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Baicalin inhibits IgG production by regulating Treg/Th17 axis in a mouse model of red blood cell transfusion

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ABSTRACT

This study was conducted to evaluate whether baicalin inhibits red blood cell (RBC) immunization and elucidate the underlying mechanism. We used human RBCs with adjuvant lipopolysaccharide (LPS) and transfused mice to induce antibodies as an experimental system for studying the effect of baicalin on RBC immunization. Mice were divided into a human RBC transfused positive control group administered with human RBC and LPS intravenously once or weekly for 4 weeks, control group administered dexamethasone (DEX) intraperitoneally daily for 4 weeks, and treatment group administered baicalin intraperitoneally daily for 4 weeks. Assessment of human RBC immunization was performed by measuring serum immunoglobulin G (IgG) and immunoglobulin M (IgM) against human RBC weekly. Lymphocyte changes in spleen were monitored by flow cytometry. We found that baicalin treatment significantly decreased serum IgG but not IgM production in a time and does dependent manner, with a concomitant reduction in Th17 cells and increase in CD4 regulatory T cells in the spleen. The percentage of CD4-positive cells in the spleen was not decreased in the baicalin-treated group but was decreased in the dexamethasone-treated group. In conclusion, baicalin inhibited RBC immunization, particularly IgG production by regulating the Treg/Th17 axis without damaging spleen function.

1. Introduction

Baicalinis, a flavonoid compound originally isolated from the root of the Chinese herb Huangqin (*Scutellaria baicalensis* Georgi), is an anti-inflammatory agent and has a good safety record in the clinic [1]. It reduces the severity of experimental autoimmune encephalomyelitis [2], asthma [3], colitis [4], systemic lupus erythematosus [5], and other immune diseases. Previous studies showed that baicalin attenuates TNBS-induced colitis [6] in rats and OVA + LPS-induced allergic asthma [7] in mice by modulating the Th17/Treg paradigm.

Scutellaria baicalensis Georgi is a main herbal component of Yin-Chen-Tang, which has been widely used to prevent hemolytic disease in newborns (HDN) caused by Rh and ABO maternal-fetal incompatibility since the 1970s in China and shows good clinical results. Numerous studies of this drug have been published in China, but the mechanism of how it reduces antibody production in pregnant women immunized by fetal red blood cells (RBCs) and newborns remains unclear. Perhaps some of the herbal monomer can participate in the inhibition of transfusion reactions.

Here, we examined if baicalin have the potential to induce transfusion tolerance and the underlying mechanism.

Dexamethasone (DEX) is a glucocorticoid drug which is widely used in the clinic to inhibit immune response in clinical transfusion, particularly urticarial transfusion reactions, but causes strong side effects [8]. Thus, a new immune inhibitor with milder side effects is needed. Many components in herbs have been shown to be effective for treating human disease. Curcumin, a major component of turmeric, can correct defects associated with homozygous expression of delta F508 cystic fibrosis [9] and suppress the growth of myeloma cells [10]. Artemisinins, extracted from sweet wormwood, are among the most potent antimalarial drugs currently available [11]. This indicates that new immunosuppressive chemical monomers can be identified in herbs.

In this study, we transfused human RBCs with adjuvant lipopolysaccharide (LPS) into mice to induce antibody production as an experimental system for studying the effect of baicalin on RBC immunization [12]. IgG and IgM in the mouse serum were detected and

