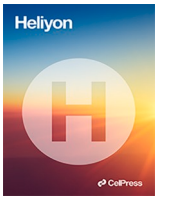
Heliyon 10 (2024) e26731

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Insufficient phosphorylation of STAT5 in Tregs inhibits the expression of BLIMP-1 but not IRF4, reduction the proportion of Tregs in pediatric aplastic anemia

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ARTICLE INFO

Keywords:
Pediatric aplastic anemia
Regulatory T cells (tregs)
B lymphocyte-induced mature protein-1 (BLIMP-1)
Interferon regulatory factor 4 (IRF4)
Signal transducer and activator of transcription 5 (STAT5)

ABSTRACT

Deficiency in regulatory T cells (Tregs) is an important mechanism underlying the pathogenesis of pediatric aplastic anemia, but its specific mechanism is unclear. In our study, we aimed to investigate whether IL-2/STAT5 can regulate the proliferation of Tregs in aplastic anemia (AA) by regulating their expression of B lymphocyte-induced mature protein-1 (BLIMP-1) or interferon regulatory factor 4 (IRF4). Through clinical research and animal experiments, we found that poor activation of the IL-2/STAT5 signaling pathway may leads to low expression of BLIMP-1 in Tregs of children with AA, which leads to defects in the differentiation and proliferation of Tregs in AA. In AA model mice, treatment with IL-2c reversed the decrease in Treg proportions and reduction in Blimp-1 expression in Tregs by increasing the phosphorylation of Stat5 in Tregs. In AA, deficiency of IRF4 expression in Tregs is closely related to the deficiency of Tregs, but is not regulated by the IL-2/STAT5 pathway.

1. Introduction

Aplastic anemia (AA) is a disease that is characterized by bone marrow failure and peripheral blood pancytopenia [1,2]. The dysregulation of the immune system is widely recognized as the main mechanism underlying the pathogenesis of AA. Regulatory T cells (Tregs), which are the main components that regulate immune tolerance, can inhibit the activation and proliferation of effector T cells (Teffs) in vivo and in vitro, and Tregs are important for maintaining a steady state of immunity [3].Many previous studies on pediatric AA have shown that decreases in Treg number and function are important immune mechanisms that are involved in the pathogenesis of this disease [4–6]. However, the specific regulatory mechanism underlying the Treg abnormalities that are observed in

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https://doi.org/10.1016/j.heliyon.2024.e26731
Received 22 September 2023; Received in revised form 19 February 2024; Accepted 19 February 2024
Available online 29 February 2024

patients with AA remains unclear. Additionally, whether there are important factors that regulate Treg cells and thus affect the ability of Tregs to suppress immune responses requires further exploration.

Tregs can be classified as central Tregs (cTregs) or effector Tregs (eTregs) according to their different activation states [7,8]. eTregs express high levels of IL-10, CTLA-4, ICOS, and TIGIT and, consequently, have stronger suppressive effects on Teff activation than do cTregs [8,9]. During the differentiation of Tregs, B lymphocyte-induced maturation protein 1 (BLIMP-1) and interferon regulatory factor 4 (IRF4) are key transcription factors that participate in promoting the differentiation of cTregs into eTregs. The impaired expression of both of these factors inhibits effector phenotype differentiation and effector function maturation in Tregs [10,11]. BLIMP-1 is encoded by the positive regulatory domain 1 (*PRDM1*) gene [12], and its expression can be induced by TCR and/or IL-2 signaling [13]. BLIMP-1 is indispensable for the differentiation of eTregs and the production of IL-10 by Tregs [14]. Erika et al. used ChIP and quantitative PCR analysis to detect the specific binding of Blimp-1 to intron 1 of the Il10 gene in Tregs, and they reported that Tregs lacking Blimp-1 exhibit decreased expression of costimulatory molecules (inducible costimulatory molecule, ICOS) and almost no secretion of IL-10 [10]. Compared with that of Tregs in Blimp-1-CKO mice, the suppressive effect of Tregs on Teffs in Blimp-1 transgenic mice is significantly enhanced [12]. Interferon regulatory factor (IRF) acts upstream of Blimp-1 and can directly regulate the expression of the Blimp-1-encoding gene Prdm1; together with Blimp-1, IRF regulates the differentiation and function of eTregs [11]. Cretney et al. [11] observed strong binding sites for Blimp-1 and Irf4 in the *Il10* gene via microarray analysis and found that Irf4 and Blimp-1 jointly regulate the expression of IL-10 in Tregs. Moreover, research by Uma et al. [15] showed that Tregs lacking IRF4 cannot secrete IL-10 and cannot inhibit Th cell-induced inflammation, leading to secondary immune disorders and even immune diseases.

IL-2 also plays key roles in the development, recruitment, and function of Tregs. IL-2 induces the expression of important transcription factors such as FOXP3 by stimulating the phosphorylation of STAT5 (p-STAT5) to mediate the differentiation of Tregs [16]. IL-2 is also an effective inducer of BLIMP-1 expression in Tregs. After binding to the IL-2 receptor (IL-2R) on Tregs, IL-2 can stimulate the phosphorylation of STAT5, thereby directly or indirectly inducing the expression of BLIMP-1 [17]. As Tregs cannot produce IL-2, these cells are inherently dependent on exogenous sources, predominantly Teffs, of this critical homeostatic factor [18]. However, because Tregs express a high-affinity receptor, they respond to low concentrations of IL-2 [18]. In vitro experiments have shown that low doses of IL-2 preferentially induce STAT5 phosphorylation (phospho-STAT5, pSTAT5) in Tregs and thus promote Treg expansion [18]. Other clinical trials have shown that low-dose IL-2 treatment has selective proliferative effects on Tregs in healthy individuals and patients with hepatitis C virus-induced vasculitis, type 1 diabetes, acute GVHD, or systemic lupus erythematosus [19–23]. Nevertheless, due to its pleiotropy, IL-2, even at low doses, may also activate potentially harmful cells, such as Teffs, which may induce or worsen autoimmunity [19]. The most recent study revealed that the combination of IL-2 and different IL-2-specific monoclonal antibodies could selectively amplify specific lymphocyte subsets [24–26]. The IL-2/JES6-1 complex induces preferential expansion of Tregs by blocking the interaction of IL-2 with CD122 (IL-2Rβ) and CD132 (common-chain or IL-2Rγ) and promoting its interaction with CD25 [27]. Therefore, we treated AA model mice with the IL-2/JES6-1 complex to evaluate its potential utility in improving the expression of Blimp-1 and Irf4 in Tregs as well as the expansion of Tregs.

Through clinical studies and animal experiment, we found that poor activation of the IL-2/STAT5 pathway in Tregs can inhibit the expression of BLIMP-1 in these cells, which may explain the decreased number and function of Tregs in AA. The low expression of IRF4 in Tregs in AA may also play an important role in decreasing the proportion of Tregs and reducing the levels of IL-10 in AA, but this change does not seem to be regulated by the IL-2/STAT5 signaling pathway.

2. Methods

2.1. Human participants

We included 17 (11 males, six females) pediatric patients with de nova-acquired AA and 18 age-matched healthy children (control, 13 males, five females), all of whom provided informed consent. The median age of the patients with AA and controls was 7.6 years (range: 1–16 years) and 8.7 years (range: 2–14 years), respectively. There were no significant differences in sex and age between the two groups (Table 1). This cohort of patients included six cases with severe AA (SAA) and 11 with non-severe AA (NSAA). The diagnosis and severity classification of AA were established according to the criteria of Camitta et al. [28,29],at least two of the following criteria must be met: hemoglobin concentration (Hb) $<$ 100 g/l, platelet count $<100 \times 10^9$ cells/L, and neutrophil count$<1.5 \times 10^9$ cells/L. The modified Camitta criteria were used to assess severity [30]. The following conditions must be met to diagnose severe aplasia: marrow cellularity $<$25% (or 25–50% with $<$30% residual hematopoietic cells) plus at least 2 of (i) neutrophils $<0.5 \times 10^9$ cells/L and (ii) platelets $<20 \times 10^9$ cells/L; otherwise, the patient is diagnosed with NSAA [29]. All the patients with AA in our study met the above diagnostic criteria, and their blood cell counts are shown in Table 2. The results of their bone

Table 1
General information of children in healthy control group and AA group.

