Dioscin Restores Immunological Tolerance of CD4+CD25+ Regulatory T Cells in Aplastic Anemia Mouse Model

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Research Article

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Abstract

Background: Aplastic anemia (AA) is an immune-mediated disease of bone marrow failure owing to activated cytotoxic T lymphocytes (CTLs). CD4+CD25+ regulatory T cells (Tregs) could negatively regulate CTLs to maintain self-tolerance and avoid excessive immune responses. This study aims to investigate the effect of Dioscin on the functions of CD4+CD25+ Tregsin the AA mouse model, which were induced by total body irradiation and allogeneic lymphocyte infusion.

Methods and Results: The collected CD4+CD25+Tregs were randomly divided into 5 groups: 1) normal control group, 2) aplastic anemia model group, 3) Dioscin group, 4) Cyclosporine A group, 5) Triptolide group. Based on flow cytometry and qRT-PCR, forkhead box P3 (Foxp3), cytotoxic T lymphocyte-associated antigen 4 (CTLA4) and glucocorticoid-induced tumor necrosis factor-related protein (GITR) were quantified to evaluate the function expression of CD4+CD25+ Tregs.

Conclusions: The result shows that the AA mice manifested loss of Foxp3 and CTLA4 expressions and overexpression of GITR. It also shows that compared with traditional AA medicine such as Cyclosporine A and Triptolide, Dioscin displays better efficacy in promoting Tregs differentiation by affecting CTLA4 and GITR on the surface of Tregs and restoring the expression of Foxp3.

1. Introduction

Aplastic anemia (AA) is an immune dysfunction of hematological disease, which is characterized by pancytopenia and bone marrow failure [1]. According to the etiopathogenesis, AA can be classified as primary and acquired forms. AA cases can also be distinguished based on the disease severity as severe aplastic anemia (SAA) or non-severe aplastic anemia (NSAA). Owing to the deficiency of neutrophils or platelets, patients with SAA are at a high risk of life-threatening infections or bleeding [2, 3], and the treatments are with less effectiveness [4]. Furthermore, patients with NSAA require long-term drug therapy and have a lower quality of life [5]. With the rapid research progress these years, the mainstream view of pathogenesis believes that cytotoxic T lymphocytes (CTLs) activate excessively and release pro-inflammatory cytokines thus causing apoptosis of hematopoietic stem cells [6].

$CD4^+CD25^+$ regulatory T cells (Tregs), a subset of $CD4^+$ T cells, are indispensable for the maintenance of immunological tolerance [7]. Tregs inhibit immune response mediated by CTLs to maintain immunity homeostasis. Reduced quantity and impaired function of Tregs in most AA patients [8] indicate that Tregs play a vital role in AA. Tregs express various surface functional receptors, such as cytotoxic T lymphocyte-associated antigen 4 (CTLA4) and glucocorticoid-induced tumor necrosis factor-related protein (GITR). CTLA4 is an analog of costimulatory signal receptor CD28 expressed by Tregs, and it has a higher appetency for CD80 and CD86 on the antigen-presenting cells (APCs) compared with CD28. Once combined with CD80/86, CTLA4 turns off APCs and further restricts T cell activation [9]. On the contrary, GITR is a positive regulator of T cell activation, which is expressed by Tregs and $CD4^+/CD8^+$ effector T cells (Teffs) [10]. After being triggered by its ligand (GITRL) on APCs, GITR promotes Teffs

proliferation and conducts Tregs fail to suppress Teffs. Lack of GITR can increase the vulnerability of activation-induced cell death (AICD). In addition, GITR actives NF-κb and MAPK signaling pathways to regulate the immune response [11].

