Effects of red clover extract on the activation and proliferation of mouse T lymphocytes and the NO secretion of mouse macrophages

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Abstract: The study investigated the effects of red clover extract (RCE) on mouse T macrophages and lymphocytes *in vitro*. The cell toxic effect of RCE was estimated by MTT assay. Multiple-fluorescence staining plus flow cytometry were used to detect the effect of RCE on CD69/CD25/CD71 expression of mouse T lymphocytes stimulated by Con A; CFDA-SE staining plus flow cytometry were used to analyze the effect of RCE on proliferation of T lymphocytes activated by Con A; The effect of RCE on nitric oxide (NO) secretion of mouse macrophages stimulated by lipopolysaccharide (LPS) for 24 h was assayed by Griess reagent system. We found that RCE had potent anti-inflammatory effects on mice. RCE had little cell toxic effect on mouse lymphocytes and macrophages. RCE strongly inhibited the excessive production of inflammatory mediators (NO, CD69, CD25, CD71), in a dose-dependent manner, like cyclosporine A injection. RCE could inhibit proliferation of $CD3^+$ T lymphocytes. These data suggested that RCE might exhibit anti-inflammatory effect by inhibiting the activation and proliferation of mouse lymphocytes and the NO secretion of mouse macrophages.

Key words: red clover extract; lymphocyte; macrophage; proliferation; activation; nitric oxide

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红车轴草提取物对小鼠淋巴细胞活化与增殖及巨噬细胞分泌 NO 的影响

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摘要：探讨红车轴草提取物(RCE)在体外对小鼠 T 淋巴细胞和巨噬细胞的影响。MTT 法检测 RCE 对细胞的毒性作用。荧光抗体染色结合流式细胞术检测 RCE 对 T 淋巴细胞在 Con A 的刺激下表达活化抗原 CD69、CD25、CD71 的影响。CFDA-SE 标记技术结合流式细胞术分析 RCE 对 T 淋巴细胞在 Con A 诱导下增殖情况的影响。Griess 法检测 RCE 对小鼠巨噬细胞在 LPS 刺激 24 h 后分泌 NO 的影响。RCE 对小鼠有潜在的抗炎作用。RCE 对小鼠淋巴细胞和巨噬细胞的细胞毒作用很小。不同质量浓度的 RCE 能够很好的抑制过量的炎症相关信号分子表达，如 NO，CD69，CD25，CD71，且呈剂量依赖性。RCE 能够抑制 T 淋巴细胞的增殖。数据显示 RCE 可能通过对小鼠淋巴细胞活化与增殖及巨噬细胞 NO 分泌的抑制展示其抗炎效应。

关键词：红车轴草提取物；淋巴细胞；巨噬细胞；增殖；活化；一氧化氮

Inflammation is a multi-step process that is mediated by activated inflammatory and immune cells, including lymphocytes and macrophages[1]. In the presence of stimuli such as lipopolysaccharide (LPS), activated macrophages induce the overproduction of proinflammatory cytokines and NO, and mediate many inflammatory diseases[2]. The development of effective herbal medicines has recently sparked renewed interest in the development of novel therapeutic strategies to suppress the production of proinflammatory cytokines and NO using macrophages[3].

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Red clover extract contains several general classes of compounds particularly rich in isoflavones. RCE were traditionally valued mainly as a respiratory antispasmodics, an anticancer and an estrogenic agent[4]. There were few reports suggesting that RCE could be a useful therapeutic approach to the treatment of inflammatory diseases. In this study, we evaluated the potential of RCE as a therapeutic modality for inflammation *in vitro* by using mouse macrophages and lymphocytes. Our results showed that RCE largely inhibited the excessive production of inflammatory mediators such as NO, CD69, CD25, CD71.