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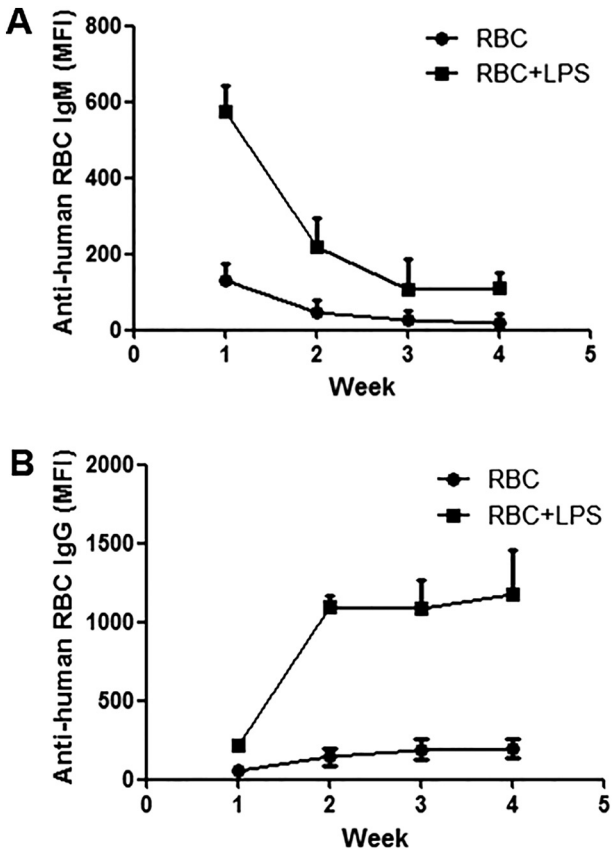

Fig. 1. Mouse transfused with human red blood cells have anti-human blood cell antibody in the serum. Groups of mice (n = 6 per group) were transfused with human RBC or human RBC + LPS i.v. Every week for 4 weeks after transfusion, whole blood was collected via orbital bleeding and the plasma was separated. The presence of IgM (A) and IgG (B) on human RBCs was measured using diluted plasma (1:50), followed by flow cytometry analysis. The anti-human RBC IgM (A) and IgG (B) development was expressed in fluorescent units on the y-axis over time (x-axis) and is shown with error bars depicting the standard error of the mean. The data represent four independent experiments.

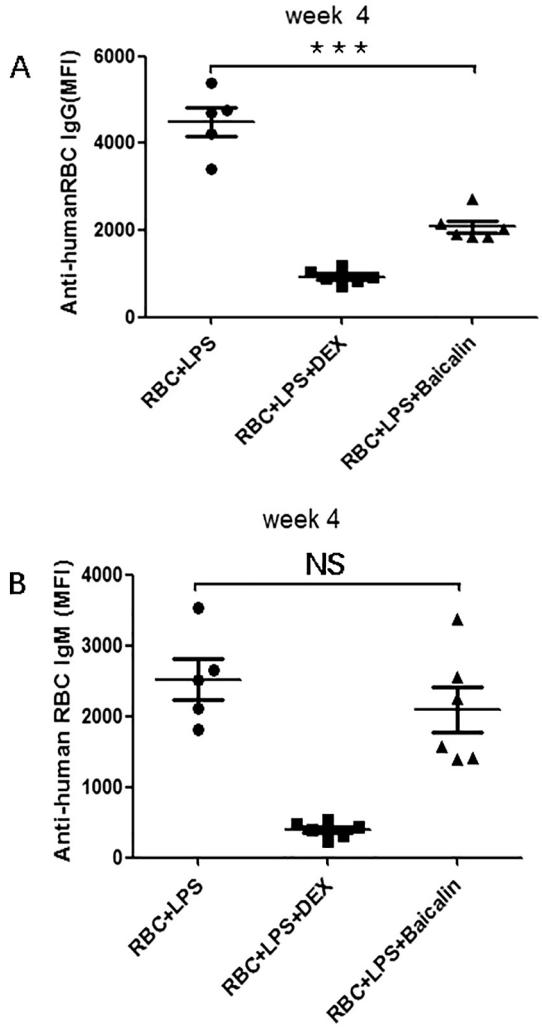

Fig. 2. Suppression of anti-human RBC IgG production by treatment with baicalin. Mice were transfused with human RBC + LPS i.v. each week for 4 weeks. Groups of mice were administered i.p. injections of PBS, DEX (10 mg/kg/day) or baicalin (250 mg/kg/day) daily for four weeks. One week after each human RBC immunization, whole blood was collected via orbital bleeding and the plasma was separated. The presence of anti-human RBC IgG (A) and IgM (B) in the plasma of mice at 4 weeks after immunization was measured using diluted plasma (1:1000), followed by flow cytometry analysis, and is expressed with fluorescent units on the y-axis. For comparison, the P value between RBC + LPS and RBC + LPS + DEX groups is shown. The data represent four independent experiments. $P < 0.05$ was considered statistically significant.

immunological indies were monitored to reveal the function of baicalin in RBC immunization and its underlying mechanism.