The therapy of AA mainly includes hematopoietic stem cell transplantation (HSCT), immunosuppressive therapy (IST) and other substitution therapies. HSCT is considered as the only way to thoroughly cure AA, while IST such as Cyclosporine A (CsA) is more widely applied to patients. However, these therapies are restricted on account of their side effects and age limitation [4]. As a result, it is necessary to identify new and effective medicaments for AA treatment. Dioscin ($C_{45}H_{72}O_{16}$), also known as *Dioscorea nipponica saponins* (DNS), is a dominant steroidal saponin from a kind of traditional Chinese medicine *Dioscorea nipponica Makino* (TCDN), has shown certain curative effects in the treatment of coronary artery disease [12], blood lipid lowering [13], anti-oxidation [14, 15], anti-inflammatory [16, 17], and anti-tumor [18, 19]. In our previous studies, it have confirmed that Dioscin has advantages in improving pancytopenia, regulating immunity and suppressing apoptosis of hematopoietic stem cells (HSCs) in AA mice [20, 21], but the affecting mechanism is undeclared. In this paper, it is investigated that whether Dioscin regulates the function of $CD4^+CD25^+$ Tregs through its effects on expressions of Foxp3, CTLA4 and GITR. Dioscin together with the other two kinds of AA medicine, CsA and Triptolide (TP) [22, 23] were treated in mouse models. Expressions of Foxp3, CTLA4 and GITR were detected based on flow cytometry and qRT-PCR to validate the mechanism of Dioscin in treating AA.

2. Materials And Methods

2.1 Chemicals and materials

Drugs, reagents and other chemical materials used on mouse models are available from commercial companies. The information of the suppliers is listed in Table 1.

Table 1
Chemical materials and their suppliers

Chemicals and materials	Suppliers
Mouse Lymphocytes Separation Kit	Tianjin haoyang Bio-Technology Co.Ltd (Tianjin, China)
RPMI 1640 medium and Cell Counting Kit-8(CCK-8)	Biosharp (Beijing, China)
Brilliant Cresyl Blue Solution, 4% Paraformaldehyde, EDTA, HE Staining Kit, 10% Fetal Bovine Serum (FBS), 1% Penicillin-Streptomycin, Phosphate Buffer Solution(PBS) and CFDA, SE Kit	Solarbio (Beijing, China)
$CD4^+CD25^+$ Regulatory T Cell Isolation Kit	Miltenyi Biotec (Bergisch Gladbach, Germany)
Dioscin, CsA and TP	Shanghai yuanye Bio-Technology Co.Ltd (Shanghai, China)
Anti-mouse CD3 Antibody, anti-mouse CD28 Antibody, CD4-FITC, CD127-APC, CTLA4-APC and GITR-PerCP	Biolegend (San Diego, California, USA)
IL-2 Protein, Mouse	MedChemExpress (State of New Jersey, USA)
TRIzol reagent	Invitrogen (Shanghai, China)
PrimeScriptTMRT Master Mix	Takara (California, USA)
UltraSYBR Mixture (Low ROX)	Cwbio (Taizhou, China)

2.2 Animals

Specific pathogen-free male Balb/c mice and DBA/2 mice, 6–8 weeks old, were obtained and bred in the laboratory animal center of Institute of Radiation Medicine, Chinese Academy of Medical Sciences (Huafukang Bioscience, Beijing, China). The laboratory animals were used following the guidelines of the National Institutes of Health of China.

2.3 Induction of bone marrow failure

Spleens and thymuses were taken from DBA/2 donor mice under aseptic procedures, then homogenized and washed with PBS, filtered through 70 μm cell strainers, separated by Mouse Lymphocytes Separation

Kit, and counted respectively. Afterwards, the splenic lymphocytes and the thymus lymphocytes were mixed up at a ratio of 2:1 to prepare the mixture cell suspension. Balb/c mice received a dose of 5Gy total body radiation (^{137}Cs source) followed by 5×10^6 DBA/2 mixture lymphocytes via tail vein injection within 4 hours.

2.4 Evaluation of aplastic anemia mouse model

The deficiency of all the three blood cell types was determined by the complete blood counts, and the extent of the bone marrow failure was estimated according to the histopathology. After duplicating the AA model for eight days, venous blood of the Balb/c mice was taken from the facial vein and collected into tubes containing anticoagulants. Complete blood counts were carried out by an auto hematology analyzer for veterinary use (Mindray BC-2800Vet, Shenzhen, China). And reticulocytes (RET) were counted after staining Brilliant Cresyl Blue Solution under an oil immersion lens. Then, the mice were sacrificed by cervical dislocation, so that spleens and thighbones were obtained. Thighbones were fixed with 4% paraformaldehyde, decalcified with EDTA solution and embedded with paraffin respectively. Bone sections were stained with HE Staining Kit and observed by an inverted microscope (Nikon eclipse Ti2, Tokyo, Japan).

2.5 $CD4^+CD25^+$Tregs isolation and culture

$CD4^+CD25^+$Tregs were separated from splenic lymphocytes using a $CD4^+CD25^+$ Regulatory T Cell Isolation Kit following the manufacturer's instructions. Then, the Tregs were cultured with RPMI 1640 medium containing 10% FBS and 1% Penicillin-Streptomycin and incubated at 37°C with 5% carbon dioxide.

