Populational change of CD4⁺CD25⁺Treg cells is responsible for the synergistic effect of the combination of RAMP2 with baicalin in treating recurrent spontaneous abortion mouse models

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Author contributions
Chen C and Guo W conceived and designed the study; Xue WY conducted the experiment; Chen C and Guo JY drafted the manuscript; Li ZL contributed to and approved the final manuscript.

Competing interests
The authors declare no conflicts of interest.

Acknowledgments
This study was supported by the National Natural Science Foundation of China (81973221), National Natural Science Foundation for Young Scientists of China (81603647), the Women and Children Health Talent Project of Jiangsu Province (FRC201785), the Chinese Clinical Medicine Innovation Center of Obstetrics, Gynecology, and Reproduction in Jiangsu Province (ZK2021020), the Women and Children Health Science Foundation of Jiangsu Province (F2020206). Thanks are due to Zeng Y, Wang YM and Zhu MJ for assistance with the experiments and to Sun Y for valuable discussion.

Peer review information
Traditional Medicine Research thanks all anonymous reviewers for their contribution to the peer review of this paper.

Abbreviations
BSYK-D, Bushen Yangxue Decoction; RSA, recurrent spontaneous abortion; TCM, traditional Chinese medicine; AM, Atractylodes macrocephala Koidz.; SB, Scutellaria baicalensis Georgi; Treg, regulatory T cells; RAMP, polysaccharides extracted from the AM rhizome; ns, no significance.

Citation

Background:
The absence of a safe and effective therapy for recurrent spontaneous abortion due to a maternofetal failure in immunological tolerance remains an intractable clinical obstacle for surgeons. Recently, traditional Chinese medicine has become a feasible alternative for certain diseases, including recurrent spontaneous abortion. However, because of the complex composition of the traditional Chinese medicine formula, its action mechanism remains unclear. Methods: We selected two isolated active ingredients (RAMP and baicalin) from the traditional Chinese medicine formula and used an abortion-prone CBA/J × DBA/2 model to simulate human RSA and compared the changes in fetal resorption rate, Treg cell percentage, and relevant cytokines before and after combination therapy. In addition, The mechanisms were preliminarily discussed using in vitro differentiation models. Results: In CBA/J × DBA/2 abortion-prone mice, the combination therapy resulted in a lower embryo resorption rate compared to that obtained with individual delivery of either RAMP or baicalin, thereby playing an embryo-protective role through the increase in Treg cells for the maintenance of maternal-fetal immune tolerance. In vitro primary cell differentiation experiments, the concentration of Treg cells significantly increased from 11% to 17.9% after the combination therapy compared to that of the single administration group. Conclusion: the synergistic effects of RAMP and baicalin were responsible for Treg differentiation. The present study provides a solid basis for improving the applicability of traditional Chinese herbs in the treatment of recurrent spontaneous abortion.

Keywords: recurrent spontaneous abortion; Atractylodes macrocephala Koidz.; Scutellaria baicalensis Georgi; CBA/J × DBA/2; regulatory T cells
Background

Maternal immune tolerance to semi-allogeneic antigens is necessary for an uncomplicated pregnancy since failure of tolerance may lead to severe pregnancy complications such as preeclampsia, premature delivery, and recurrent spontaneous abortion (RSA) [1]. RSA is defined as the occurrence of two or more spontaneous abortions with the same partner [2]. Generally, abnormal immune tolerance and excessive antigen-antibody responses lead to more than 50% of abortion cases being classified as RSA, thereby posing a formidable challenge for clinicians [3]. Currently, treatment for RSA with immune dysfunction mainly involves the enhancement of immune tolerance of the maternal-fetal interface coupled with anticoagulant drugs and hormones [4, 5]. Immunotherapy and mesenchymal stem cell therapy are also commonly employed [6, 7]. However, immunotherapy and mesenchymal stem cell therapy are complex, expensive, and exhibit poor patient compliance, while anticoagulant therapy carries the risk of maternal bleeding and fetal deformities [8]. Therefore, the need for safer and more effective therapies for immunological RSA is of utmost importance.