2. Materials and methods

2.1. Drug

Baicalin (7-D-glucuronic acid-5, 6-dihydroxy flavone, molecular weight = 446.36) was supplied by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The purity of baicalin as measured by high-performance liquid chromatography was 92.8%. Baicalin was dissolved in dimethyl sulfoxide and then diluted with phosphate-buffered saline (PBS) for intraperitoneal injection.

2.2. Mice and transfusion model with drug administration

Wild-type female C57BL/6 mice (B6, H-2Kb) (age 6–8 weeks) were obtained from the Shanghai Laboratory Animal Center of the Chinese Academy of Science (Shanghai, China). Animals were housed in a specific pathogen-free environment with free access to drinking water supplemented with gentamicin sulfate. Experiments were conducted according to the guidelines of the Institutional Animal Care and Use Committee of the Chinese Association for Laboratory Animal Sciences. This study was carried out in strict accordance with the recommendations in the guidelines of the Institutional Animal Care and Use Committee of the Chinese Association for Laboratory Animal Sciences. The protocol was approved by the Committee on the Ethics of Animal Experiments of Shanghai Blood Center. All surgeries were performed under diethyl ether, and all efforts were made to minimize animal suffering.

C57/BL6 mice were transfused intravenously with 30 µl Ficoll-treated packed RBCs from healthy blood donors (equivalent to approximately 1.2×10^8 cells) without or with 25 µg LPS (Sigma-Aldrich, St. Louis, MO, USA) once or weekly for 4 weeks.

2.3. Flow cytometry analysis

30 µl 2% human red blood cell (2 µl packed red blood cell in 98 µl PBS buffer) were incubated with 30 µl 1:50 diluted mouse serum in 37 °C for 30 min, washed by PBS for three times, and stained with secondary antibodies goat anti-mouse IgG Fab-FITC, goat anti-mouse

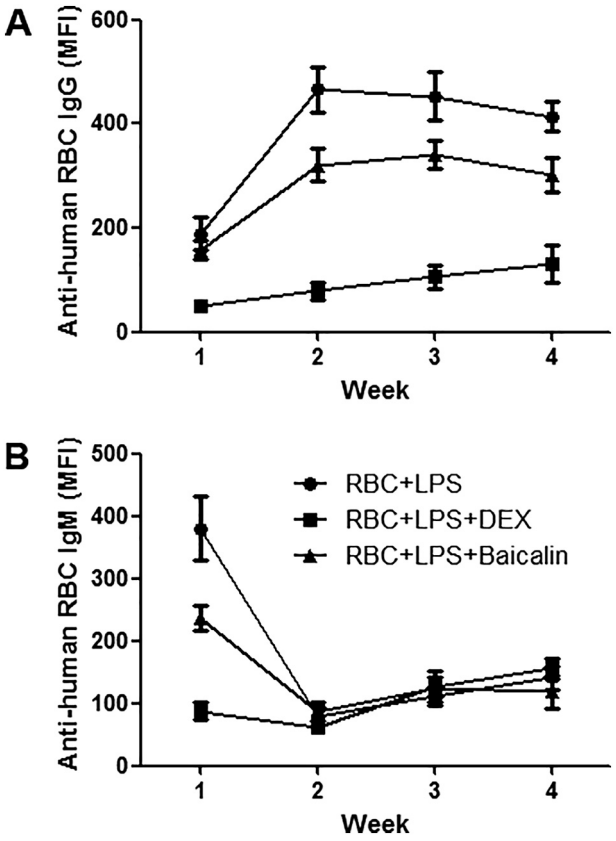

Fig. 3. Effect of baicalin on mouse blood transfusion model in four weeks.
Mice were transfused with human RBC + LPS i.v. each week once. Groups of mice were administered i.p. injections of PBS, DEX (10 mg/kg/day) or baicalin (250 mg/kg/day) daily for four weeks. One week after each human RBC immunization, whole blood was collected via orbital bleeding and the plasma was separated. The presence of anti-human RBC IgG (A) and IgM (B) in the plasma of mice for 4 weeks was measured using diluted plasma (1:100), followed by flow cytometry analysis, and is expressed with fluorescent units on the y-axis. The data represent four independent experiments.