2.6 Proliferation of $CD4^+CD25^+$Tregs by CFSE staining assay

To analyze the proliferation, $CD4^+CD25^+$Tregs were labeled with CFDA-SE, seeded into 96-well plates at an initial density (4×10^5 cells per well) and treated with anti-mouse CD3 antibody (5ug/ml), anti-mouse CD28 antibody (2ug/ml) and IL-2 protein (20ng/ml) for 5 days. Then, the cells were collected and detected by NovoCyte Flow Cytometer (NovoCyte 2000, Agilent Technologies, CA, USA).

2.7 Preparation of drug by CCK-8 Assays

$CD4^+CD25^+$ Tregs were plated in 96-well plates at a density of 4×10^5 cells per well. They were divided into three groups, which were respectively treated with different concentrations of Dioscin (1.25, 2.5, 3.75 and 5μM), CsA (1.25,2.5,3.75 and 5μM) and TP (1.25,2.5,10,20 and 30nM) for 24h. After being added with the CCK-8 reagent, the plates were incubated at 37℃ for 2h. Next, the absorbance value at 450 nm was measured with a microplate reader. The effects of these drugs were confirmed according to the cell viability calculation based on the CCK-8 reagent:

$$\text{cell viability} = \frac{\text{OD value in drug experiments well - OD value in medium well}}{\text{OD value in control well} - \text{OD value in medium well}} \quad (1)$$

2.8 Flow cytometry

After being treated with anti-mouse CD3 antibody (5ug/ml), anti-mouse CD28 antibody (2ug/ml) and IL-2 protein (20ng/ml) for 5 days, the $CD4^+CD25^+$ Tregs were treated with Dioscin 2.5μM, CsA 1.25μM and TP 20nM respectively and incubated in 37°C for 24h. Next, the Tregs were collected and stained with CD4-FITC, CD127-APC, CTLA4-APC and GITR-PerCP in dark for 30min at room temperature, then washed with PBS. Finally, the cells were filtered for analysis by NovoCyte Flow Cytometer.

2.9 Quantitative real-time PCR

To quantify and compare the changes of Foxp3, CTLA4 and GITR in $CD4^+CD25^+$Tregs after being treated with the three different drugs. Total RNA was extracted from $CD4^+CD25^+$Tregs using TRIzol reagent. cDNA was synthesized from 500ng of the total RNA using 5X PrimeScript RT Master Mix and RNase Free dH2O following the manufacturer's instructions. Foxp3, CTLA4 and GITR were designed by Primer Premier Software (PREMIER Biosoft International, USA) and were synthesized by Bioengineering (Shanghai) Co., Ltd (Table 2). qRT-PCR was performed to detect the target genes using UltraSYBR Mixture (Low ROX) on QuantStudio^{tm} Design & Analysis Software (Thermo Fisher Scientific, Waltham, MA USA). Reactions were performed in a 50ul reaction mixture containing 25 ul of 2× UltraSYBR Mixture (Low ROX), 2ul of cDNA, 1ul of each primer (10uM) and 21ul ddH2O. The reaction mixture was then subjected to a thermal profile of denaturation as follows: 1 cycle at 95°C for 10min, 40 cycles at 95°C for 15s and 60°C for 1 min. mRNA expression was normalized to Beta-actin and calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 2
Primer sequences of PCR

Gene	Forward primer(5'- to 3')	Reverse primer(5'- to 3')
FOXP3	ATGTTCGCCTACTTCAGAA	TCATCTACGGTCCACACT
CTLA4	CGATGGTGATGGAGTGTAA	CCTACTGGTCTCAGGAATG
GITR	GTGGAGACCCTCAGTGCAAG	CGAATGGCTGGGTCTTTGGTC
Beta-actin	GCACCACACCTTCTACAA	TACGACCAGAGGCATACA

2.10 Statistical analysis

Commercial software *Graphpad* was utilized to analyze the data and plot the graphs, and the results were expressed as mean ± standard error of the mean. The comparison among different groups was based on variance (ANOVA) and *t*-test, and $P < 0.05$ was believed statistically significant.

