diseases. The role of B cells in the pathogenesis of rheumatoid arthritis (RA) (11), type I diabetes (12), systemic lupus erythematosus (SLE) (13), and several other autoimmune inflammatory diseases has been described fully. Auto-reactive B cells can present self-antigens to auto-reactive T cells, produce auto-antibodies and pro-inflammatory cytokines, such as TNF-α and interleukin-6 (IL-6), in autoimmune diseases (14). Autoantibodies can greatly amplify inflammatory responses and promote tissue damage. Immune complexes containing IgM or IgG autoantibodies can initiate complement-mediated inflammation and promote antibody-dependent cellular cytotoxicity by NK cells or macrophages (15–17). Thus, inhibiting the abnormal activation of B cells is required for therapeutic efficacy in patients with autoimmune diseases; however, maintaining the normal function of B cells as far as possible should be considered for preventing the occurrence of secondary infection in the treatment of autoimmune diseases.

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Total glucosides of peony (TGP) is extracted from the root of Paeonia lactiflora pall (18, 19). Paeoniflorin (PF) is the main active substance of TGP for treatment of autoimmune diseases, e.g., RA, SLE, Diabetic nephropathy (DN) and Sjogren's Syndrome (SS) etc., for many years (20). Mechanismly, PF has been shown to affect anti-inflammatory and immune-regulatory functions by attenuating the functional maturation of dendritic cells (21), inhibiting Th1/Th17 cells development in RA patients (22), altering the methylation of regulatory T cells (Treg) of SLE patients (23), and up-regulating the Treg population in an animal model of SS (24–26). Moreover, PF was reported to be significantly efficient in decreasing the anti-SSA/SSB produced in experimental SS mice, suggesting that PF possibly altered B cells function *in vivo*. However, whether and how PF affect the activities of B cells remains unclear.

In this study, we analyzed the effects of PF on activities of murine B cells *in vitro*. We stimulated B cells with LPS, anti-CD40 and IL-4 in the presence of different concentrations of PF. The results showed that LPS, anti-CD40 and IL-4 provoked B cell activation, which was characterized by higher expression levels of molecules such as CD69 and CD86 and a significant increase in proliferation. However, we found that PF could inhibit CD69 and CD86 expression and the B cell proliferation stimulated by LPS, but did not alter the B cell activation and proliferation provoked by anti-CD40 and IL-4. Furthermore, we found that PF reduced the formation of antibody-secreting cells ($B220^{low}CD138^{+}$ B cells) derived from B cells activated by LPS; consistently, PF impaired the immunoglobulin production (IgM, IgG1, IgG2a, IgG2b and IgG3) of B cells stimulated by LPS. Our study indicates for the first time that PF inhibits B-cell activation and proliferation by blocking the LPS/TLR4 signaling pathway selectively, suggesting that PF provides an advantage for autoimmune disease treatment but does not alter CD40/CD40L- and IL-4-provoked B-cell function.

2. Material and methods

2.1. Animals

Female C57BL/6J mice, 6–8 weeks of age, were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Science and were kept under pathogen-free conditions at the animal core facility of Shanghai Jiao Tong University School of Medicine. All experiments were performed according to the Animal Care and Use Committee guidelines.

2.2. B cell purification

To obtain purified murine B cells, spleens from naïve mice were removed and freshly prepared for single-cell suspensions by mincing through cell strainers, and the erythrocytes were lysed with an ammonium chloride solution. B cells were negatively isolated from the resulting splenocytes using magnetic bead separation. Briefly, splenocytes were depleted for T cells, monocytes/macrophages, dendritic cells, NK-cells, granulocytes, platelets, and CD43-positive B cells (activated B cells, plasma cells, $CD5^{+}$ B1a cells) using biotin-labeled specific mAb and anti-biotin magnetic beads (Invitrogen Life Technologies, Gaithersburg, MD) and an LD magnetic bead column (MiltenyiBiotec, Auburn, CA, USA). The purity of the isolated cells was verified by flow cytometric analysis; the percentage of $B220^{+}$ cells was approximately 95%.

2.3. B-cell culture

Purified resting B cells were cultured in complete RPMI-1640 medium (Gibco, New York, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Gibco), 100 μg/ml streptomycin (Gibco), 100 units/ml penicillin and 2 mM L-glutamine (Gibco). B cells were seeded at $1–5 \times 10^5$ cells/well in a final volume of 200 μl in 96-well flat-bottom plates (for proliferation and activation assay) or 1×10^7cell/well in a final volume of 1 ml in 24-well plates (for antibody-secreting cell transformation and immunoglobulin production assay).

2.4. B cell stimulation

LPS was purchased from Sigma–Aldrich (St. Louis, MO). Anti-mouse CD40 mAb (clone HM-40.3) was obtained from BD Pharmingen (San Diego, CA). Mouse IL-4 was purchased from PeproTech (Rocky Hill, NJ). Purified murine B cells were stimulated with LPS (1 μg/ml), anti-mouse CD40 mAb (50 ng/ml) and mouse IL-4 (10 ng/ml).

2.5. Drugs

PF was supplied from Liwah Plant Extraction Technology Co., Ltd. (Ningbo, China) (21). The molecular weight of PF is 480.05. PF was dissolved in double-distilled water and filtered. In this study, 1 μg/ml, 10 μg/ml, 100 μg/ml and 500 μg/ml of PF were added into cultured and stimulated B cells to analyze the inhibiting titration of PF *in vitro*.

2.6. Flow cytometry analysis

Cells were washed with PBS containing 2 mM EDTA and then stained with various fluorochromes using standard methods provided by the manufacturers. Antibodies used for surface staining of FITC-labeled anti-mouse CD86, PE-labeled anti-mouse CD69, and APC-labeled anti-mouse B220 Abs were purchased from e-Bioscience. For the detection of antibody-secreting cells, cultured cells were incubated with APC-labeled anti-mouse B220 and PE-Cy7-labeled anti-mouse CD138 (e-Bioscience, San Diego, CA, USA). Isotype-matched antibodies were used as controls, and cells were pre-incubated with anti-Fc receptor (CD16/CD32) Ab 2.4G2 (BD Pharmingen) to reduce Fc receptor-mediated non-specific binding. To examine B cell activation, double-positive signals of B220 and CD19 (e-Bioscience, San Diego, CA, USA) were used to gate B cells. All samples were detected using a BD FACS Canto II flow cytometer (Becton Dickinson, San Jose, CA, USA) and analyzed by FlowJo 7.6.2 software (Treestar Inc., California, USA).

2.7. B cell activation assay

To analyze B-cell activation, purified murine B cells were stimulated with LPS, anti-mouse CD40 mAb, or IL-4 in presence of different concentrations of PF. After 24 h, the cells were harvested and stained with anti-mouse B220, anti-mouse CD19, anti-mouse CD69 and anti-mouse CD86 monoclonal antibodies (described above). Then, the labeled cells were examined by flow cytometry. Activated B cells were evaluated according to the percentage of $B220^{+}CD19^{+}CD69^{+}$ or $B220^{+}CD19^{+}CD86^{+}$ populated as previously described (27).

2.8. B cell division assay

Purified B cells were stimulated by LPS, and cell division was detected using flow cytometry as described previously (3). In brief, 1×10^7/ml of B cells were washed with PBS and subsequently labeled with carboxy fluorescein succinimidyl ester (CFSE) and incubated for 10 min at 37 °C. Then, RPMI1640 containing 10% FCS was added to the labeled cells to stop the reaction. Furthermore, the cells were washed another twice with PBS, the profile of CFSE-