Materials and methods

Chemicals and materials The powder of RCE obtained from Phytoway Inc. of China contains abiochanin A 21%, formononetin 18%, daidzein 18%, genistein 9%, sission 9%, ononin 8%, daidzin 7% and genistein 6%. It was dissolved in dimethyl sulphoxide (DMSO; Sigma, USA) prior to use, stock solution 200 g · L^{-1}. It was diluted with PBS during assays to a working solution 400 mg · L^{-1} and 2 g · L^{-1}. Cyclosporine A injection (CsA, 50 g · L^{-1}) obtained from Shuanglu Inc. of Beijing, with a final concentration 5 mg · L^{-1}. Penicillin G 8×10^5 u, streptomycin sulfate 1×10^6 u, RPMI-1640, Dulbecco's modified Eagle' smedia (DMEM) and fetal bovine serum (FBS), were obtained from Gibco (Grand Island, NY, USA). Anti-mCD3-FITC, anti-mCD69-PE, anti-mCD25-PE and anti-mCD71-PE were obtained from Pharmingen Inc. of USA. Griess reagent was obtained from Promega Corporation (USA). Phosphate buffered saline (PBS, pH 7.2) by autoclaved sterilization (121 ℃, 20 min), was prepared in our Lab. CO_2 incubator was from Rheoyne (Rheoyne, USA). Male or female BALB/c mice, weighing 20 – 30 g, were supplied by the Experimental Animal Center of Guangdong Province (Guangzhou, CN).

Cell culture and isolation Eight-week-old male BALB/c mice were sacrificed and primary lymphocytes were collected from lymph nodes. The lymphocytes were washed with PBS and plated with RPMI-1640 containing 10% heat-inactivated FBS, 100 u · mL^{-1} of penicillin and 100 mg · L^{-1} of streptomycin for the experiments.

RAW264.7 cells (American Type Culture Collection) were maintained in DMEM supplemented with 10% FBS, 100 u · mL^{-1} of penicillin and 100 mg · L^{-1} of streptomycin at 37 ℃ in a 5% CO_2 incubator.

Primary macrophages were collected from the peritoneal cavities of mice (8-week-old female BALB/c) after an intraperitoneal (IP) injection of 3 mL of 1% thioglycolate broth (Sigma, USA) 3 days before harvesting. The peritoneal macrophages were washed with PBS and plated with DMEM containing 10% FBS, 100 u · mL^{-1} of penicillin and 100 mg · L^{-1} of streptomycin overnight for the experiments.

MTT assay analysis the cell toxic effect of RCE A commercially-available cell viability assay was employed to evaluate the cell toxic effect of RCE using the MTT-based colorimetric assay (Sigma, USA). Lymphocytes (2×10^5 cells per well), RAW264.7 cells (2×10^4 cells per well) and peritoneal macrophages (5×10^4 cells per well) were plated with a variety of concentrations of RCE in 96-well plates (Becton Dickinson Inc, USA), and were then cultured for 72 h at 37℃ in a 5% CO_2 incubator. At the termination of cultures, 20 μL of the MTT solution was added to each well, then cultured another 4 h at 37 ℃ in a 5% CO_2 incubator. 100 μL of solubilized solution was added to each well; the plate was allowed to stand overnight in the incubator after evaluation for complete solubilization of the purple formazan crystals and the measurement of the optical density (OD) at 570 nm by a microplate reader (Bio-Rad, USA).

Measurement of the CD69/CD25/CD71 expression of lymphocytes Prepare mouse lymphocytes suspension under germ free condition. After pre-incubation of the lymphocytes (5×10^5 cells per well) with a variety of concentrations of RCE or 5 mg · L^{-1} cyclosporine (CsA) for 4 h in a 96-well plate, either with or without concanavalin A (Con A; Sigma, USA) (5 mg · L^{-1}), the cells were incubated at 37 ℃ in a 5% CO_2 incubator. Cells were collected and stained with Anti-mCD3-FITC and anti-mCD69-PE or anti-mCD3-FITC and anti-mCD25-PE or anti-mCD3-FITC and anti-mCD71-PE after 12/24/36 h separately, then measured them with flow cytometry (Becton Dickinson Inc, USA).

Measurement of the proliferation of lymphocytes Mouse lymphocytes (1×10^7 cells · mL^{-1}) stained with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; Molecular Probes, USA) (1 μmol · L^{-1}), were pre-incubated with a variety of concentrations of RCE or 5 mg · L^{-1} CsA for 4 h in a

96-well plate (5×10^5 cells per well), either with or without Con A ($5\ mg \cdot L^{-1}$), the cells were incubated for 72 h at 37 ℃ in a 5% CO_2 incubator. Then collected cells and detected the proliferation of mouse $CD3^+$ T lymphocytes stimulated by Con A with Flow cytometry.