	Control group	AA group	p value
Number	18	17	
Sex(male:female)	13:5	11:6	$p = 0.632$
Median age,y(range)	8.7(2,14)	7.6(1,16)	$p = 0.478$

AA: aplastic anemia.

marrow smear and bone marrow biopsy showed that the proliferation of bone marrow nucleated cells was decreased, and the amount of hematopoietic tissue was decreased. Inherited bone marrow failure syndrome (IBMFS) was excluded in all the AA patients in our study by the mitomycin C-induced chromosome break test and second-generation sequencing of the gene. This study was conducted with the approval of Ethics Committee of Sun Yat-Sen University (approval number 2019SYSUSH-023), and informed consent was provided by the guardians of the included patients.

2.2. Mice

Fifty-four male SPF grade C57BL/6 mice, 8–10 weeks old, were purchased from the Animal Experiment Center of Bojin Biotechnology. The animal use protocol listed below has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUCA) of Bojin Biotechnology (approval number BG-AMS-20210223-LN01-Y). The mice were randomly divided into the following three groups: the control group (CTRL), untreated AA group (UAA), and IL-2c-treated AA group (TAA). All the animal experiments were approved by the Ethics Committee of Sun Yat-Sen University. A mouse AA model was established by intraperitoneal injection of recombinant murine IFN-γ (7500 U/d, Popretech, New Jersey,USA) and oral administration of busulfan (18 mg/kg/d, China) for 10 consecutive days [31]. To validate this model, we have conducted comprehensive evaluations including blood routine tests and bone marrow analyses in AA mice. These assessments revealed a significant reduction in three-line cells (Table S1) and a decrease in bone marrow hematopoietic capacity (Table S2; Fig. S1), aligning closely with clinical observations in human aplastic anemia patients. During the experiment, survival rates were as follows: 100% in the control group, 66.7% (12 out of 18) in the UAA group, and 66.7% (12 out of 18) in the TAA group. The mice in the control group received an intraperitoneal injection and oral administration of an equal volume of saline. Recombinant murine IL-2 was purchased from eBioscience (California, USA), and anti-mouse IL-2 monoclonal antibodies (JES6-1) were obtained from PeproTech (New Jersey, USA). IL-2c was mixed with anti-IL-2 (JES6-1) at a ratio of 1:5 (1 mg of recombinant murine IL-2 and 5 mg of JES6-1) and incubated at 37 °C for 30 min. The day after the model was established, one group of AA model mice was administered an intraperitoneal injection of IL-2c (6 mg/d) for 7 days to establish the TAA group, while the other group of AA model mice was administered an intraperitoneal injection of an equal volume of saline to establish the UAA group [27,32,33]. All the mice were sacrificed on the second day after administration, and the materials were collected for the experiment.

2.3. Measurement of interleukin-10 levels in peripheral blood (PB) plasma

The levels of interleukin-10 (IL-10) in plasma samples were determined by immunostaining using a LEGENDplexTM kit (BioLegend, California, USA) according to the manufacturer's protocol. The data were obtained using flow cytometry (Beckman, California, USA) and analyzed with LEGENDplex version 8.0 software.

2.4. Detection of T cells and treg cells in human PB

Brilliant Violet 421 (BV421)-conjugated anti-human CD8 (Biolegend, USA), fluorescein isothiocyanate (FITC)-conjugated anti-human CD4 (Biolegend, USA), phycoerythrin (PE)-conjugated anti-human CD25 (Biolegend, USA), PE/Cyanine7 (PC7)-conjugated anti-human CD127 (Biolegend, USA), and matched isotype control IgG antibodies were used for surface staining. After a 20-min incubation at 4 °C in the dark with these antibodies, the cells were incubated with red blood cell lysis buffer (BioLegend, USA) for 10 min at room temperature. The cells were subsequently washed three times with phosphate-buffered saline (PBS). Finally, $CD4^{+}CD8^{-}$ T cells, $CD4^{-}CD8^{+}$ T cells and $CD4^{+}CD25^{+}CD127^{dim}$ Tregs were detected in human PB samples on a Cytoflex Lx flow cytometer (Beckman, USA), and the data were analyzed by CytExpert version 2.3.0.84 software (Beckman, USA).

2.5. Detection of BLIMP-1 and IRF4 expression levels in tregs

PB mononuclear cells (PBMCs) were isolated by density gradient sedimentation of whole blood. PBMCs were washed with PBS and stained with FITC-conjugated anti-human CD4 and PE-conjugated anti-human CD25 antibodies or matched isotype control IgG. After surface staining, the cells were fixed and permeabilized with a Transcription Factor Staining Buffer Set (eBioscience, California, USA) according to the manufacturer's instructions. Fixed cells were then stained with an anti-BLIMP-1 mAb (Cell Signaling Technology, Boston, USA) or an anti-IRF4 mAb (Cell Signaling Technology, USA). Finally, the labeled cells were examined on a Cytoflex Lx flow cytometer, and analysis was conducted using CytExpert version 2.3.0.84 software.

Table 2
Blood cell counts in children with aplastic anemia.

	WBC ($\times 10^9$ cells/L)	ANC ($\times 10^9$ cells/L)	Hb (g/L)	PLT ($\times 10^9$ cells/L)	Ret ($\times 10^9$ cells/L)
NSAA	4.36 ± 0.25	1.11 ± 0.10	93.1 ± 6.3	35.7 ± 5.0	41.5 ± 4.8
SAA	2.72 ± 0.41	0.20 ± 0.04	54.3 ± 4.2	8.7 ± 6.5	19.4 ± 4.3
p value	0.003	< 0.001	0.001	< 0.001	0.008

NSAA:non-severe aplastic anemia; SAA: severe aplastic anemia; WBC: white blood cell; ANC: absolute neutrophil count; Hb: hemoglobin; PLT: platelet; Ret: reticulocyte.