IgM Fab-APC or isotype control monoclonal antibodies (Jackson ImmunoResearch). The spleen was ground and then filtered through a 40 μM cell strainer to obtain a splenocytes suspension. Splenocytes were stained with anti-mouse CD4-FITC mAb, fixed, permeabilized, and stained with anti-mouse FoxP3-PE, IL-17-PE, IL-4-PE, IFN γ-PE or isotype control monoclonal antibodies (BD Biosciences Pharmingen, San Jose, CA, USA). Flow cytometry was done with a FACSCalibur (BD Bioscience), and data were analyzed with CellQuest Version 3.3 software (BD Bioscience).

2.4. Statistical analysis

The results were analyzed with GraphPad Prism 5.0 software (GraphPad, Inc., La Jolla, CA, USA) and SPSS 24.0.0.0 (IBM, Inc.), and data were expressed as the mean ± SEM. Two-tailed Student's *t*-test was used to assess significant differences between two paired groups.

3. Results

3.1. LPS induced robust antibody production in mouse model of blood transfusion

We utilized LPS, a Toll-like receptor 4 agonist, as an adjuvant of human RBCs to transfuse mice [13]. This significantly increased the level of antibody production compared to human RBCs alone (Fig. 1). Anti-human blood cell IgM in the mouse serum reached a peak at one week after blood transfusion, and then decreased over time (Fig. 1A). Anti-human blood cell IgG increased in the first two weeks and reached a plateau (Fig. 1B). Similar results were observed when traditional methods such as the tube test and gel test were utilized (data not shown).