3. Results

3.1 Pancytopenia and bone marrow failure in AA mouse model

In order to evaluate the establishment of the AA model, blood counts and bone marrow biopsies were examined. The counts of white blood cell (WBC), hemoglobin (HGB), platelet (PLT) and RET were significantly lower in the AA model group than in the normal control (NC) group (Fig. 1A,B). Bone marrow in the AA model group was hypoplastic (Fig. 1C), which was consistent with the bone marrow histopathological findings of AA patients. In addition, as the Fig. 1D shows, the spleen length of the AA group was 1.2 ± 0.03 cm, which was significantly smaller than that of the NC group (1.5 ± 0.02 cm) (P 0.05).

3.2 Anti-CD3 + CD28 + IL2 stimulation induces Treg proliferation.

The positive rate of $CD4^+CD25^+$ Tregs after purification was more than 90% (Fig. 2A). Compared with non-stimulated $CD4^+CD25^+$ Tregs, the number of the cells stimulated with anti-CD3, CD28 antibody and IL-2 protein were microscopically observed to increase by four-fold after being culturing for five days. The result of the flow cytometry demonstrated that a significant proportion of non-stimulated $CD4^+CD25^+$ Tregs are undivided ($^*P$ 0.05) (Fig. 2B).

3.3 Effect of Dioscin on the cell viability of $CD4^+CD25^+$Tregs

The criterion was identified that the cell viability was over 80% for selecting the optimal drug concentration. The optimal concentrations of Dioscin, CsA and TP were 2.5μm, 1.25μm and 20nm respectively (Fig. 2C-E).

3.4 Effect of Dioscin on the expression of CTLA4/GITR on Tregs in AA Mouse model

In the AA model group, CTLA4 expression on Tregs was lower (7.33 ± 0.23%) than that in the NC group (19.57 ± 0.87%) (P< 0.05). As compared with the AA model, CTLA4 levels on Tregs increased in the Dioscin group (13.43 ± 1.18%), the CsA group (10.25 ± 0.38%) and the TP group (9.07 ± 0.08%). Furthermore, Tregs in the AA model group showed significantly higher expression of GITR (46.60 ± 0.97%) than those in the NC group (6.58 ± 0.49%) (P< 0.05). After the treatments, the GITR expression on Tregs elevated in the Dioscin (35.27 ± 0.99%), the CsA (43.93 ± 1.07%) and the TP (38.17 ± 1.13%) groups (Fig. 3A-D).

3.5 Effect of Dioscin on the mRNA expressions of CD4 + CD25 + Tregs

To investigate the effect of the Dioscin on the $CD4^+CD25^+$ Tregs, the mRNA expressions of Foxp3, CTLA4 and GITR were examined through the qRT-PCR analysis, and the results were presented in Fig. 6. Firstly, it could be found that the AA group expressed lower levels of Foxp3 and CTLA4 (1.00 ± 0.03% and 1.00 ± 0.07%, respectively) but a higher level of GITR (1.00 ± 0.24%) compared with those in the NC group, whose above three indexes were 3.60 ± 0.44%, 10.11 ± 1.03% and 0.07 ± 0.01, respectively. After being treated with the drugs including Dioscin, CsA and TP, the expression levels were all approaching those of the NC group, especially those in the Dioscin group, which were respectively 2.05 ± 0.03%, 9.343 ± 0.36% and 0.35 ± 0.06% (Fig. 3E-G).

4. Discussion

AA, a refractory blood disease, has complex causes of disease and multiple pathogenic mechanisms. More and more researches suggest that AA is an immune-mediated disease caused by dysregulation of immune cell subsets, especially T lymphocytes. The main mechanism is that the abnormal activation of CTLs damages HSCs to induce bone marrow failure. While, $CD4^+CD25^+$Tregs inhibit the cytotoxic effect of the activated CTLs, which play an important role in maintaining immunological tolerance. Both bone marrow and peripheral blood Tregs ratio are significantly decreased in AA patients, and Tregs function is impaired, diminishing the ability to suppress CTLs [8]. The number of Tregs in AA correlates with disease severity, and an increase in the number of Tregs predicts a better response to IST. $CD4^+CD25^+$ Tregs specifically overexpress Foxp3, which is vital in the developmental maturation and functional refinement of Tregs [24]. The results of this study showed that the Foxp3 mRNA level of $CD4^+CD25^+$ Tregs in the spleen of AA mice was significantly lower than that in the NC group, which was consistent with the above findings. At the same time, Foxp3 mRNA levels in Tregs increased after the treatments with Dioscin and CsA, suggesting that upregulation of Treg-targeted therapy may increase the benefit of AA patients.