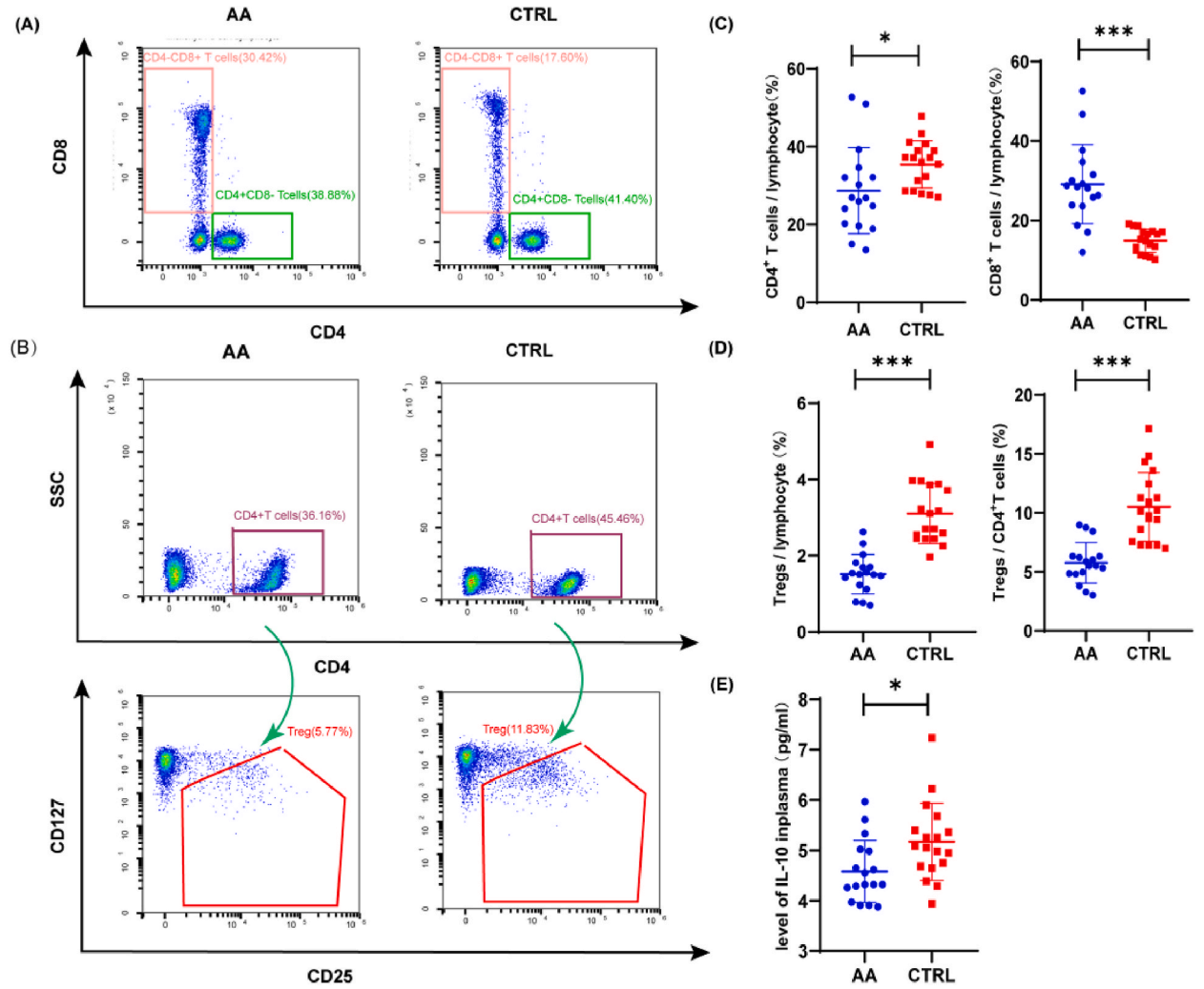

Fig. 1. Abnormal proportions of T-cell subsets, decreased frequencies of Tregs and decreased levels of IL-10 in children with AA. (A) Detection of $CD4^+$ T cells and $CD8^+$ T cells among lymphocytes by flow cytometry. (B) Detection of Tregs among $CD4^+$ T cells by flow cytometry. (C) The percentages of $CD4^+$ T cells and $CD8^+$ T cells in the lymphocyte population of the AA (n = 17) and normal control (CTRL) (n = 18) groups. (D) The percentage of Tregs in the lymphocytes population and in the $CD4^+$ T cell population in the AA (n = 17) and CTRL (n = 18) groups. (E) The level of IL-10 in the plasma of children in the AA (n = 17) and CTRL (n = 18) groups. The data are expressed as the mean ± SD. $*p < 0.05$; $***p < 0.001$.

2.6. Assessment of p-STAT5 expression in $CD4^+$ T cells

PBMCs were isolated by density gradient sedimentation of whole blood before being resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Biosera, France) supplemented with 10% fetal bovine serum (FBS) (Biosera, France) and 1% penicillin/streptomycin (Biosera, France). Subsequently, the PBMCs (5×10^5 cells/ml) were stimulated with IL-2 (100 ng/ml) for 15 min at 37 °C in 5% CO2. The cells were washed with PBS and then stained with a FITC-conjugated anti-human CD4 antibody. After surface staining, 4% formaldehyde was used to fix the cells in the dark at room temperature for 15 min. The fixed cells were washed twice with PBS and then permeabilized with ice-cold methanol for 10 min at 4 °C. Intracellular staining was performed with an APC-conjugated anti-human/mouse p-STAT5 antibody (eBioscience, USA) for 1 h at 4 °C, after which the stained cells were washed three times with PBS. The percentages of stained cells were measured using a Cytoflex Lx flow cytometer (Beckman, USA) and analyzed by CytExpert version 2.3.0.84 software.

2.7. Mouse cell preparation

Spleens were harvested and pooled from 8- to 10-week old C57BL/6 mice and poolied in RPMI 1640 medium (Mediatech, Manassus, VA, USA). Cell suspensions were prepared by dicing the spleens with a razor blade and digesting them with a solution containing 0.1 mg/mL DNase I (Roche, Indianapolis, IN, USA) and 1 mg/mL Collagenase D (Roche, Indianapolis, IN, USA) in HBSS (Cellgro,

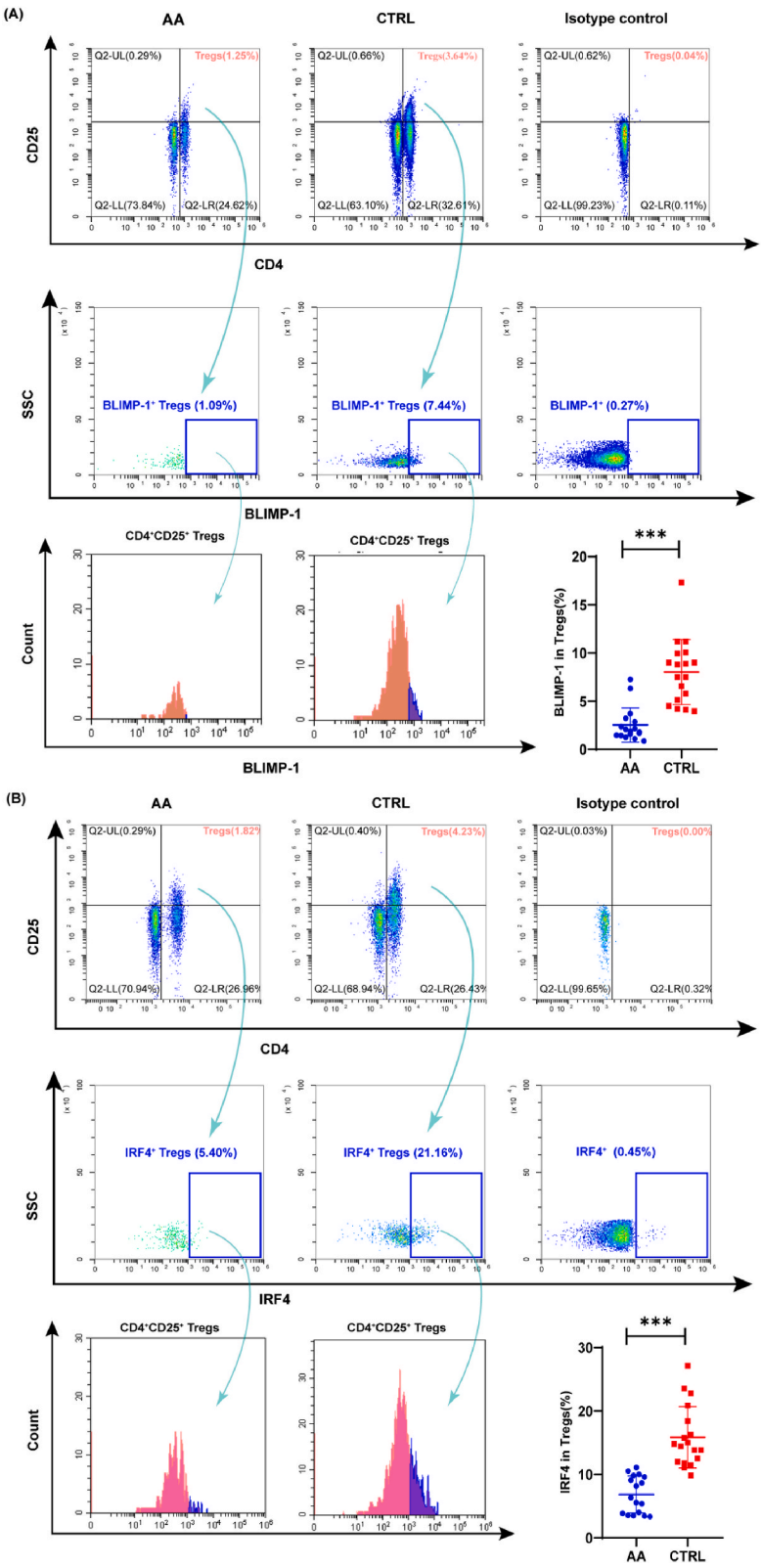

Fig. 2. Decreased expression of BLIMP-1 and IRF4 in Tregs from children with AA. (A) Detection of BLIMP-1-positive Tregs by flow cytometry and the level of BLIMP-1 expressed in Tregs from the AA group (n = 17) and CTRL group (n = 18). (B) Detection of IRF4-positive Tregs by flow

cytometry and the level of IRF4 in Tregs from the AA group (n = 17) and CTRL group (n = 18). The data are expressed as the mean ± SD. ***$p < 0.001$.

Manassas, VA, USA) for 30 min at 37 °C. Then, the cells were passed through a 40-μM nylon filter (BD Falcon, Bedford, MA, USA). Red blood cell lysis was performed using 2 ml of 1x red blood cell lysis buffer (BioLegend, USA) per spleen. The cells were then washed in PBS containing 5% heat-inactivated FBS and counted using a Countess automated cell counter (Invitrogen, Carlsbad, CA, USA). For flow cytometry immunophenotyping experiments, 1×10^6 cells per tube were stained as described below. For cell sorting, approximately 1×10^8 cells per cocktail were prepared in MACS buffer (Miltenyi Biotech) rather than staining buffer.