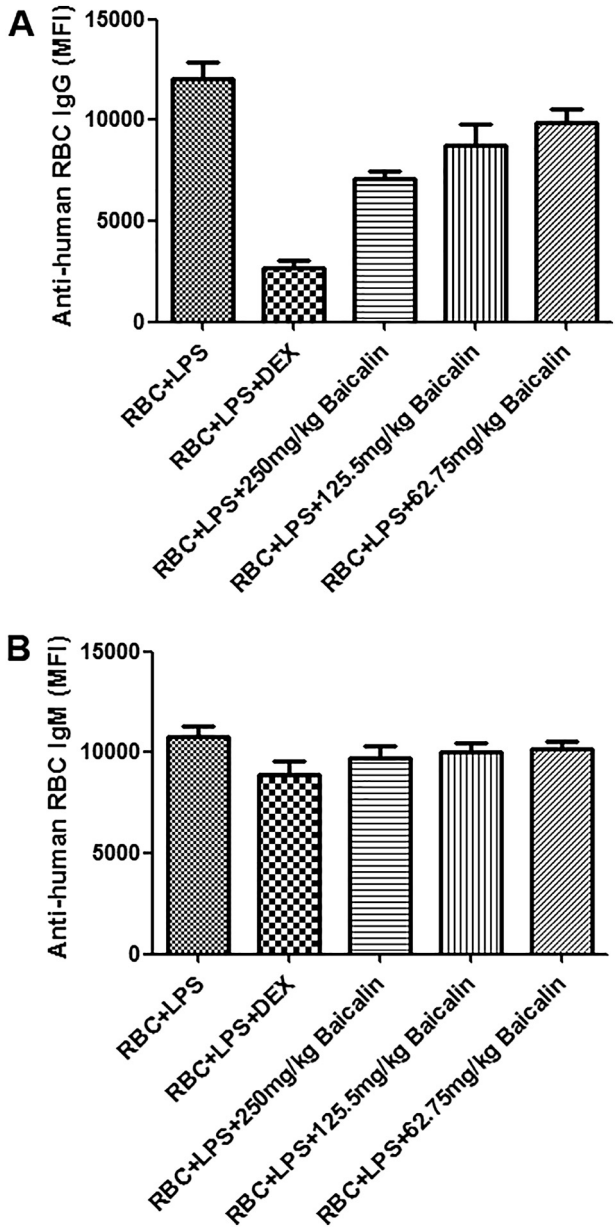

Fig. 4. Effect of different dose of baicalin on antibody production.
Mice were transfused with human RBC + LPS i.v. each week once. Groups of mice were administered i.p. injections of PBS, DEX (10 mg/kg/day) or baicalin (250 mg/kg/day), baicalin (125.5 mg/kg/day), baicalin (62.75 mg/kg/day) daily for two weeks. The presence of anti-human RBC IgG (A) and IgM (B) in the plasma of mice at week 2 was measured using diluted plasma (1:100), followed by flow cytometry analysis, and is expressed with fluorescent units on the y-axis. The data represent four independent experiments.

3.2. Baicalin treatment inhibited serum IgG but not IgM development in a time and dose dependent manner

To test whether baicalin suppresses antibody responses against human RBC antigens, mice were divided into a human RBC-transfused positive control group administered human RBCs and LPS intravenously

