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# Immunoenhancement effect of rehmannia glutinosa polysaccharide on lymphocyte proliferation and dendritic cell

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ABSTRACT

The aim of this study is to investigate immunomodulatory effect of rehmannia glutinosa polysaccharide (RGP) on murine splenic lymphocyte and bone marrow derived dendritic cells (DCs). Splenic lymphocytes obtained from mice were co-cultured with RGP for 48 h and then harvested for analyzing with MTT method. The cytokine production of T lymphocytes was measured by ELISA. Effects of RGP treatment on DCs were investigated and assessed by MTT method. The results showed RGP significantly stimulated lymphocyte proliferation and the growth rate of T cell was more significant. The IL-2 and IFN-γ production of T lymphocyte were significantly upregulated after being stimulated with RGP. DCs stimulating on proliferation of T cells and the ability of antigen presenting of DCs have been enhanced under the stimulation of RGP. In conclusion, these findings provided valuable information that RGP possessed strong immunoenhancement activity, which provided the theoretical basis for the further experiment.



## 1. Introduction

*Rehmannia glutinosa* (RG) has been used as traditional Chinese herbal medicine for thousands of years. Rehmannia refers to the root of RG, one herb belonging to the Scrophulariaceae family. It was recorded in Chinese medical classics "Shennong's Herba" and was thought as a "top grade" herb in China (Zhang et al., 1993). Many clinical and experimental studies show that RG possesses various pharmacological properties.

RG was associated with ameliorate of progressive renal failure and diabetic nephropathy (Harari, Vallelian, Meylan, & Pantaleo, 2005; Kang, Sohn, Moon, Lee, & Lee, 2005; Lee, Choi, Cho, & Kim, 2009; Yokozawa, Kim, & Yamabe, 2004). And it could be used as a hypoglycemic and the treatment of various diabetic disorders (Huang, Niu, Lin, Cheng, & Hsu, 2010; Zhang, Zhou, Jia, Zhang, & Gu, 2004). It was also reported that RG extract could enhance the bone metabolism in osteoporosis (Oh et al., 2003), inhibit liver inflammation and fibrosis (Wu, Wu, Tsai, Lin, & Chao, 2011). Besides, it has anti-fatigue (Tan et al., 2012), anti-depressant (Zhang, Wen, Wang, Shi, & Zhao, 2009), neuroprotective effect (Zhang et al., 2008) and so on. Furthermore, RG could inhibit inflammatory responses (Baek et al., 2012), reduce the syndromes of inflammation (Lau et al., 2009; Liu, Tang, Xu, Xia, & Xie, 2007; Sung, Yoon, Jang, Park, & Kim, 2011; Waisundara, Huang, Hsu, Huang, & Tan, 2008) as well as inhibit nitric oxide production (Lau et al., 2012) and protect against cell damage via scavenging free radicals (Yu et al., 2006). Aqueous extract from a steamed root of RG was reported to suppress the production of TNF-α and IL-1 in mouse astrocytes (Kim et al., 1999).

About 70 monomeric compounds have been separated from *Rehmannia glutinosa*, including iridoids, saccharides, phenol glycoside ionone, flavonoid, amino acid, inorganic ions, as well as other trace elements (Oshio & Inouye, 1982; Tomoda, Kato, & Onuma, 1971). Researches have also indicated that polysaccharides are the main chemical components related to the bioactivities and pharmacological properties of the herb (Li, Yu, & Wang, 2004).

In the past few years, pharmacological researches on RG and its active principles mainly focused on wide actions on the blood system, endocrine system, cardiovascular system and the nervous system. This study focused on Rehmannia glutinosa polysaccharide (RGP) function on immune system. The aim of this research was to investigate the effect of RGP on the proliferation of mouse lymphocyte from spleen, and the activity of bone marrow derived

*Abbreviations:* RG, *Rehmannia glutinosa*; RGP, rehmannia glutinosa polysaccharides; IL-2, interleukin-2; IFN-γ, interferon-γ; DCs, dendritic cells; PHA, phytohemagglutinin; LPS, lipopolysaccharide; ELISA, enzyme linked immunosorbent assay; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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dendritic cells (DCs), which provide the theoretical basis for the further experiment in vivo to study whether RGP could promote immune response.

## 2. Materials and methods

### 2.1. Reagents

The purified RGP (≥98% purity) was obtained from Ci Yuan Biotechnology Co. Ltd., Shanxi, China. RPMI-1640 (Gibco) supplemented with benzylpenicillin 100 IU $mL^{-1}$, streptomycin 100 IU $mL^{-1}$ and 10% fetal bovine serum (FCS, Hyclone), was used for re-suspending the lymphocytes, diluting the mitogen and culturing the lymphocytes. RPMI-1640 supplemented with recombinant murine granulocyte macrophage colony stimulating factor (GM-CSF, Amgen, Thousand Oaks, CA) (20 ng/mL), rmIL-4 (R&D Systems, Minneapolis, MN) (20 ng/mL), 10% FCS, benzylpenicillin 100 IU $mL^{-1}$, streptomycin 100 IU $mL^{-1}$, $NaHCO_3$ 2.0 g, HEPES 2.38 g, was used for re-suspending and culturing DCs. Phytohemagglutinin (PHA, Sigma), as the T-cell mitogen, was dissolved into 0.1 mg $mL^{-1}$ with RPMI-1640. Lipopolysaccharide (LPS, Sigma), as the B-cell and DCs mitogen, was dissolved into 0.05 mg $mL^{-1}$ with RPMI-1640. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Amresco Co.) was dissolved into 5 mg $mL^{-1}$ with calcium and magnesium-free (CMF) phosphate-buffered saline (PBS, pH 7.4). These reagents were filtered through a 0.22 μm syringe filter. PHA and LPS solution was stored at −20 °C, MTT solution at 4 °C in dark bottles. Dimethyl sulfoxide (DMSO) was produced by Zhengxing Institute of Chemical Engineering in Suzhou, China.