Traditional Chinese medicines (TCMs) have been used to treat abortions for over 3,000 years and have recently become a prevalent alternative therapy to Western medicine [9]. TCMs are usually combined into different formulae to produce a more coordinated effect for systemic treatment. Owing to the complexity of the components of TCM formulae, studying the pairing of two specific herbs is a popular strategy in pharmacotherapeutics. Thus, the screening of active components from herb pairing is a predominant approach to investigate the mechanism of TCM formulae. Bushen Yangxue Decoction (BSXY-D) is a fetal-protective TCM formula approved by the Jiangsu Province Hospital of Chinese Medicine, China. It is composed of Cuscutae Semen, Eucommiae Cortex, Taxilli Herba, Dipsaci Radix, Angelicae Sinensis Radix, Parochiae Radix Alba, Codonopsis Radix, Atractylodis Macrocephalae Rhizoma, Dioscoreae Rhizoma, Scutellariae Radix, Atractylodis macrocephalae Koidz (AM) and Scutellariae baicalensis Georgi (SB) are the two core ingredients of BSXY-D. In this study, we selected this pairing (AM and SB) to demonstrate their embryo-protective effect. Polycyclicarboxylates extracted from the AM rhizome (RAMP) and baicalin extracted from SB are the main active components of immune regulation [10, 11]. Moreover, RAMP and baicalin are known to regulate the differentiation of CD4+ T cells, especially regulatory T cells (Treg). The high-purity RAMP identified in a previous study (RAMP2) upregulates Treg cells via the IL-2/STAT5 pathway [12]. In addition, baicalin was found to regulate Treg/Thf2 balance by activating the aryl hydrocarbon receptor [13]. Another study revealed that baicalin improves the inflammatory response by regulating the Treg/Th17 balance [14]. However, we observed that the TCM formulae had marked protection during pregnancy with AM and SB as core components. Therefore, the molecular mechanisms underlying the efficacy are possibly related to the immune modulation of Treg cells.

Materials and methods

Reagents

The rhizome of AM (20170205) was obtained from Fenyuan Tongling Traditional Chinese Medicine Co., Ltd. (Tongling, China). The extraction method, quality, and structural identification results of RAMP (including basic chemical properties, FT-IR, NMR analysis, and advanced structural analysis) were obtained from previous studies [12]. Baicalin (572667) and progesterone (P0130) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Purified anti-mouse CD3 (no azide and low-endoxin; 145-2C11), anti-mouse CD28 (no azide and low-endoxin; 37,51), and fluorochrome-conjugated anti-mouse CD4 (RM4-5) antibodies were purchased from BD Pharmingen (New York, NY, USA). Purified anti-human CD3 (OKT3) and anti-human CD28 (CD28.2) were purchased from eBioscience (San Diego, CA, USA). Fluorochrome-conjugated anti-mouse CD4 (GK.1), anti-mouse CD25 (3C7), and anti-mouse Foxp3 (MF-14) antibodies were purchased from BioLegend (San Diego, CA, USA). Anti-mouse Foxp3 (D608R), anti-STAT5 (D206Y), and anti-Phospho-STAT5 (Tyr694) (D47E7) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Recombinant mouse IL-2 (402-ML) and TGF-β1 (7666-MB) were purchased from R&D Systems (Amoryville, CA, USA).

Mice

The Laboratory Animal Ethics Committee of China Pharmaceutical University approved the animal experiments and the corresponding protocol (Approving number: 2020-12-008). All animals were kept under controlled temperature (20 ± 2 °C), humidity, and light (12/12 h light/dark) conditions for 1 week. Eight-to-nine-week-old SPF female CBA/J (laboratory animal quality certificate number: 11400700299084) and male DBA/2 (laboratory animal quality certificate number: 11401300077874) mice (18–22 g) were obtained from Beijing HFK Bioscience Co., Ltd. (Beijing, China). All animal experiments were performed in accordance with the protocol of the Institutional Animal Care and Use Committee of China Pharmaceutical University.