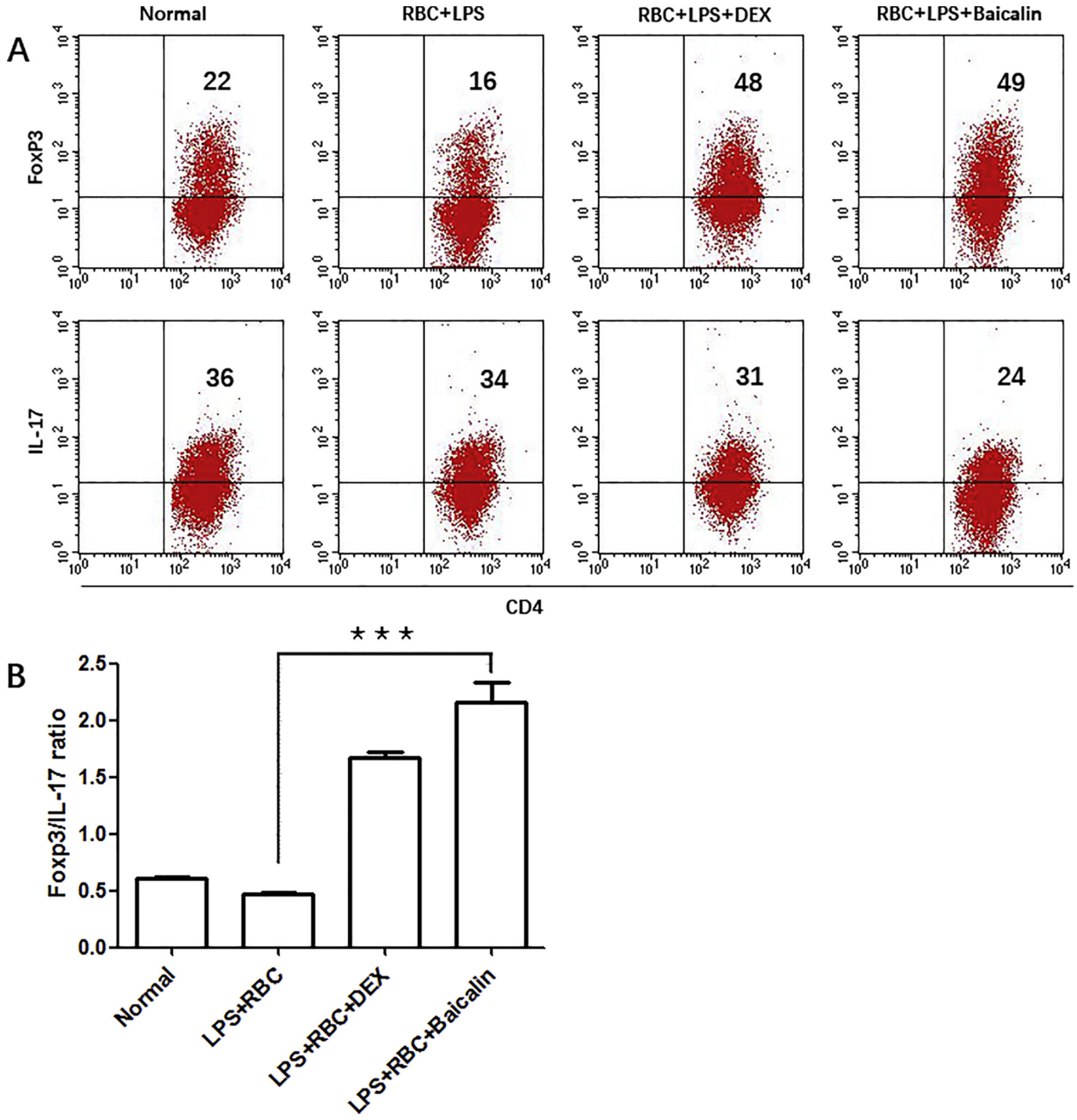

Fig. 5. Inhibition of IgG production is associated with polarization of the Treg/Th17 balance. At week four after serum collection via orbital bleeding, splenocytes from all groups were stained with anti-mouse CD4-FITC mAb, and then stained intracellularly with anti-Foxp3 mAb and anti-IL-17 mAb. Flow cytometry was used to measure the percent frequency of positively stained cells (A), and the frequency of Treg/Th17 cells was expressed as the mean ± SEM of three independent experiments. (B) $P < 0.05$ was considered statistically significant.

weekly for 4 weeks, control group administered DEX, and treatment group administered baicalin. Baicalin treatment decreased serum IgG but not IgM production, as levels in the four week showed a significant difference compared to the control model group. DEX treatment also inhibited serum IgG but not IgM production (Fig. 2). To examine if baicalin treatment inhibited serum IgG but not IgM in a time dependent manner, we immunized mouse with human RBC once, and then give them DEX or baicalin as the regimen above. It is observed baicalin inhibit IgG production since week 2 but not IgM (Fig. 3), To examine if baicalin treatment inhibited serum IgG but not IgM in a dose dependent manner, we immunized mouse with human RBC once, and then give them DEX or different dose of baicalin for two weeks. We found baicalin inhibited serum IgG in a dose dependent manner but not IgM (Fig. 4).

3.3. Baicalin inhibited Th17 development and increase Treg proportion

We examined if CD4 T cell subsets including Th1, Th2, Treg, and Th17 could participate in the regulation of \antibody production by

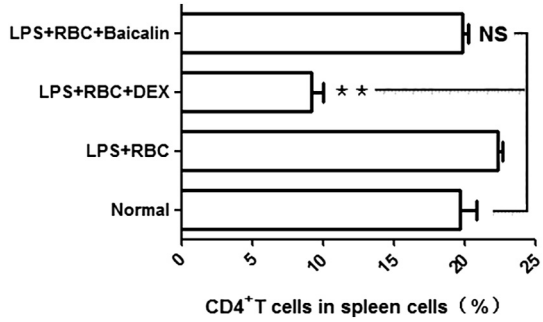

Fig. 6. The percentage of CD4 positive T cells in groups of mouse. At week four, the splenocytes of all groups were stained with anti-mouse CD4-FITC mAb, Flow cytometry was used to measure the percent frequency of CD4 stained cells in the whole splenocytes. For comparison, the *P* value between Normal group, RBC + LPS + DEX group and RBC + LPS + baicalin group is shown. The data represent four independent experiments. $P < 0.05$ was considered statistically significant.

baicalin. Baicalin inhibited Th17 cells and increased CD4 Treg in spleen (Fig. 5) and mesenteric lymph nodes. There are no significant differences in the percentage of Th1, Th2 CD4 subpopulations among all groups (data not shown).