2.8. Detection of Blimp-1, Irf4 and p-Stat5 expression in tregs in mouse spleen

Splenocytes were obtained from mice in each group by erythrocyte lysis and cryopreserved for further evaluation by flow cytometry. The percentage of Tregs in the splenocyte population was determined via flow cytometry according to the methods used for human specimens mentioned above. Tregs were sorted from mouse spleens using a $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec, Germany) according to the reagent manufacturer's instructions [34–39]. Partial Tregs were fixed and permeabilized with a Transcription Factor Staining Buffer Set (eBioscience, USA) and then stained with an anti-BLIMP-1 mAb (Cell Signaling Technology, USA) or anti-IRF4 mAb (Cell Signaling Technology, USA). The labeled cells were examined on a Cytoflex Lx flow cytometer and analyzed using CytExpert version 2.3.0.84 software. The other Tregs were stimulated with IL-2 (100 ng/ml) for 15 min at 37 °C in 5% CO2 before staining with an APC-conjugated anti-human/mouse p-STAT5 antibody (eBioscience, USA). Finally, the level of p-STAT5 in Tregs was measured using a Cytoflex Lx flow cytometer (Beckman, USA) and analyzed with CytExpert version 2.3.0.84 software.

2.9. Statistical analysis

The statistical significance of differences was calculated with GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). The sex characteristics of the two groups were analyzed by the chi-square test. Quantitative data are presented as the mean ± standard deviation (SD). Quantitative data displaying a normal distribution pattern were analyzed by t tests to compare two groups and analysis of variance to compare multiple groups. The Pearson correlation model was used to analyze the correlation between two particular

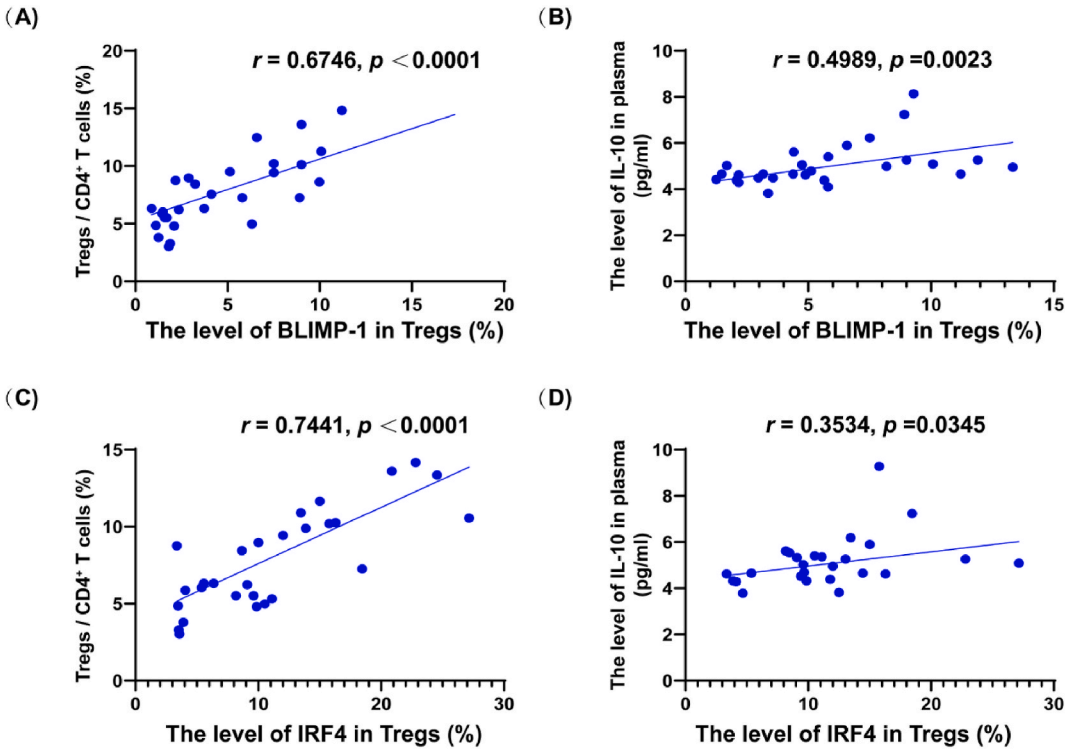

Fig. 3. Positive correlation between BLIMP-1 or IRF4 expression in Tregs and the percentage of Tregs among $CD4^+$ T cells or the IL-10 plasma level. (A) Correlation between the expression of BLIMP-1 in Tregs and the percentage of Tregs among $CD4^+$ T cells. (B) Correlation between the expression of BLIMP-1 in Tregs and the level of IL-10 in plasma. (C) Correlation between the expression of IRF4 in Tregs and the percentage of Tregs among $CD4^+$ T cells. (D) Correlation between the expression of IRF4 in Tregs and the level of IL-10 in plasma. *r*: Pearson correlation analysis coefficient.

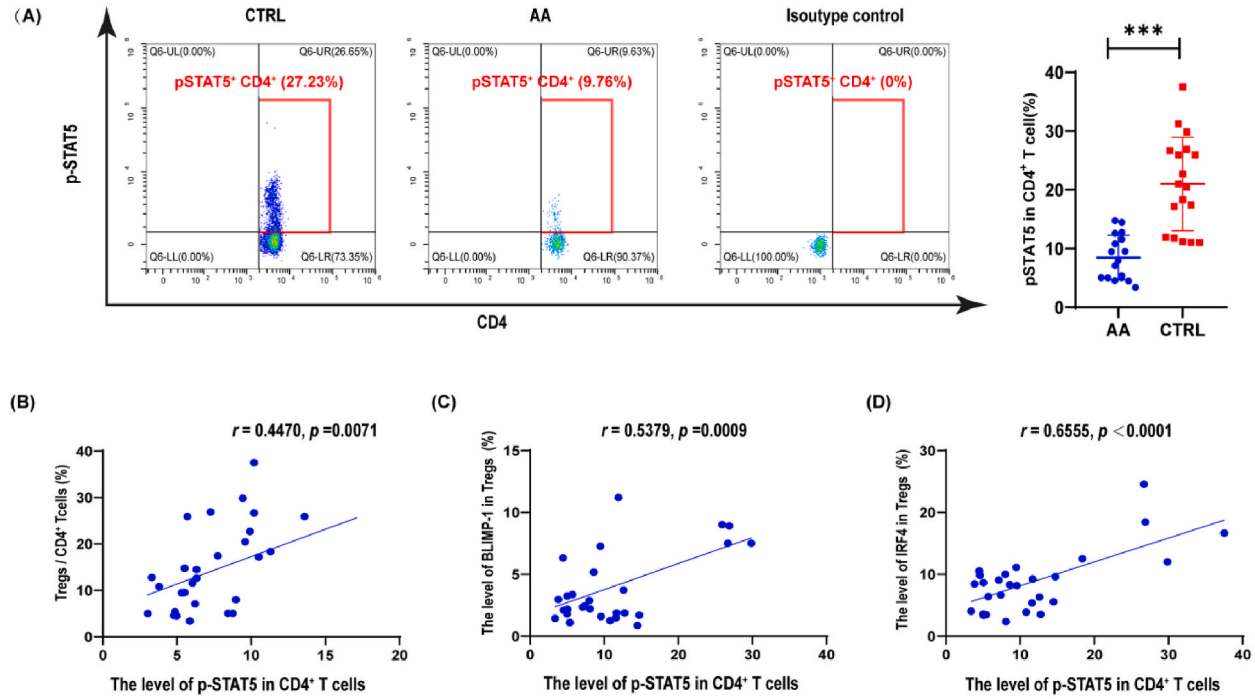

Fig. 4. (A) Analysis of p-STAT5-positive $CD4^+$ T cells by flow cytometry (left) and the level of p-STAT5 expressed in $CD4^+$ T cells from the AA group (n = 17) and CTRL group (n = 18) (right). The data are expressed as the mean ± SD. ***$p < 0.001$. (B) Correlation between the expression of p-STAT5 in $CD4^+$ T cells and the percentage of Tregs among $CD4^+$ T cells. (C) Correlation between the expression of p-STAT5 in $CD4^+$ T cells and the expression of BLIMP-1 in Tregs. (D) Correlation between the expression of p-STAT5 in $CD4^+$ T cells and the expression of IRF4 in Tregs.

metrics, and the data from a control group (n = 18) alongside the AA group (n = 18) were included in our correlation analysis. P values ≤ 0.05 were considered to indicate statistical significance as follows: *p < 0.05, **p < 0.01, and ***p < 0.001.