Meanwhile, CTLA4 and GITR, the surface proteins of Tregs, are also important to maintaining the immune tolerance function of Tregs. CTLA4 has been linked to many autoimmune diseases, such as Diabetes mellitus type 1, rheumatoid arthritis, systemic lupus erythematosus and autoimmune thyroid disease [25]. CTLA4 regulates T cell immunity by combining CD80 and CD86 in competition with CD28. CTLA4 also produces inhibitory signals to block the activation of autoreactive T cells. Specifically, CTLA4 blockade promotes the activation of Teffs and reduces the inhibitory activity of Tregs, thereby enhancing anti-tumor immunity. It can also cause immune-related adverse reactions (irAEs) [26]. GITR, a receptor of the TNFR superfamily, is expressed at low levels in resting T lymphocytes and upregulated in Tregs and activated T cells. Its ligand GITRL is expressed in endothelial cells and APCs. The cytoplasmic region of GITR has significant homology to 4-1BB, CD27, OX40, which are involved in T lymphocyte activation and bind TRAF molecules and Siva [27]. GITR binds to TRAF-2 and activates the NF-κB pathway, which inhibits T cell apoptosis. Siva is a cytoplasmic molecule containing a death domain that is involved in apoptotic responses to viral infection and oxidative stress, and murine GITR binds Siva and induces apoptotic activation [28].

In our previous studies, the positive therapeutic effect of TCDN has been shown in AA patients [29]. In addition, TCDN has a positive effect in raising the percentage of CD3 + and CD4 + T cells in bone marrow mononuclear cells (BMNCS), and inhibits pro-inflammatory factors such as IL-2 and IFN-γ, which consequently suppresses Fas/FasL mediated apoptosis[30]. Dioscin is a major effective ingredient extracted from TCDN, and its effects are gradually being studied. Dioscin promotes the proportions of $CD4^+CD25^+Foxp3^+$Tregs and upregulates the SUMO-specific protease 1 in the rat model of Hashimoto's thyroiditis [31]. The transcription factor Foxp3, a molecule central to Tregs differentiation and function, is essential for maintaining immune tolerance, as well as regulating the immune response to pathogens and tumors [32]. To determine whether Dioscin promotes Tregs differentiation by regulating Foxp3, CTLA4 and GITR, we detected the mRNA level in Tregs of AA mice. Our results showed that the Foxp3 expression on Tregs in AA mice was significantly decreased. The data from the flow cytometry and the qRT-PCR confirmed that CTLA4 and GITR were negatively correlated and aberrantly expressed in AA mice, which was consistent with the pathogenesis of AA immune dysfunction. After the treatment with Dioscin, the expression of CTLA4 was elevated and GITR decreased, suggesting that Dioscin may exert immunomodulatory effects by influencing CTLA4 and GITR expression on the surface of Tregs. Increased Foxp3 levels suggested a high abundance of Tregs differentiation. To the best of our knowledge, our study elucidates for the first time the effect of Dioscin on the molecular mechanism of Tregs in AA mice. The present study lays the foundation for further characterization of the immunomodulatory effects of Dioscin, especially in the treatment of AA. In fact, the effects of Dioscin against AA are complex, and two surface functional receptors of Tregs cannot fully demonstrate the molecular mechanism. In the future, the regulations associated with multiple immune cells and multiple signaling pathways will be studied to declare the molecular mechanisms of Dioscin more deeply.

5. Conclusion

In this paper, the abilities of Dioscin on the functions of $CD4^+CD25^+$ Tregs are investigated by analyzing the expressions of Foxp3, CTLA4 and GITR on AA mouse models. Based on flow cytometry and qRT-PCR, it finds that the AA mouse model shows downregulation of Foxp3 and CTLA4 expressions but increase of GITR expression. Compared with CsA and TP, Dioscin showed better efficacy in improves the functions of $CD4^+CD25^+$ Tregs by affecting the expressions of Foxp3, CTLA4 and GITR. This work provides support to the efficacy of Dioscin in treating AA and more knowledge of its therapeutic mechanism.