3.4. Baicalin treatment didn't decrease the percentage of CD4 positive cells in spleen

Here we compared the size of spleens and the percentage of CD4 cells in spleen cells in DEX treated mouse and baicalin treated mouse in week 4. It was found the percentage of CD4 cells in spleen cells was significantly decreased by DEX but not baicalin in mouse (Fig. 6), and the size of spleen wasn't affected by baicalin.

4. Discussion

Our results indicate that baicalin inhibited RBC immunization, particularly IgG production, without decreasing the percentage of CD4 positive T cells in spleen. While DEX, a widely used immune-suppressive drug in blood transfusion, damaged spleen function. Considering its good safety record in the clinic [1], baicalin may be useful for suppressing transfusion immunization events.

It has been demonstrated that inflammatory signals including both polyinosinic polycytidylic acid [poly(I:C)], a synthetic double-stranded RNA molecule and unmethylated bacterial CpG dinucleotides (CpG ODN), induce robust antibody production in a mouse model of blood transfusion [12,16,17]. In our experiment, we utilized a different inflammatory signal LPS, we also found enhanced RBC alloimmunization in our mouse model (Fig. 1). Possible mechanisms that contribute to the adjuvant activity of LPS include their effect on antigen-presenting cells as well as T cells. Specifically, LPS treatment results in increased antigen uptake [18], enhanced maturation and differentiation of antigen-presenting cells, which in turn result in activation of T cells and B cells [19]. The results has shown anti-human blood cell IgM in the mouse serum reached a peak at one week after blood transfusion, and then decreased over time; anti-human blood cell IgG increased in the first two weeks and reached a plateau (Fig. 1). This result is similar to the phenomenon observed in human blood transfusion, and thus this model could mimics human RBC immunization.

Treg could control the rate and the frequency of red blood cell alloimmunization in mouse models [12,20]. Adoptive transfer of Th17 cells heightened the initial anti-rat RBC antibody responses and concomitantly increased the onset of Autoimmune hemolytic anemia (AIHA) in mouse. This disease is defined as the increased destruction of red blood cells (RBCs) in the presence of anti-RBC autoantibodies [21]. In human, researchers has found reduced Treg activity in alloantibody responders compared with nonresponders in chronically transfused patients with sickle cell disease [22]. Several reports have shown that baicalin could regulate immune diseases including experimental autoimmune encephalomyelitis (EAE) [2], asthma [3], colitis [4], systemic lupus erythematosus (SLE) [5] by modulating the Th17/Treg paradigm. Our results had shown baicalin could inhibit RBC alloimmunization especially IgG production in a time and dose dependent manner. The IgG and IgM production in red blood cell transfusions are now only discussed in phenomena, but the mechanism of their production, as well as differences in the effects of drugs on them, are still less studied. Baicalin might inhibit newly generated Th17 cells via reducing RORγt expression, and together with up-regulating Foxp3 expression to suppress RORγt-mediated IL-17 expression in established Th17 cells in vitro [23]. The Possible mechanism about baicalin promote Treg development may be that it could induced Foxp3 protein expression, promoted Treg cell differentiation and regulatory activity [5].

Immunotoxicity refers to any effect on the structure or function of the immune system, or on other systems as a result of immune system dysfunction. An effect is considered to be immunotoxic if it impairs the humoral or cellular immunity needed by the host to defend itself, or if it causes unnecessary tissue damage, such as autoimmunity, chronic inflammation or hypersensitivity. CD4 + T cells, often referred to as Th or T helper cells, are essential for immunity [14,15]. Our results have shown the percentage of CD4 cells in spleen cells was significantly decreased by DEX but not baicalin in mouse, indicating baicalin have milder immunotoxicity.

In conclusion, in this study we have shown that baicalin inhibits transfusion-mediated RBC antibody responses with mild immunotoxicity, opening up the possibility that baicalin may be applied for induction of transfusion tolerance.

Competing interest statement

The authors declare no conflict of interest.

Acknowledgments

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