3. Results

3.1. Dysregulated T-cell subsets and decreased treg frequency in children with AA

To determine the frequencies of T cells and Tregs in AA patients, we analyzed the frequencies of $CD4^-CD8^+$ T cells, $CD4^+CD8^-$ T cells (gating on the lymphocyte population), and $CD4^+$ $CD25^+$ $CD127^{dim}$ Tregs (gating on the $CD4^+$ T-cell population and lymphocyte population) in peripheral blood samples that were obtained from pediatric patients with AA (AA) and normal controls (CTRL). The percentages of $CD4^+$ T cells in the lymphocyte population of the AA and CTRL groups were 28.70% ± 11.10% and 35.42% ± 6.04%, respectively ($p = 0.032$) (Fig. 1A and C). The percentages of $CD8^+$ T cells in the lymphocyte population of the AA and CTRL groups were 29.14% ± 9.98% and 14.90% ± 2.93%, respectively ($p < 0.001$) (Fig. 1A and C). The percentage of Tregs in the $CD4^+$ T cell population of patients with AA was 5.77% ± 1.72%, which was obviously lower than that of the CTRL group (10.50% ± 2.98%) ($p < 0.001$) (Fig. 1B and D). Moreover, the percentages of Tregs in the lymphocyte population of the AA and CTRL groups were 1.51% ± 0.52% and 3.10% ± 0.79%, respectively ($p < 0.001$) (Fig. 1B and D). In pediatric patients with AA, the proportion of effector T cells, such as $CD8^+$ T cells, was significantly increased, while that of Tregs was decreased, which indicates that T-cell subsets are dysregulated in patients with AA.

4. Decreased plasma IL-10 levels in children with AA

Interleukin-10 (IL-10) is an important cytokine that is used by immune cells to suppress inflammation [8]. $CD4^+Foxp3^+$ regulatory T cells (Tregs) can negatively regulate the immune system by secreting IL-10 [8]. We measured the levels of IL-10 in plasma samples from children with AA and healthy children by flow cytometry. The results showed that the plasma levels of IL-10 in children with AA were lower than those in healthy children (4.58 ± 0.62 vs. 5.17 ± 0.76, p = 0.018; Fig. 1E).

4.1. Decreased BLIMP-1 and IRF4 expression levels in tregs from children with AA

BLIMP-1 and IRF4 are important transcription factors that are expressed by eTregs [11]. We measured the levels of BLIMP-1 and IRF4 in Tregs by flow cytometry. The results showed that 2.54% ± 1.78% of Tregs in the AA group expressed BLIMP-1, which was

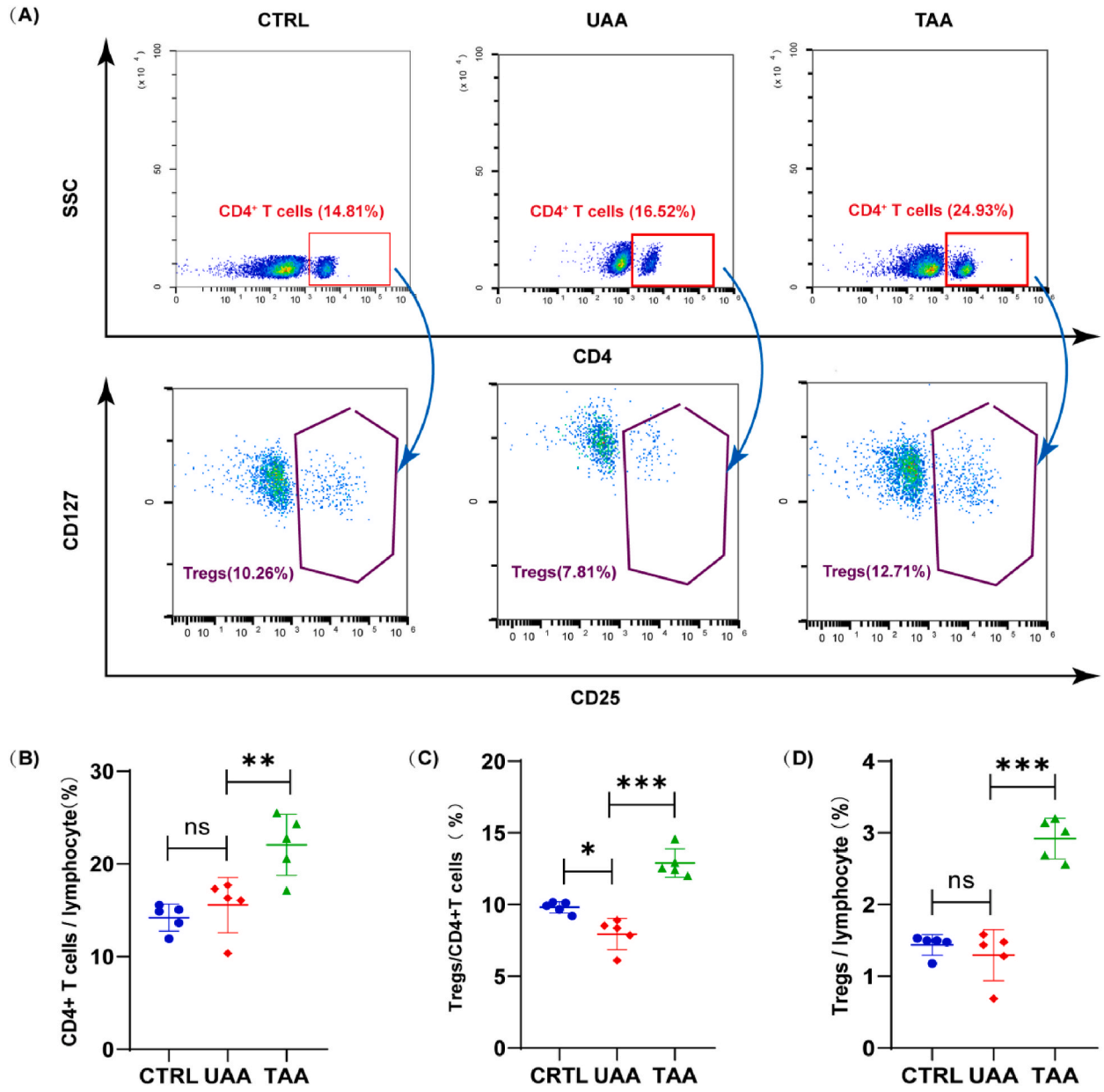

Fig. 5. Decreased percentage of Tregs in splenocytes from AA model mice and the effect of IL-2c treatment on ameliorating Treg damage in AA model mice. (A) Determination of the proportion of $CD4^+$ T cells (gating on lymphocytes) and the proportion of Tregs (gating on $CD4^+$ T cells) in splenocytes from the CTRL, UAA, and TAA groups. (B) The percentage of $CD4^+$ T cells among lymphocytes in splenocytes from the CTRL (n = 5), UAA (n = 5), and TAA groups (n = 5). (C) The percentage of Tregs among $CD4^+$ T cells in splenocytes from the CTRL (n = 5), UAA (n = 5), and TAA groups (n = 5). (D) The percentage of Tregs among lymphocytes in splenocytes from the CTRL (n = 5), UAA (n = 5), and TAA groups (n = 5). The data are expressed as the mean ± SD. ns: not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

significantly lower than that in the CTRL group (8.05% ± 3.37%) ($p < 0.001$; Fig. 2A). Similarly, 6.83% ± 2.90% of Tregs in the AA group expressed IRF4, which was significantly lower than that in the CTRL group (15.87% ± 4.85%) ($p < 0.001$; Fig. 2B). Accordingly, the expression of BLIMP-1 and IRF4 in Tregs was inhibited in children with AA.