Declarations

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Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Animal Management and Ethics Committee of Tianjin Medical University.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Liwei Fan, Runfeng Ni and Le Zhang performed experiments, collected and analyzed the data, and wrote the paper. Wuxia Yang, Meng Li and Runjie Li interpreted the data. Aidi Wang provided key reagents, conceived and designed the study. Baoshan Liu supervised the study and approved the final version of the manuscript.

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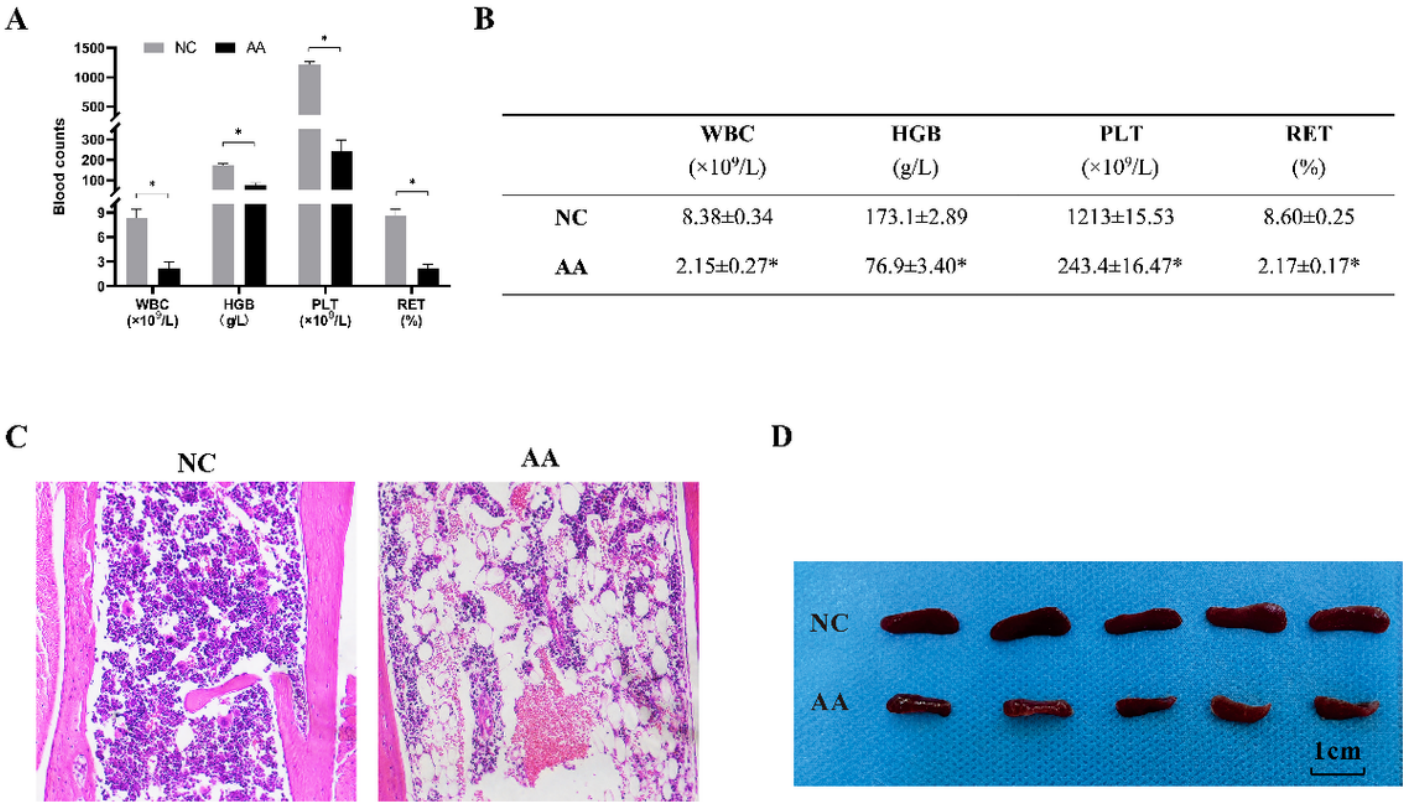
Figures



	WBC (×10⁹/L)	HGB (g/L)	PLT (×10⁹/L)	RET (%)
NC	8.38±0.34	173.1±2.89	1213±15.53	8.60±0.25
AA	2.15±0.27*	76.9±3.40*	243.4±16.47*	2.17±0.17*

Figure 1

A,B. Blood counts in the NC and AA groups. The blood counts of the AA model group showed pancytopenia compared with those of the NC group. Data were mean±SEM, n=10 (*P 0.05). **C.** Bone marrow biopsies were stained with HE in the NC and AA groups (200X). The bone marrow biopsy of NC group showed densely packed hematopoietic cell distribution in normal conditions. The AA bone marrow biopsy showed a significant decrease in bone marrow hematopoietic tissue and an increase in adipose tissue. **D.**Spleens in the NC and AA groups.