CBA/J mice were mated with DBA/2 in the evening, and the next morning, vaginal plugs were examined. The first day of pregnancy was regarded as the day the vaginal plug appeared. Pregnant female CBA/J mice were randomly divided into the following groups via intragastric administration on day 1 of pregnancy for 10 days: Group 1 received phosphate-buffered saline (PBS, vehicle, n = 5), Group 2 received baicalin (300 mg/kg, n = 5), Group 3 received progesterone (30 mg/kg, n = 5) (labeled as P4), and Group 4 received RAMP2 (300 mg/kg) + baicalin (300 mg/kg) (n = 5). On day 14 of pregnancy, pregnant CBA/J mice were euthanized by cervical dislocation; their uterus were removed, and the locations of implantation were noted. When compared to normal embryos and placentas, abortion sites were distinguished by their tiny size along
with a necrotic and hemorrhagic appearance. The ratio of resorption sites to implantation sites was used to compute the percentage of abortions.

Sample collection
On day 14 of pregnancy, blood samples were extracted from the CAB/J’s retro-orbital plexus and centrifuged for 10 min at 3,000 × g. The sera were kept at −80 °C until the ELISA examination. In both the abortion and therapy groups, normal placental units close to resorbing embryos were preserved as samples. They were then meticulously cleaned in cold, sterile PBS (pH 7.40), snap-frozen, and kept at −80 °C for real-time RT-PCR. Samples of the thymus and kidney were chopped into tiny pieces and kept in RPMI at 4 °C. Thymus and spleen tissues were crushed with a 5 ml syringe plunger to create single-cell suspensions, and uterine tissues were digested for 2 h at 37 °C using 1 mg/ml collagenase A (Roche) in RPMI medium.

Isolation of PBMCs
Blood was collected and diluted with PBS at a ratio of 1:1. The diluted blood was carefully placed above the lymphocyte separation solution (Cedarlane Laboratories, Hornby, Canada), centrifuged at 400 × g for 30 min, and peripheral blood mononuclear cells (PBMCs) were collected from the interface. The samples were washed once with PBS and centrifuged at 300 × g for 10 min. Cells were stored at −80 °C for flow cytometry analysis.

In vitro differentiation of mouse Treg cells
CD4+ T cells were purified by magnetic cell sorting (Miltenyi Biotec, Bergish Gladbach, Germany) from C57BL/6 mice spleens and cultured at 1 × 10^6 cells per well in 24-well plates with plate-bound anti-CD3 (2 μg/mL) and soluble anti-CD28 (2 μg/mL), with or without recombinant mIL-2 (2 ng/mL) and mTGF-β (5 ng/mL), at 37 °C with 5% CO2. The cells were cultured in the IMDM medium. After three days, the cells were analyzed using FACS or real-time RT-PCR.

Flow cytometry analysis
Intracellular cytokine staining was performed using fixation/permeabilization buffer solution (BD Biosciences, New York, NY, USA) according to the manufacturer’s instructions. For intracellular cytokine staining, cells were stimulated using Leukocyte Activation cocktail with BD GolgiPlug (1:500 dilution, BD Pharmingen, New York, NY, USA) and 5 μg/mL monensin (1:1,000 dilution, Multi Sciences, Hangzhou, China) at 37 °C under 5% CO2 for 5 h. They were then fixed with the fixation/permeabilization buffer solution according to the manufacturer’s instructions. Stained cells were analyzed on Accuri C6 (BD Biosciences, New York, NY, USA), and data were analyzed using FlowJo.

Real-time RT-PCR
Real-time RT-PCR was performed as described previously [12]. The results were normalized to β-actin mRNA expression levels.

Enzyme-linked immunosorbent assay
Serum from mice was separated by centrifuging at 3,000 × g for 10 min. Culture medium of mouse CD4+ T cells was set to 3,000 × g for 10 min at 4 °C. Levels of IL-10 (Dakewei, Shenzhen, China), IL-2 (Dakewei, Shenzhen, China), IL-35 (CUSABIO, Wuhan, China) and TGF-β (Dakewei, Shenzhen, China) were measured using ELISA kits (Lianke, Hangzhou, China).