Measurement of nitric oxide secretion of macrophages After pre-incubation of the RAW264.7 cells (5×10^4 cells per well) and peritoneal macrophages (1×10^5 cells per well) with a variety of concentrations of RCE or $5\ mg \cdot L^{-1}$ CsA for 24 h in 96-well plates, either with or without lipopolysaccharide (LPS; Sigma, USA) ($2\ mg \cdot L^{-1}$), the cells were incubated for 24 h at 37 ℃ in a 5% CO_2 incubator. Nitric oxide was measured by Griess reagent system, 50 μL of 1% sulfanilamide in 5% phosphoric acid and 50 μL of 0.1% *N*-1-napthylethylenediamine dihydrochloride in water to 50 μL of culture supernatant for 15 min at room temperature in the dark. The absorbance at 540 nm was determined on a microplate reader (Bio-Rad, JP). Another standard curve was generated by the same way using $NaNO_2$ (Promega Corporation, USA).

Statistics Data was presented by the means ± standard error (SE). Statistical analysis was performed by one-way ANOVA. A $P < 0.05$ was considered statistically significant.

Results

1 Cell toxicity of RCE

For excluding the possibility that reductions of activation, proliferation and the levels of NO from the cells were due to direct toxicity of RCE to the cells, we evaluated cell toxicity at a variety of concentrations of RCE ($5-200\ mg \cdot L^{-1}$) by MTT assay (Figure 1). RCE-induced cell toxicity was negligible at concentrations of $5-40\ mg \cdot L^{-1}$ in lymphocytes (Figure 1A), RAW264.7 cells (Figure 1B) and peritoneal macrophage cells (Figure 1C).

2 RCE inhibited the CD69/CD25/CD71 expression of $CD3^+$ lymphocytes

CD69/CD/25/CD71 are the key markers of T lymphocytes' activation. The T lymphocytes from lymph node express little cluster of differentiation in quiescent condition, CD69/CD/25/CD71 expression rate increase notably in the group stimulated by Con A. The rate depress in a dose-dependent manner with the intervention of RCE (Figure 2a,b,c). The depression of $40\ mg \cdot L^{-1}$ RCE near $5\ mg \cdot L^{-1}$ CsA. The group

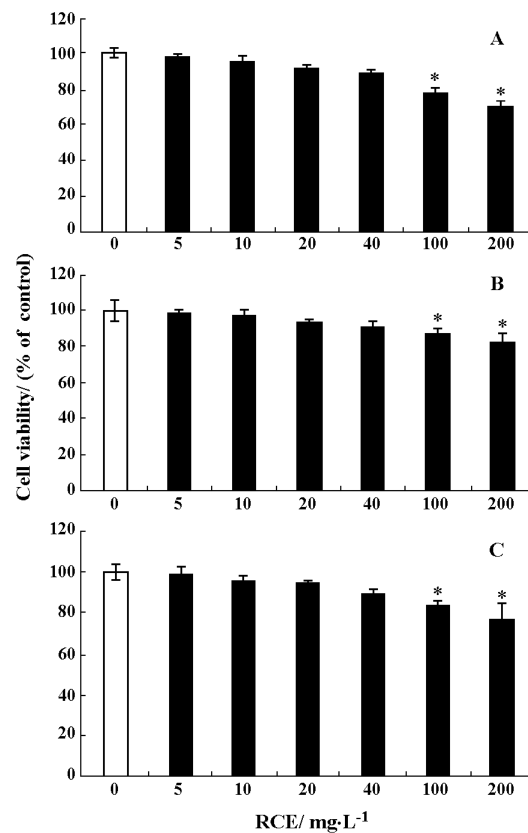

Figure 1 Cell toxic effect of RCE in lymphocytes and macrophages. The cell toxic effects of RCE in lymphocytes (A), RAW264.7 cells (B) and peritoneal macrophages (C) were determined by MTT assay. Six independent experiments were performed, and data shown represent the mean ± SE; $^*P < 0.05$ *vs* control

with $40\ mg \cdot L^{-1}$ RCE only almost as the same as control.