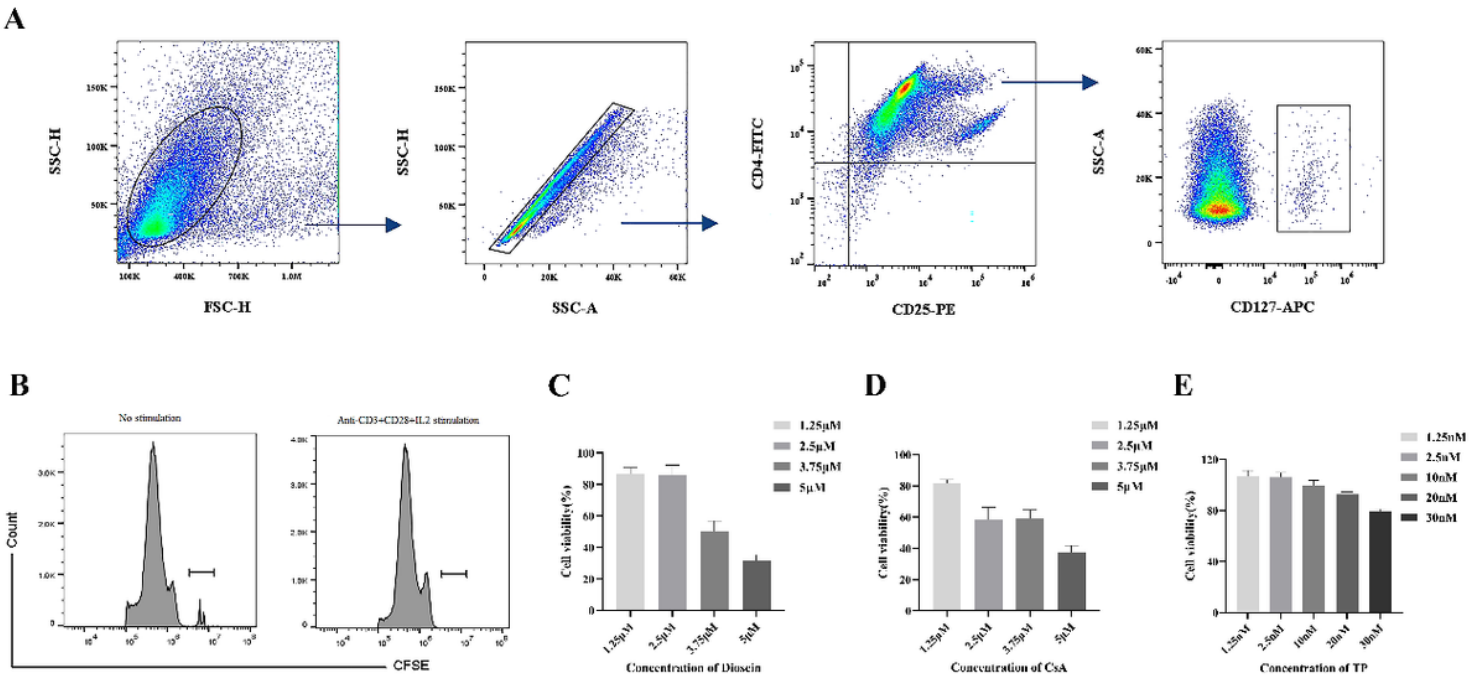


Figure 2

A. Flow cytometry strategy for purification of CD4+ CD25+ Tregs. CD4+ CD25+Tregs have a purity of 97.47±0.32%, and low expression of CD127(1.36±0.27%), n=3. **B.** Histograms show the number of events (Y-axis) and CFSE intensity (X-axis) for cells gated on live lymphocytes (day 5 of culture). The right side indicates the population of the undivided cells, n=3. **E, F, G.** The cell viability of CD4+ CD25+ Tregs 24 hours after being treated with Dioscin, CsA and TP. n=3.