Western blotting
The tissues were collected and processed as described previously [12]. Equal amounts of protein were separated using SDS-PAGE, transferred onto NC membranes (PALL), and blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) containing 0.01% Tween-20 (TBST) for 3 h at room temperature. Membranes were incubated overnight at 4 °C with the appropriate diluted antibody (1:1,000 dilution, anti-mouse Foxp3, anti-STATS, anti-Phospho-STAT5 (Tyre694)) in 5% BSA. The membranes were treated with a secondary antibody (1:5,000 dilution, Abcam, Cambridge, UK) coupled with horseradish peroxidase after being washed with TBST. Images were detected using an enhanced chemiluminescence detection system (ECL, Tanon).

Statistics
Unless otherwise stated, comparisons between two groups were performed using an unpaired two-tailed Student’s t-test; one-way ANOVA (with Tukey’s multiple-comparison post-tests) was used for comparisons between groups constituting more than two elements. Statistical significance was set at P < 0.05. Statistical analyses were performed using the GraphPad Prism 8.

Results
RAMP2 combined with baicalin reduces embryo resorption rate in RSA model mice
Owing to the complex composition of BSYX-D, we focused on exploring the action mechanism of its core components: AM and SB. RAMP and baicalin were combined to treat RSA model mice. This was ensured by extracting a novel polysaccharide, RAMP2, from AM and baicalin from SB. The CBA/J × DBA/2 abortion-prone model was used to evaluate the effect of combination therapy in vivo [15]. The detailed experimental groupings are presented in Table 1. The uterus of female CBA/J mice were dissected on the 13.5th day from the date of confirmation of vaginal suppository, and the absorption number as well as the total number of embryos in each group were recorded. The embryo absorption rate was determined using the following Equation (1):

\[ \text{Embryo Absorption Rate} = \frac{\text{Number of Embryos Absorbed}}{\text{Number of Surviving Embryos}} \times 100\% \]

Figure 1A presents the detached pregnant uterus. The absorption site showed a significant reduction in embryo size and obvious bleeding or necrosis. After combination treatment, the embryo absorption rate decreased from 24.39% to 16.33% (a decrease of 8.06%) (Table 2). In the baicalin single administration group, embryo absorption decreased by 7.00%, while that in the progesterone group, as a positive control decreased by 5.64%. These results indicate that combination therapy reduced the resorption rate is more effective than monotherapy and positive control.

Combination of RAMP2 with baicalin increased the proliferation of Tregs in mouse models
To further confirm whether the combination therapy had a similar effect in vivo, especially at mother-fetus interface, the spleen (Figure 1B, 1C), thymus (Figure 1D), decidua (Figure 1E), and uterus implants (Figure 1F) of CBA/J female mice were dissected, and the proportion of Treg cells was evaluated using FACS. Figure 1C illustrated that the ratio of Treg cells in the spleen of combination therapy group had increased from 8.37% to 10.30% and was significantly different from that of the control group, indicating that combination therapy could

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Treatment</th>
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</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>5</td>
<td>Spontaneous abortion model group without treatment</td>
</tr>
<tr>
<td>Group 2</td>
<td>5</td>
<td>Treat with baicalin</td>
</tr>
<tr>
<td>Group 3</td>
<td>5</td>
<td>Treat with P4</td>
</tr>
<tr>
<td>Group 4</td>
<td>5</td>
<td>Treat with RAMP2 + baicalin</td>
</tr>
</tbody>
</table>
Table 2 Embryo resorption rate of RSA model mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of mice</th>
<th>Total embryos number</th>
<th>Resorbed embryos</th>
<th>Resorption rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>5</td>
<td>41</td>
<td>10</td>
<td>24.39%</td>
</tr>
<tr>
<td>Group 2</td>
<td>5</td>
<td>46</td>
<td>8</td>
<td>17.39%</td>
</tr>
<tr>
<td>Group 3</td>
<td>5</td>
<td>48</td>
<td>9</td>
<td>18.75%</td>
</tr>
<tr>
<td>Group 4</td>
<td>5</td>
<td>49</td>
<td>8</td>
<td>16.33%</td>
</tr>
</tbody>
</table>

![Figure 1](https://www.tmrjournals.com/tmr)  