3 RCE inhibited the proliferation of $CD3^+$ lymphocytes

CFDA-SE labeling technique is cellular dye (CFDA-SE)'s fluorescence intensity weaken a half in next generation in the detection of flow cytometry, CFDA-SE can stain living cell[5]. The result displayed that $(87.81 \pm 0.27)\%$ of the lymphocytes that from lymph node were $CD3^+$ lymphocytes, most T lymphocytes were parental generation in the control group (Figure 3A). With the stimulated of Con A, T lymphocytes appear three filial generations except the parental generation (Figure 3B), proliferation index (PI, total generations cell number *vs* parental generation cell number) is 2.62 ± 0.005 (Figure 4). All RCE group's PI smaller than the Con A group's (Figure 4),

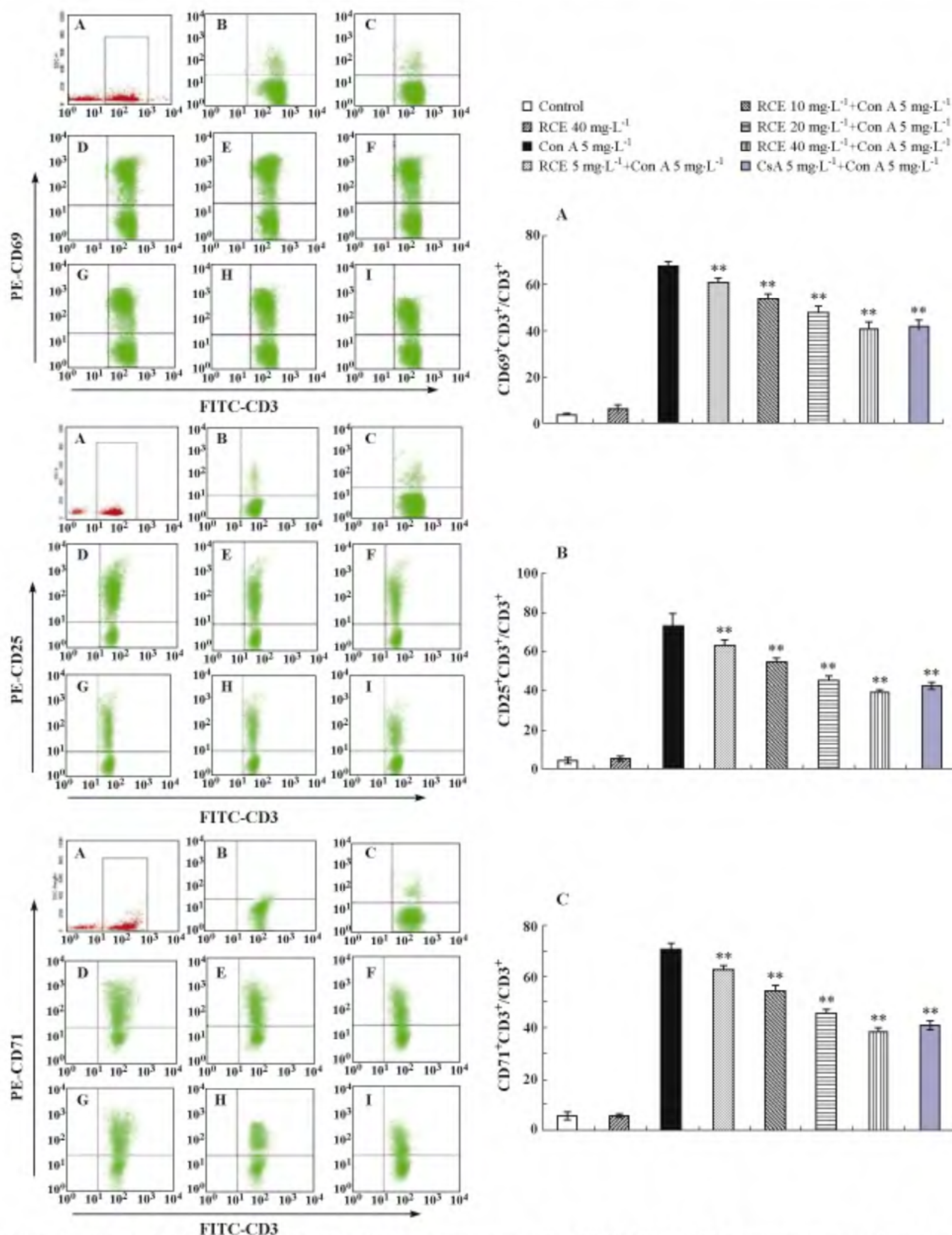

Figure 2 RCE inhibited the CD69/CD25/CD71 expression. The CD69/CD25/CD71 expressions of lymphocytes were detected by multiple-fluorescence staining plus flow cytometry. CD69/CD25/CD71 expression rate depresses in a dose-dependent manner with the intervention of RCE. The depression of 40 mg · L^{-1} RCE near 5 mg · L^{-1} CsA. It is a typical example in the left of figure in six independent experiments. A: The cell region acquired on flow cytometry; B: Control; C: 40 mg · L^{-1} RCE only; D: 5 mg · L^{-1} Con A; E: 5 mg · L^{-1} Con A + 5 mg · L^{-1} RCE; F: 5 mg · L^{-1} Con A + 10 mg · L^{-1} RCE; G: 5 mg · L^{-1} Con A + 20 mg · L^{-1} RCE; H: 5 mg · L^{-1} Con A + 40 mg · L^{-1} RCE; I: 5 mg · L^{-1} Con A + 5 mg · L^{-1} CsA. Data shown represent the mean ± SE; $^{**}P < 0.01$ *vs* Con A alone

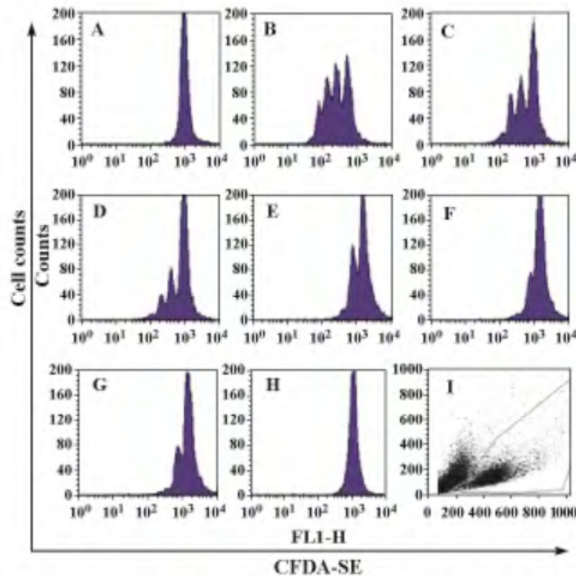