4.2. Positive correlation between BLIMP-1 or IRF4 expression in tregs and the percentage of tregs among $CD4^+$ T cells or IL-10 plasma level

Pearson correlation analysis was performed with the 17 children who were newly diagnosed with AA and 18 healthy children who were described above. The results showed that the expression of BLIMP-1 in Tregs was significantly positively correlated with the percentage of Tregs among $CD4^+$ T cells ($r = 0.6746$, $p < 0.0001$; Fig. 3A) and the level of IL-10 in plasma ($r = 0.4989$, $p = 0.0023$;

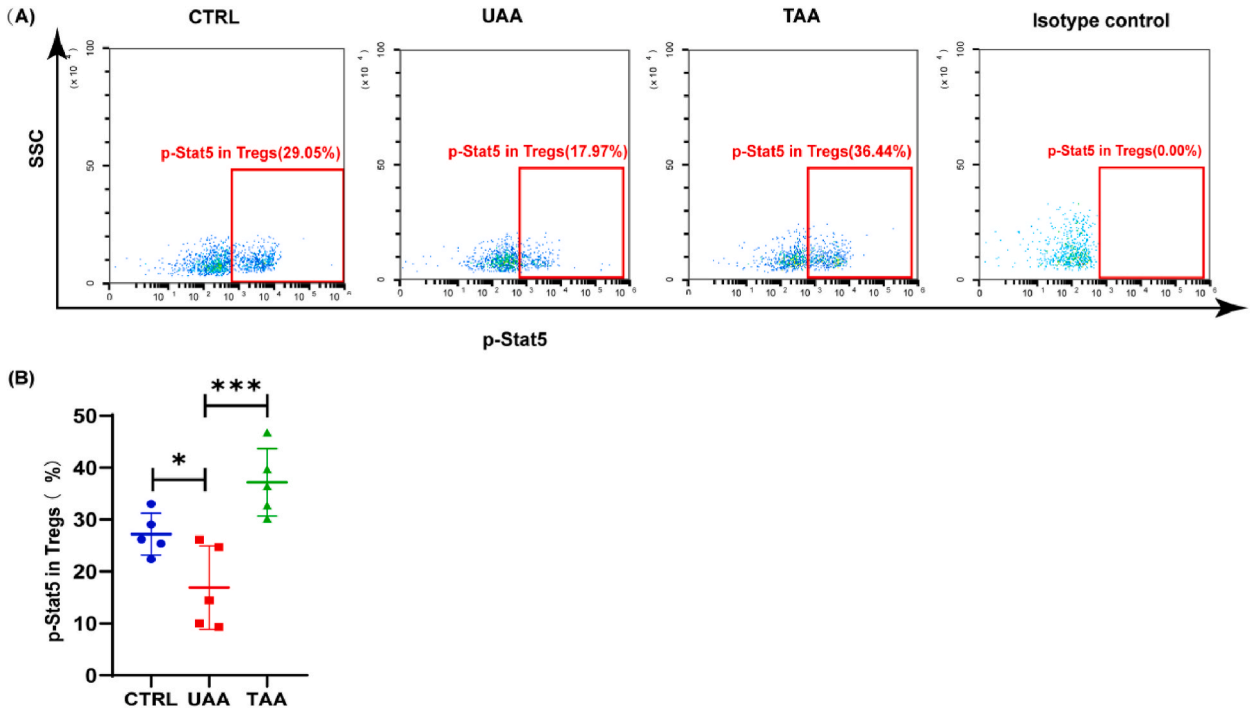

Fig. 6. Decreased p-Stat5 levels in Tregs from the spleens of AA model mice and the effect of IL-2c treatment on ameliorating poor p-Stat5 activation. (A) Detection of p-Stat5-positive cells among Tregs sorted from mice in the spleens of CTRL, UAA, and TAA groups by flow cytometry. (B) Levels of p-Stat5 in Tregs sorted from the spleens of mice in the CTRL (n = 5), UAA (n = 5), and TAA groups (n = 5). The data are expressed as the mean ± SD. *$p < 0.05$; ***$p < 0.001$.

Fig. 3B). The expression of IRF4 in Tregs was also positively correlated with the percentage of Tregs among $CD4^+$ T cells ($r = 0.7441$, $p < 0.0001$; Fig. 3C) and the level of IL-10 in plasma ($r = 0.3534$, $p = 0.0345$; Fig. 3D). Accordingly, the low expression of BLIMP-1 and IRF4 in Tregs may lead to decreased numbers Tregs in the peripheral blood and reduced levels of IL-10 in plasma in children with AA.

4.3. Decreased p-STAT5 expression levels in $CD4^+$ T cells from children with AA

The proliferation, survival, and function of Tregs depend on the IL-2/STAT5 signaling pathway [40,41], and the level of phosphorylated STAT5 can indicate the activation of this pathway. To analyze whether the activation of STAT5 in pediatric patients with AA is abnormal, we measured the expression of pSTAT5 in $CD4^+$ T cells stimulated with IL-2. After stimulation with 100 ng/ml IL-2 for 15 min, the percentage of $pSTAT5^+$ $CD4^+$ T cells in children with AA was 8.47% ± 3.81%, which was significantly lower than that in the CTRL group (21.00% ± 7.9%) ($p < 0.001$) (Fig. 4A). The results showed that the STAT5 pathway was poorly activated in the $CD4^+$ T cells from children with AA.

4.4. Positive correlation between p-STAT5 expression in $CD4^+$ T cells and the percentage of tregs among $CD4^+$ T cells and BLIMP-1 or IRF4 expression in tregs

Pearson correlation analysis demonstrated that the expression of p-STAT5 in $CD4^+$ T cells was positively correlated with the percentage of Tregs among $CD4^+$ T cells ($r = 0.4470$, $p = 0.0071$; Fig. 4B). We also discovered that the expression of p-STAT5 in $CD4^+$ T cells was significantly positively correlated with the expression of BLIMP-1 in Tregs ($r = 0.5379$, $p = 0.0009$; Fig. 4C) and the expression of IRF4 in Tregs ($r = 0.6555$, $p < 0.0001$; Fig. 4D). These data demonstrated that poor activation of STAT5 in pediatric patients with AA may be responsible for the low expression of BLIMP-1 in Tregs and the decreased numbers of Tregs in children with AA.

4.5. Decreased percentage of tregs in splenocytes from AA model mice and the effect of IL-2c treatment on ameliorating treg damage in AA model mice

JES6-1 is a selective anti-IL-2 antibody, and its interaction with IL-2 can promote the expansion of Tregs, increase the expression of IL-2Rα (CD25), and inhibit the proliferation of Teffs [33,42]. In our study, AA model mice were generated by the combined administration of IFN-γ and busulfan, and the mice were evenly divided into two groups: treated AA model mice (TAA) and untreated AA model mice (UAA). The mice in the TAA group were injected with the IL-2/JES6-1 complex (IL-2c), while those in the UAA group were