**Figure 1** Combination of RAMP2 with baicalin increase proliferation of Tregs in vivo. (A) Uteri from normal pregnant mice (left) and abortion mice (right); the arrow indicates the resorbed fetus. (B) The proportion of CD4+ T cells in lymphocytes. Representative plots for T cells and frequency of CD25’Foxp3’Treg in CD4’ T cells of splenic (C), thymic (D), decidual (E) and uterus implants (F) after treatments with baicalin, P4, and RAMP2 + baicalin, and without any treatment in RSA model mice. *P < 0.05 vs model group, **P < 0.01 vs model group and ***P < 0.001 vs model group (Student’s t-test). RAMP, polysaccharides extracted from the AM rhizome; ns, no significance.

also increase the proportion of Treg cells in vivo. Figure 1E, 1F represent immune microenvironment at the local mother-fetus interface. The ratio of Treg cells in the decidual of combination therapy group had increased from 23.2% to 29.8% and the ratio of Treg cells in the uterus implants of combination therapy group had increased from 1.91% to 2.34% which indicates the combination therapy could improve the proportion of Treg cells at mother-fetus interface too.

To further study the mechanism underlying the fetal protective effect of the combination treatment, the levels of IL-2, IL-10, IL-35, and TGF-β1 in the serum of each experimental group were measured 13.5 days after pregnancy. Compared to the RSA model group, the expression levels of IL-10, IL-2, and IL-35 in the combination therapy group significantly increased after treatment, but that of TGF-β1 was unaffected (Figure 2A). These increasing cell factors are considered to enhance the ability of Treg cells to support pregnancy [16]. The mRNA expression levels of Foxp3, IL-2, and IL-10 in the spleen and decidua were detected using RT-PCR. RAMP2 combined with baicalin upregulated the Foxp3 mRNA levels in the spleen (Figure 2B), as well as the Foxp3 and IL-10 mRNA levels in the decidual uterus (Figure 2C). Thus, these two components promote the proliferation of Treg cells in vivo.

**Synergistic effect of RAMP2 and baicalin in promoting differentiation of Treg cells**

In the RSA mouse model, we observed that RAMP2 combined with baicalin upregulated Tregs. The pathway by which the combination therapy induces the regulation of Treg cell differentiation attracted
our interest. In isolated mouse CD4+ T cells, the ratio of CD25+Foxp3+ Tregs to CD4+ T cells was detected by FACS. As shown in Figure 3A, the proportion of CD25+Foxp3+ Tregs was increased by RAMP2, baikalin, combination therapy, rmIL-2, and rmTGF-β1 induction compared with that in the control group. The concentration of CD25+Foxp3+ Tregs in the combination therapy group (17.9%) was significantly higher than that in the single-drug group (P < 0.01). Foxp3 also showed an upregulated trend in mRNA levels (Figure 3B). In the combination group, a significant elevation in IL-10, IL-2, and IL-35 was detected after treatment (Figure 3C), which was similar to the RT-PCR results.

RAMP2 increased Foxp3 expression by activating the IL-2/STAT5 pathway, as described previously [12]. The phosphorylation level of STAT5 in the baikalin or combination therapy group showed no significant increase compared to the control group (Figure 4A, 4B), indicating that RAMP2 acts in combination with baikalin through other pathways.

**Discussion**

Pregnancy is a complex process, with a special immune environment at the materno-fetal interface. Growing evidence reveals that recurrent pregnancy loss may be related to failed maternal-fetal immune tolerance. Moreover, ancient Chinese medical works recorded that Huang Qin (Scutellaria baicalensis Georgi) and Bai Zhu (Atractylodes macrocephala Koidz.) were the best cure for miscarriages. RAMP and baikalin have individually been shown to have immunomodulatory activity [10, 13, 17, 18]. However, studies on their individual effects have not demonstrated the molecular action mechanism of complex formulae, and research on the combined use of common herb pairing is still lacking. In our previous study, we isolated and identified a novel polysaccharide from AM and named it RAMP2. In the present study, we analyzed the specific regulatory effects of RAMP2 combined with baikalin on Treg cells. Our findings provide new information on the combination of AM and SB in vivo treatment, thereby providing a new feasible approach for the treatment of immune recurrent abortion.