Figure 3 RCE inhibited the proliferation of CD3$^+$ lymphocytes. The proliferation of lymphocytes were detected by CFDA-SE staining plus flow cytometry. Figure three shows a typical example in experimental results. A: Control; B: 5 mg · L^{-1} Con A; C: 5 mg · L^{-1} Con A + 5 mg · L^{-1} RCE; D: 5 mg · L^{-1} Con A + 10 mg · L^{-1} RCE; E: 5 mg · L^{-1} Con A + 20 mg · L^{-1} RCE; F: 5 mg · L^{-1} Con A + 40 mg · L^{-1} RCE; G: 5 mg · L^{-1} Con A + 5 mg · L^{-1} CsA; H: 40 mg · L^{-1} RCE only; I: The cell region acquired on flow cytometry

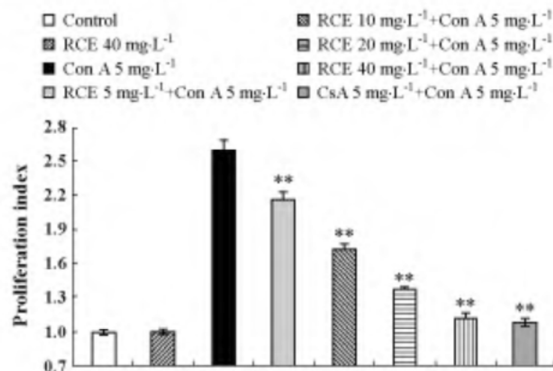

Figure 4 The effect of RCE on proliferation index of CD3$^+$ lymphocytes. Six independent experiments were performed, and data shown represent the mean ± SE; $^{**}P < 0.01$ *vs* Con A alone

the filial generation rate depresses in a dose-dependent manner with the intervention of RCE (Figure 3 C, D, E, F). It appears just one weak filial generation and the parental generation in 40 mg · L^{-1} RCE or 5 mg · L^{-1} CsA group (Figure 3 F, G). The group with 40 mg · L^{-1} RCE only almost as the same as Control. (Figure 3H).

4 RCE inhibited the NO secretion of macrophages induced by LPS

NO is known to be a proinflammatory mediator in inflammatory diseases, we investigated whether the RCE inhibited NO secretion of macrophages that were activated by LPS. As shown in Figure 5, the secretion of NO increased after LPS stimulation in both RAW264.7 cells (Figure 5A) and peritoneal macrophages (Figure 5B). While the secretion of NO in the following group which was pretreated with the RCE was significantly inhibited in both cell types in a dose-dependent manner.