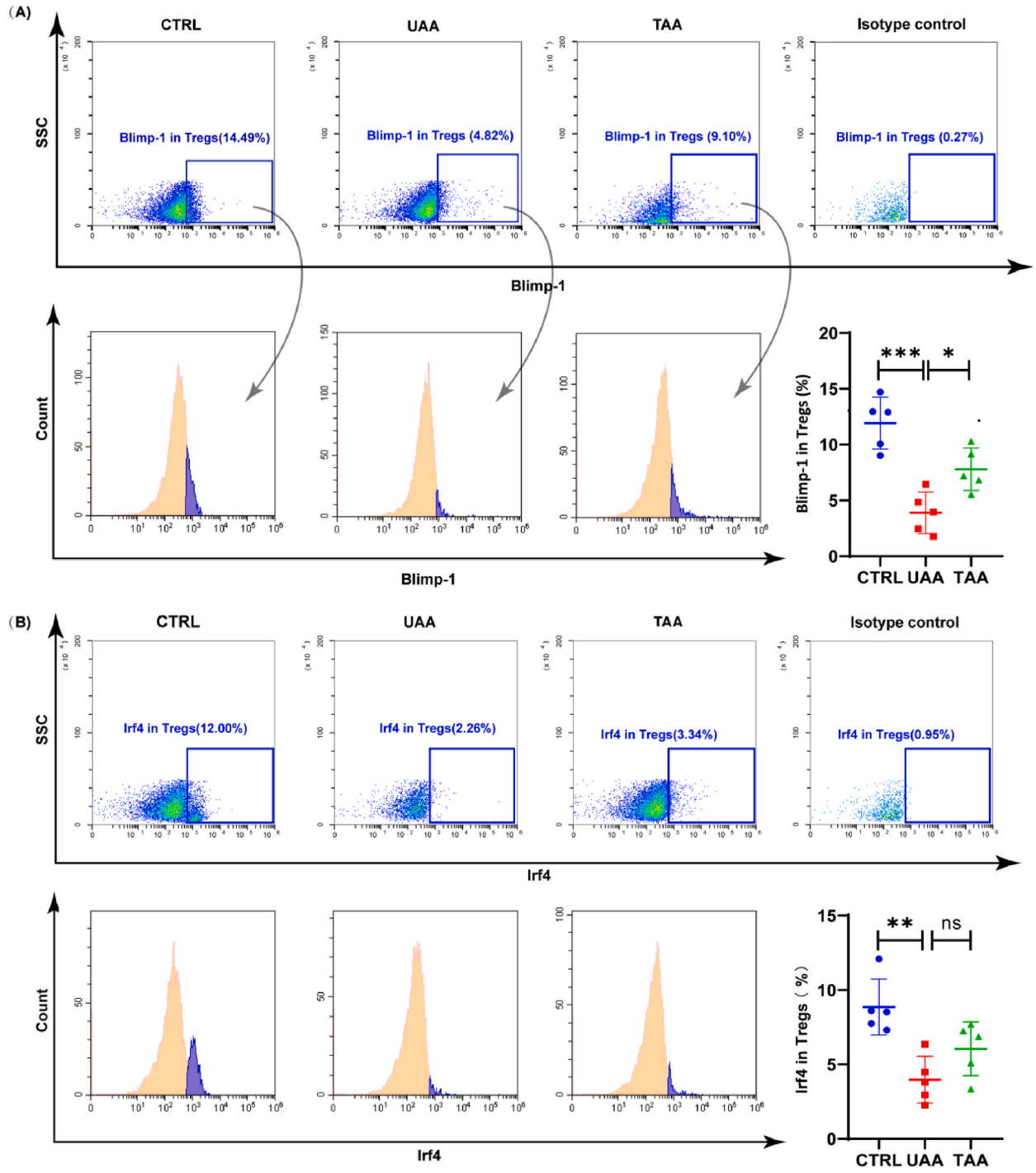

Fig. 7. Low expression levels of Blimp-1 and Irf4 in Tregs from AA model mice were restored by IL-2c. (A) Detection of Blimp-1-positive Tregs by flow cytometry and the level of Blimp-1 expression in Tregs from CTRL mice (n = 5), UAA model mice (n = 5), and TAA model mice (n = 5). (B) Detection of Irf4-positive Tregs by flow cytometry and the level of Irf4 in Tregs from CTRL mice (n = 5), UAA model mice (n = 5), and TAA model mice (n = 5). The data are expressed as the mean ± SD. ns: not significant; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

injected with an equal volume of normal saline. Mice in the blank control group (CTRL) were administered an equal volume of normal saline in the same way during modeling and intervention.

To obtain evidence for the decreased frequency of Tregs in AA model mice and the effect of IL-2c on ameliorating this damage, we analyzed the differences in the frequencies of Tregs (gating on $CD4^+$ T cells or lymphocytes) in spleens from the CTRL, UAA, and TAA groups by flow cytometry. First, the results showed that the frequencies of $CD4^+$ T cells among lymphocytes in splenocytes from the CTRL, UAA, and TAA groups were 22.24% ± 3.72%, 17.65% ± 0.84%, and 20.97% ± 4.75%, respectively (Fig. 6A and B). The difference between the CTRL group and the UAA group was not significant ($p = 0.443$), but that between the UAA and TAA groups was significant ($p = 0.003$) (Fig. 5A and B). Then, we determined the frequency of Tregs (gating on $CD4^+$ T cells or lymphocytes). The frequency of Tregs among $CD4^+$ T cells in splenocytes from the UAA group was 6.45% ± 1.12%, which was obviously lower than that in the CTRL group (10.77% ± 1.38%) (Fig. 5A and C). However, the proportion of Tregs among $CD4^+$ T cells in the TAA group (11.45% ± 1.05%) was significantly higher than that in the UAA group ($p < 0.001$) (Fig. 5A and C), which indicates that the percentage of Tregs among $CD4^+$ T cells from AA model mice can increase with IL-2c intervention. We also determined the percentage of Tregs among lymphocytes. The results showed that the percentages of Tregs among the lymphocytes of the CTRL, UAA, and TAA groups were 2.22% ± 0.39%, 1.14% ± 0.24%, and 2.55% ± 0.50%, respectively (Fig. 5A and D). Although the decrease in the percentage of Tregs among lymphocytes in the UAA group compared with that in the CTRL group was not significant ($p = 0.424$), the percentage of Tregs among lymphocytes in the TAA group was obviously greater than that in the UAA group ($p < 0.001$) (Fig. 5A and D), which further confirmed the ability of IL-2c intervention on increasing the percentage of Tregs in AA model mice.

4.6. Decreased p-Stat5 levels in tregs from the spleens of AA model mice and the effect of IL-2c intervention on ameliorating poor p-Stat5 activation

Activation of STAT5 signaling depends on IL-2 and is essential for the proliferation and differentiation of Tregs [43,44]. Splenocytes were obtained from mice in the UAA, TAA, and CTRL groups for cell sorting and flow cytometry. To analyze whether STAT5 phosphorylation is inhibited in AA model mice and the effect of IL-2c intervention, we analyzed the level of p-STAT5 in Tregs that were sorted from the spleens of mice in each group by flow cytometry. The results showed that 16.92% ± 8.02% of Tregs in the UUA group expressed p-Stat5, which was significantly lower than that of the CTRL group (27.20% ± 4.05%) ($p = 0.026$) (Fig. 6A and B). However, the proportion of p-Stat5-positive Tregs from TAA model mice (37.18% ± 6.49%) was higher than that from UAA model mice ($p < 0.001$) (Fig. 6A and B), demonstrating that treatment with IL-2c can ameliorate the poor activation of STAT5 signaling in Tregs from AA model mice.

4.7. The low expression of Blimp-1 and Irf4 in tregs from AA model mice was rescued by IL-2c

Blimp-1 and Irf4 are transcription factors that are crucial for the differentiation and function of Tregs [8]. We studied the abnormal expression of Blimp-1 and Irf4 in Tregs from AA model mice and explored changes in the expression of these factors Tregs from AA model mice after treatment with IL-2c. Tregs were isolated from the spleens of mice in each group, and the expression of Blimp-1 and Irf4 was determined via flow cytometry. The expression of Blimp-1 in Tregs from UAA model mice (4.34% ± 1.67%) was lower than that in Tregs from CTRL mice (11.93% ± 2.33%) ($p < 0.001$), while the expression of Blimp-1 in Tregs from TAA model mice (7.91% ± 1.73%) was significantly increased compared with that in Tregs from UAA model mice ($p = 0.013$) (Fig. 7A). However, although the level of Irf4 in Tregs from UAA model mice (3.64% ± 1.49%) was significantly lower than that in Tregs from CTRL mice (9.09% ± 1.88%), the slight increase in Irf4 expression in Tregs from TAA model mice (4.22% ± 0.78%) compared with UAA model mice was not significantly different ($p = 0.540$) (Fig. 7B). Based on these observations, we concluded that the expression of both Blimp-1 and Irf4 in Tregs from AA model mice was suppressed compared with that in Tregs from control mice; moreover, IL-2c treatment could restore the expression of Blimp-1 in Tregs from AA model mice to a certain extent, but IL-2c had no obvious effect on the expression of Irf4 in Tregs from AA model mice.

5. Discussion

AA is an autoimmune disease that is caused by immune disorders, especially abnormal immune responses that are mediated by T lymphocytes, and it seriously threatens the health of children [5]. Overactivation of effector $CD4^+$ (e.g., Th1, Th17) and $CD8^+$ T cells in AA can disrupt the hematopoietic microenvironment, and these overactive T cells can attack hematopoietic stem and progenitor cells, leading to bone marrow failure [1,45]. Tregs can directly inhibit the proliferation and differentiation of Teffs and indirectly inhibit the activity of Teffs by blocking the effect of antigen-presenting cells, such as macrophages, on activating Teffs [3].IL-10 is one of the main anti-inflammatory cytokines that is secreted by Tregs [46]. Either quantitative or functional impairment of Tregs can lead to increased activation and proliferation of Teffs, thereby disrupting the immune balance and resulting in the occurrence of autoimmune diseases [41,47]. Similar to other autoimmune diseases, childhood AA is characterized by reduced numbers of Tregs and decreased abilities of these cells to suppress the proliferation of Teffs [4–6]. Our study showed that compared with that in healthy children, the frequency of $CD8^+$ T cells in the PB of children with AA was higher, while the frequency of Tregs was lower. In light of the changes in cytokine secretion in children with AA, we found that the level of IL-10, which is an anti-inflammatory cytokine, was decreased. Based on these results, we confirmed the deficiency of Tregs and the hyperactivation of Teffs in children with AA, providing evidence that the pathogenesis of AA is mediated mainly by T cell immune dysfunction.

cTregs are quiescent and exhibit a naive phenotype, while eTregs exhibit an effector phenotype. The latter has stronger suppressive

activity and proliferation ability than the former and plays a more important role in inhibiting autoimmunity [8,9,48]. In 2016, Kordast et al. proposed that Tregs can be divided into two subgroups, namely, the Treg A and Treg B subgroups. According to the phenotypic and functional differences outlined in that study, it was determined that Treg A cells correspond to cTregs, while TregB cells correspond to eTregs [49]. This study revealed that Treg B cells (i.e., eTregs) are the predominant Treg subset that is negatively affected during the pathogenesis of AA [49]. In Tregs, the expression of BLIMP-1 is restricted to mature eTregs. As BLIMP-1 is an essential transcription factor for the differentiation of eTregs and the production of IL-10, it has been shown to serve as a phenotypic marker of eTregs [14,50,51]. IIRF4 is an upstream regulatory molecule of BLIMP-1; it is highly expressed in Tregs after its expression is induced and activated by TCR signaling, and then, it directly activates the expression of downstream BLIMP-1 [11,52]. IRF4 and BLIMP-1 coregulate the differentiation of eTregs and the production of IL-10. Assessment of BLIMP-1 and IRF4 expression in Tregs is helpful for analyzing the differentiation of eTregs. Here, we verified that the expression of BLIMP-1 and IRF4 in Tregs from children with AA was lower than that in Tregs from healthy children, indicating impaired differentiation of eTregs in patients with AA. In animal experiments, we observed lower expression of Blimp-1 and Irf4 in Tregs from the spleens of AA model mice than in those from control mice, which provides further support for the above-mentioned conclusion. In addition, the results of Pearson correlation analysis further showed that low expression levels of BLIMP-1 and IRF4 in Tregs may lead to decreased numbers of Tregs in PB and decreased levels of IL-10 in children with AA.