### 2.2. Splenic lymphocyte proliferation assay

Following the dissection of the abdomens, the spleens were harvested, and spleens from ICR mouse were gently mashed by pressing with the flat surface of a syringe plunger against a stainless steel sieve (200 mesh). After the red blood cells were disrupted, the splenocytes were washed twice. The resulting pellet was resuspended and diluted to $2.5 \times 10^6$ $mL^{-1}$ with RPMI-1640 with fetal bovine serum after the cell viability was assessed by trypan blue exclusion. The solution was divided into two parts: one part was added with PHA or LPS, and respectively incubated into 96-well culture plates, 100 μL per well. Then, RGP at series of concentrations were added, in cell control group and PHA or LPS control group, RPMI-1640 medium and PHA or LPS respectively, 100 μL per well, four wells each concentration. The final concentration of PHA or LPS reached to 10 μg $mL^{-1}$ or 5 μg $mL^{-1}$. The plates were respectively incubated in a humid atmosphere with 5% $CO_2$ (Thermo) at 37 °C for 48 h. Briefly, 30 μL of MTT (5 mg $mL^{-1}$) was added into each well at 4 h before the end of incubation Then the plates were centrifuged at 1000 × *g* for 10 min at room temperature. The supernatant was removed carefully and 100 μL of DMSO was added into each well. The plates were shaken for 5 min to dissolve the crystals completely. The absorbance of cells in each well was measured by microliter enzyme-linked immunosorbent assay reader (Model RT-6000, Leidu Co., Ltd., Shenzhen City) at a wave length of 570 nm ($A_{570}$ value) as the index of lymphocytes proliferation (Fan et al., 2012). Meanwhile, the lymphocytes proliferation rate was calculated as follows:

$$\text{Proliferation rate}\,(\%) = \frac{\bar{A}_{(\text{test group})} - \bar{A}_{(\text{control group})}}{\bar{A}_{(\text{control group})}} \times 100\%$$

### 2.3. Measurement of IL-2 and IFN-γ

Lymphocytes were collected as in Section 2.2. Cells were incubated for 48 h. Culture supernatants were collected and the concentrations of interferon γ (IFN-γ) and interleukin 2 (IL-2) were assayed by ELISA kit (R&D, Co., USA).

### 2.4. Analysis of a range of RGP doses on DCs stimulating proliferation of T cells

#### 2.4.1. Generation of immature DCs

The femurs and tibiae of ICR mouse (4–6 weeks old) were removed and isolated from surrounding muscle tissue. Intact bones were washed twice in PBS. Bone marrow cells were flushed from the femur and tibiae of ICR mouse and treated with ACK lysis buffer (Sigma) to lyze erythrocytes. DCs were cultured at a starting concentration of $2.0 \times 10^6$/mL in round-bottomed 12-well plates with RPMI-1640 supplemented with GM-CSF, rmIL-4, 10% FCS, 2 mL per well. Cells were cultured in a humidified chamber at 37 °C and 5% atmospheric $CO_2$. After incubation for 24 h, the medium with non-adherent cells was replaced with fresh medium. The culture medium was removed and replenished with an equal volume of fresh medium every two days. On the 7th day, full matured DCs were harvested for stimulation of T cell proliferation assays.

#### 2.4.2. RGP on mature DCs

On the 7th day of incubation, different concentrations (200, 100, 50, 25 and 12.5 μg $mL^{-1}$) of RGP, serum-free RPMI-1640 or 5 μg $mL^{-1}$ LPS were added into DCs incubated for another 48 h. After 30 min treatment with 50 μg $mL^{-1}$ mitomycin C at 37 °C, cells were washed with PBS twice, and then resuspended in complete RPMI-1640 at a concentration of $5 \times 10^5$/mL.

#### 2.4.3. Allogeneic mixed lymphocyte reaction

Spleens from ICR mouse were harvested sterility and gently mashed by pressing with the flat surface of a syringe plunger against a stainless steel sieve (200 mesh). After the red blood cells were lyzed, the splenocytes were washed twice and resuspended in complete RPMI-1640. Splenocytes ($1.0 \times 10^6$/mL) were cultured in 96-well plates in a volume of 100 μL/well. The mature DCs were added into each well, four wells each group.

#### 2.4.4. Analysis of T cell proliferation

The cultures were incubated at 37 °C and 5% $CO_2$ for 72 h. In the last 4 h of incubation, MTT (5 mg $mL^{-1}$, 30 μL $well^{-1}$) was added into each well. Then supernatant were discarded before DMSO (100 μL $well^{-1}$) were added. Finally, $A_{570}$ was tested as the index of BMDCs stimulating the proliferation of T cells.

### 2.5. Ability of antigen presentation assessment of RGP on DCs

#### 2.5.1. Generation of immature DCs

The immature DCs were collected as Section 2.4.1.

#### 2.5.2. Preparation of allogeneic lymphocyte

OVA solution mixed with aluminium adjuvant, each mouse was immunized subcutaneous with 100 μg OVA. On the 7th, 14th day, the mice were separately boostered. Three days after the last immunization, the responding cells were collected as in Section 2.4.3. Lymphocytes were resuspended in complete RPMI-1640 at a concentration of $1.0 \times 10^6$/mL, supplemented with OVA (100 μg $mL^{-1}$), in round-bottomed 96-well plates.

#### 2.5.3. Analysis of antigen presentation ability

DCs having been treated with mitomycin C were added into lymphocytes, four wells each group. The cultures were incubated