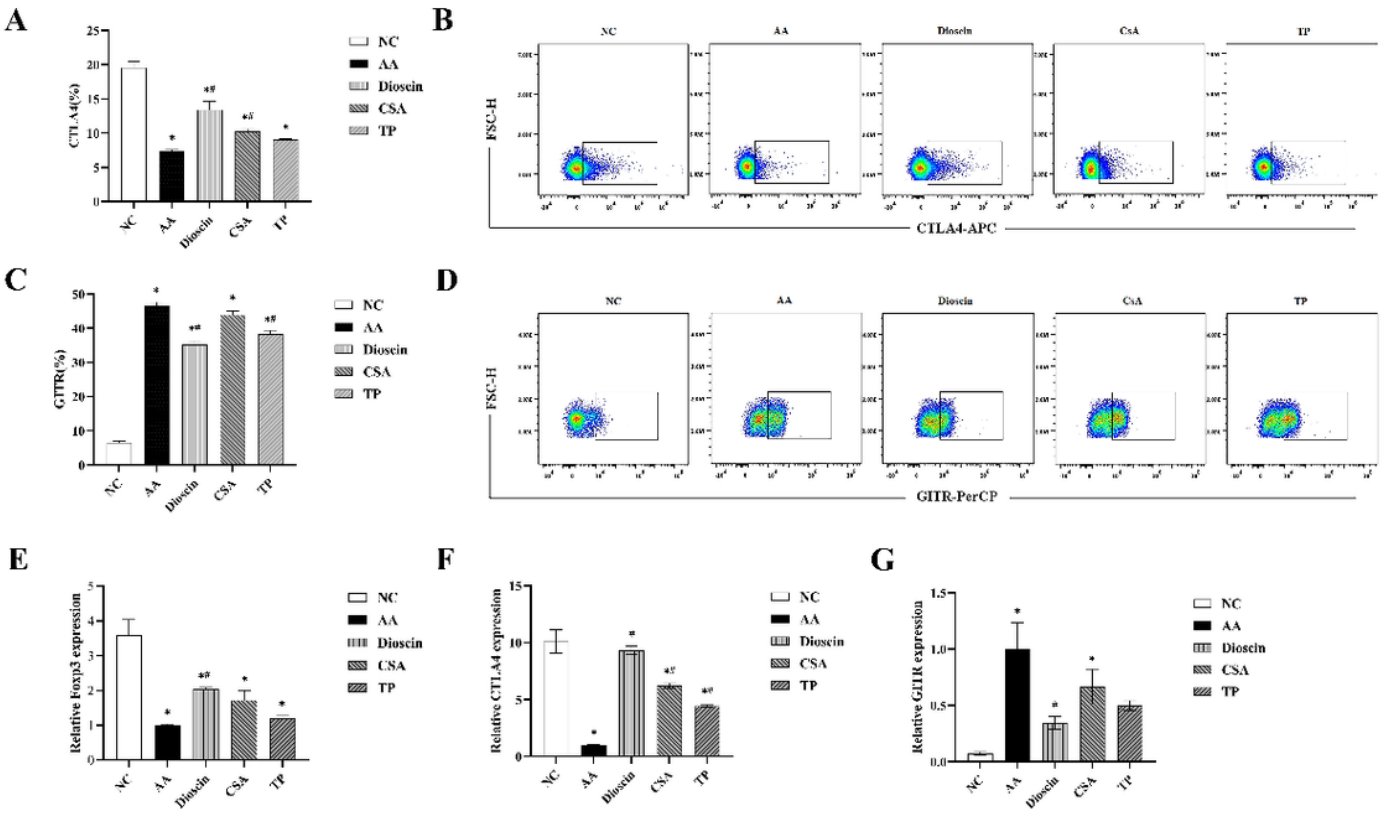


Figure 3

A, B. The proportion of CTLA4 on Tregs among groups. NC, Dioscin, CsA and TP groups had higher expression of CTLA4 compared with the AA model group. **C, D.**The proportion of GITR on Tregs among groups. NC, Dioscin, CsA and TP groups had lower expression of GITR compared with the AA model group. Data were mean±SEM, n=3 (**P*<0.05 vs. NC group, #*P*<0.05 vs. AA model group). **E, F, G.** The relative mRNA expression of Foxp3, CTLA4 and GITR among groups. Data were mean±SEM, n=3(**P*<0.05 vs. NC group, #*P*<0.05 vs. AA model group).