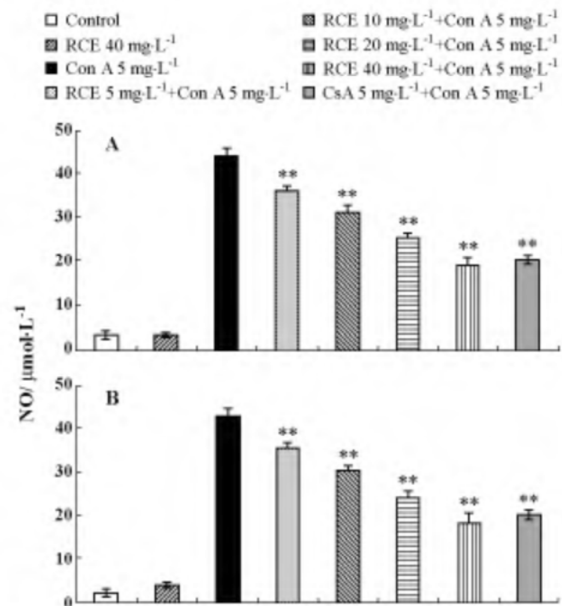

Figure 5 Effects of RCE on the NO secretion of macrophages induced by LPS. RAW264.7 cells (A) and peritoneal macrophages (B) were incubated with different concentrations of RCE in the presence of LPS (2 mg · L^{-1}) for 24 h, respectively. NO was measured by the Griess reaction. The depression of 40 mg · L^{-1} RCE near 5 mg · L^{-1} CsA. Six independent experiments were performed, and data represent are the mean ± SE; $^{**}P < 0.01$ *vs* LPS alone

Discussion

Over several years, semipurified isoflavone supplements made from red clover have been studied for use in menopause, maintenance of bone health, and improvement of cardiovascular health[6]. Despite the identified effects in these diseases, the immunological activities of the RCE have not been fully

explored in effects. Therefore, compared with cyclosporine A we prepared RCE for the treatment of the patients with inflammatory diseases, identified its effects on Con A-stimulated lymphocytes and LPS-activated mouse macrophages.

Recently, new approaches to the use of Chinese herbal plants to prevent and treat inflammatory responses have become an important area of investigation[7]. Here, we confirmed that RCE could remarkably suppress the activation and proliferation of $CD3^+$ lymphocytes (Figure 2; Figure 3). RCE blocked Con A combine with cell surface receptor (TCR/CD3), interfered the early activation incident, then the early activation related protein tyrosine kinase (PTK)-Fyn and PTK-Lck can not be activated, RCE may inhibit the activation of T lymphocytes by this way[8]. T lymphocytes are the key cells in the whole immune response, it is an effective way to handle harmful immune responses by interfering T lymphocytes activation, such as transplant rejection and autoimmune diseases[9]. Existing immunosuppressant mainly interfere the behavior of T lymphocytes such as antigen identification, activation, proliferation, clonal expansion and differentiation, *etc*. The activation and proliferation of T lymphocytes were the foundation of immune function, but excessive activation and proliferation might induce autoimmune disease or organ transplant rejection. Therefore, the drugs which could inhibit the activation and proliferation of T lymphocyte might have a positive role in the effective control of harmful immune responses. For example, the clinical applied immunosuppressant cyclosporine A achieved immune pharmacological effect mainly by inhibited the activation and proliferation of T lymphocyte[10].

The secretion of nitric oxide increased notably with the activation by LPS in macrophages, NO may contribute to the pathological process in various acute and chronic inflammatory conditions, the reduction of NO production may present a useful strategy for the treatment of a variety of inflammatory diseases[11]. In this study, we demonstrate that RCE significantly inhibited NO production (Figure 4) in LPS-stimulated macrophages without showing any cell toxicity (Figure 1). Although no reports have yet addressed the anti-inflammatory effect of Red Clover extract through the modulation of inflammatory molecules, the RCE appears to have an anti-inflammatory action based on our study results.

In conclusion, we demonstrate here that RCE is a potent suppressor against activation and proliferation of Con A-stimulated lymphocytes, and secretion of an inflammatory mediator (NO) in LPS-activated macrophages. Thus, RCE may have therapeutic potential for the modulation and regulation of immune system, and may provide safe and effective treatment options for a variety of inflammation-mediated diseases.

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