![Figure 2](image-url)

Figure 2 Expression levels of IL-2, IL-10, IL-35, and TGF-β1 in CBA/J × DBA/2 mouse serum with the treatments of baikalin, P4 and RAMP2 + baikalin. (A) mRNA expression of Foxp3, IL-2, IL10, and TGF-β1 in mouse splenic (B) and decidual (C) CD4+ T cells after treatments with baikalin, P4, and RAMP2 + baikalin. *P < 0.05 vs model group, **P < 0.01 vs model group and ***P < 0.001 vs model group (Student’s t-test). RAMP, polysaccharides extracted from the AM rhizome; ns, no significance.
The ability of combination therapy to improve pregnancy outcomes is based on its immunomodulatory effects in the CBA/J × DBA/2 abortion-prone pregnancy model. During human pregnancy, the enhancement of Treg cell function suppresses immune activation and allows for fetal growth and development [19]. We aimed at demonstrating the role of BSXY-D in preventing miscarriages through the same immune mechanism. Therefore, we selected BSXY-D, RAMP, and baicalin as combination therapies for subsequent experiments. Consistent with clinical studies, animal studies have shown similar results. Combination therapy reduced fetal loss and increased the proportion of CD25⁺Foxp3⁺ Treg cells in the spleen, thymus, decidua, and uterus of female mice. Furthermore, the CD4⁺ T cells of the mice were separated to evaluate the efficacy of combination therapy. In combination-treated CD4⁺ T cells, more CD25⁺Foxp3⁺ Treg cells were detected by flow cytometry; this result was significantly different from that obtained for the RAMP2-treated and baicalin-treated groups, thereby implying a synergistic effect of the combination of RAMP2 and baicalin.

Tregs are CD4⁺CD25⁺T-cells that express the Foxp3 as a specific biomarker. Zenclussen showed that the proportion of Tregs is negatively correlated with the probability of RSA in a spontaneous abortion mouse model, implying that the abnormal expression of Tregs is related to the occurrence and development of this disease [20]. Tregs act on T-cells and maintain immune tolerance to autoantigens in vivo through two possible mechanisms: 1) secretion of IL-10, TGF, and other cytokines after antigen stimulation to inhibit T-cell activation, thereby regulating immunity [16, 21, 22]; 2) Treg cell membranes express CTLA-4 and competitively bind to B7, thereby inhibiting T-cell activation [23–25]. Meanwhile, they also downregulate other lymphocytes in the body, such as B-cells, NK cells and antigen-presenting cells, including dendritic cells and macrophages to further regulate immune function [20, 26–29].

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According to earlier research, IL-2 could be crucial for preserving Foxp3 expression [30, 31]. And that IL-2 binding to IL-2R regulates the expression by Foxp3 via activating STAT5 [25, 32]. Foxp3, a characteristic transcription factor of Treg cells, is crucial in the regulation of Treg cytokine secretion [23]. Our previous study showed that RAMP2 has good immunomodulatory activity and regulates Treg cells by activating the IL-2/STAT5 pathway. Surprisingly, the combination group's STAT5 phosphorylation level was marginally lower than that of the RAMP2-treated group and did not significantly rise above that of the control group. We speculate that the effect of RAM2 on the STAT5 pathway is offset by baicalin because it inhibits the activation of JAK2 [33]. However, we encountered certain limitations in this study. Our previous study showed that there was no difference on JAK activation after RAMP2 stimulation, suggesting that the signaling pathway is not related to JAK and its pathway, which could be another subject for further investigation. Except for the drug pair AM and SB, the action mechanism of other drug combinations on the compound remains unknown. Thus, conducting further research on the compatibility of other drugs with the BSYX-D is highly recommended.

Conclusion

In conclusion, our study combined animal and in vitro differentiation experiments to investigate the immunological principles of the combination of two traditional Chinese herbs, AM and SB, in the treatment of RSA. We found that the combination of RAMP2 and baicalin can protect against fetal loss by inducing Treg cell proliferation, and the effect is more significant than that of RAMP2 or baicalin alone. Therefore, spreading the application of these traditional Chinese herbs for the treatment of RSA is of great importance.

References

http://doi.org/10.1038/nri.2017.75