IL-2 is a growth factor for all T cells, and the survival, differentiation and proliferation of Tregs depend on stimulation by IL-2 [53,54]. After the binding of IL-2 to the IL-2 receptor (IL-2R) on Tregs, STAT5 is phosphorylated; p-Stat5 can induce Tregs to upregulate the expression of transcription factors such as BLIMP-1, thereby mediating the further differentiation and functional maturation of Tregs [17]. As IL-2 mainly regulates Tregs through the STAT5 signaling pathway, the pSTAT5 expression level in $CD4^+$ T cells reflects the activation of the IL-2/STAT5 signaling pathway in Tregs to a certain extent. Our current analysis showed that after stimulation with IL-2, the expression of pSTAT5 in $CD4^+$ T cells from children with AA was significantly lower than that in $CD4^+$ T cells from normal controls, indicating poor activation of the IL-2/STAT5 signaling pathway in $CD4^+$ T cells. We also observed that the expression of pSTAT5 in $CD4^+$ T cells was positively correlated with the proportion of Tregs and the expression of BLIMP-1 and IRF4 in Tregs, suggesting that IL-2/STAT5 signaling pathway activation may play an important role in the low expression of BLIMP-1 and IRF4 in Tregs as well as the decreased number of Tregs in children with AA.

Effective activation of the IL-2/STAT5 signaling pathway is an important condition for the differentiation and functional maturation of Tregs [43,44,53,55]. It has been proven that administration of low-dose exogenous IL-2 can expand Tregs in vivo or in vitro and promote the expression of functional molecules, such as pStat5 and Foxp3, in Tregs, thereby effectively improving autoimmune disorders [18,21–23]. However, IL-2 not only can effectively expand Tregs but also is an effective trophic factor for proinflammatory Teffs. After activation and proliferation, Teffs can compete with Tregs for IL-2 to further drive their overactivation. Accordingly, IL-2 alone cannot prevent the negative effects that are caused by the additional proliferation of Teffs [56]. Recent studies have shown that complexes the mouse anti-IL-2 antibody JES6-1 and IL-2 (IL-2c) can preferentially activate the STAT5 signaling pathway in CD25-high Tregs and further promote their proliferation and differentiation while inhibiting the expansion of CD25-low Teffs [26]. In our animal experiment, we proved that, similar to humans, AA model mice had a lower frequency of Tregs in the spleen, poorer activation of Stat5 in Tregs, and lower expression of Blimp and Irf4 in Tregs than control mice. To elucidate the effect of IL-2c on ameliorating Treg inhibition in AA, we established an AA mouse model and administered IL-2c. After treatment of the AA model mice with IL-2c, the level of phosphorylated Stat5 in Tregs was significantly higher in TAA model mice than in UAA model mice, indicating that IL-2c can effectively activate the Stat5 signaling pathway in the Tregs of AA model mice. By comparing the proportions of Tregs in UAA and TAA model mice, we found that the proportion of Tregs in the spleens of TAA model mice was significantly higher, which provided evidence that IL-2c promotes Treg expansion in AA model mice. We also demonstrated that the expression of Blimp-1 in the Tregs of TAA model mice was noticeably higher than that in the Tregs of UAA model mice. Combined with the IL-2c-induced STAT5 activation in Tregs, we reasoned that IL-2c could promote the expression of its downstream target Blimp-1 by enhancing STAT5 phosphorylation in Tregs. However, we found that IL-2c treatment did not effectively reverse the decreased expression of Irf4 in the Tregs of AA model mice; these results indicated that Irf4 may not be a downstream factor of the IL-2/STAT5 signaling pathway and therefore may not be regulated by this pathway. Previous studies have confirmed that Irf4 acts upstream of Blimp-1 and upregulates the expression of Blimp-1 in Tregs. Combined with the effect of the IL-2/STAT5 pathway on Blimp-1 in Tregs, we speculate that during the pathogenesis of AA, Irf4 may cooperate with IL-2/STAT5 to regulate the expression of Blimp-1 in Tregs and subsequently regulate the proliferation and differentiation of Tregs. The positive correlation between the expression of IRF4 in human Tregs and the expression of p-STAT5 in human $CD4^+$ T cells provides some support for this hypothesis.

In conclusion, activation of the IL-2/STAT5 signaling pathway is an important mechanism for promoting the expression of BLIMP-1 in Tregs in AA and subsequently promoting the proliferation and differentiation of these cells. More importantly, exogenous IL-2c can improve the poor activation of Stat5 in Tregs and the low expression of Blimp-1 in Tregs, increasing the frequency of Tregs in AA model mice. Therefore, exogenous IL-2c is expected to be an effective means for treating AA in children, and this study provides important ideas and experimental support for further research on AA. Moreover, the low IRF4 expression in Tregs is closely related to the low numbers of Tregs in AA, but IRF4 expression is not regulated by the IL-2/STAT5 pathway. Further study of the specific mechanism underlying this phenomenon will help to elucidate the pathogenesis of AA.

Funding

This research was funded by the Sanming Project of Medicine in Shenzhen (SZSM202011004), the National Natural Science Foundation of China (81570105, 82370164 and 82372312), and the Science, Technology and Innovation Commission of Shenzhen

Municipality (JCYJ20180307150419435, JCYJ20210324123004011, JCYJ20220530144604011, JCYJ20210324123003009 and JCYJ20220530144814032)Sanming Project of Medicine in Shenzhen (SZSM202011004),.

Ethics statement

The study of the human subject was conducted with the approval of Ethics Committee of Sun Yat-Sen University (approval number 2019SYSUSH-023), and informed consent was provided by the guardians of the included patients. The animal experiments have been approved by the Animal Care and Use Committee (IACUCA) of Bojin Biotechnology (approval number BG-AMS-20210223-LN01-Y).

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Lifen Huang: Writing – original draft, Investigation, Formal analysis, Data curation. **Junbin Huang:** Validation, Methodology, Formal analysis, Data curation. **Nannan Tang:** Resources, Investigation. **Hongman Xue:** Methodology, Conceptualization. **Shaofen Lin:** Resources, Methodology. **Su Liu:** Resources. **Qihui Chen:** Resources, Methodology. **Yinsi Lu:** Software, Methodology. **Qian Liang:** Methodology. **Yun Wang:** Methodology. **Qingqing Zhu:** Methodology. **Guoxing Zheng:** Methodology. **Yun Chen:** Writing – review & editing. **Chengming Zhu:** Supervision, Methodology, Conceptualization. **Chun Chen:** Supervision, Resources, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We wish to thank Dr. Chun Chen, Dr. Chengming Zhu, and Dr. Yun Chen for their assistance and suggestions related to this article. We also thank all the patients, families, and nursing and medical staff for their collaboration. Lifen Huang and Junbin Huang should be considered co-first authors.

List of abbreciations

AA	Aplastic anemia
Tregs	regulatory T cells
BLIMP-1	B lymphocyte-induced mature protein-1
IRF4	interferon regulatory factor 4
STAT5	signal transducer and activator of transcription 5
Teffs	effector T cells
cTreg	central regulatory T cells
eTreg	effector regulatory T cells
PRDM1	the positive regulatory domain 1
ICOS	inducible costimulatory molecule
p-STAT5	phosphorylation of STAT5
IL-2c	IL-2/JES6-1 complex

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e26731.

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