

# Preliminary investigation on the abnormal mechanism of CD4<sup>+</sup>FOXP3<sup>+</sup>CD25<sup>high</sup> regulatory T cells in pediatric B-cell acute lymphoblastic leukemia

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**Abstract.** The current study aimed to investigate the changes and regulatory mechanism of cluster of differentiation (CD)4<sup>+</sup>CD25<sup>high</sup> forkhead box protein 3 (Foxp3<sup>+</sup>) regulatory T cells (Tregs) in childhood B-cell acute lymphocytic leukemia (B-ALL). A total of 18 children with B-ALL and 15 age-matched healthy children were included. Reverse-transcription quantitative polymerase chain reaction was used to evaluate the mRNA levels of Foxp3, cytotoxic T-lymphocyte associated protein 4 (CTLA4), glucocorticoid-induced tumor necrosis factor receptor (GITR), lymphocyte activation gene 3 (LAG3), interleukin (IL)-2 receptor (R)β/γ, IL-6Rα/β, mothers against decapentaplegic homolog (Smad)3/4 and runt-related transcription factor (RUNX)1/3 in CD4-positive cells. The concentration of cytokines in plasma were measured using a cytometric bead array. Additionally, the proportion of CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> Tregs and levels of associated proteins was analyzed using flow cytometry. The results demonstrated that the proportion of CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> and expression of Foxp3 in children with B-ALL was significantly higher compared with healthy controls (P<0.05) and that transcription levels of CTLA4, GITR and LAG3 were also significantly elevated (P<0.05). Compared with healthy controls, the expression of IL-2Rα/β and its downstream molecule phosphorylated signal transducer and activator of transcription 5 (pSTAT5) in CD4-positive cells significantly increased (P<0.05); however, no significant difference of IL-2Rγ levels was identified between the two groups. Correlation analysis demonstrated a significant positive correlation between the expression of phosphorylated (p) signal transducer and activator of transcription factor (STAT)5 and CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> Tregs in children

with B-ALL ( $r=0.17$ ;  $P<0.05$ ). The plasma concentration of TGF-β, the expression of its receptor TGF-βRI/II and downstream molecules Smad3/4 were significantly upregulated in children with B-ALL ( $P<0.05$ ), whereas the expression of RUNX1/3 was lower compared with healthy controls ( $P<0.05$ ). Furthermore, the expression of Smad3 and RUNX1 was positively correlated with CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> Tregs in children with B-ALL ( $r=0.87$  and  $0.60$ , respectively;  $P<0.05$ ). Additionally, the expression of pSTAT3 in CD4-positive cells decreased significantly in pediatric patients with B-ALL when compared with healthy controls; however, plasma concentrations of IL-6 was significantly higher ( $P<0.05$ ). Furthermore, a negative correlation was identified between pSTAT3 and CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> Tregs in pediatric patients with B-ALL ( $r=-0.39$ ;  $P<0.05$ ). However, no significant differences in IL-6Rα/β expression were identified between the two groups. The results demonstrated that the excessive activation of IL-2/pSTAT5 and TGF-β/Smad signaling, and insufficiency of pSTAT3 may be correlated with increased CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> Tregs in pediatric B-ALL.

## Introduction

Acute lymphoblastic leukemia (ALL) is a heterogeneous disease, which is characterized by massive proliferation, extensive infiltration and inhibition of normal hematopoiesis (1). ALL is the most common cancer in children, accounting for 25% of cancer diagnosed among children <15 years of age (2). Lymphocyte phenotyping reveals that ALL has two subtypes: B cell (B-) and T cell (T-) ALL, with 85% of cases being B-ALL and 15% of T-ALL (3). Various therapeutic protocols have been applied in treatment and management of ALL, including chemotherapy, targeted therapy and bone marrow transplantation (4). Among children with ALL, ~95% of patients achieved complete remission following targeted therapy and 15-20% achieved an initial remission followed by a relapse (5). The etiology and pathogenesis of ALL is yet to be fully elucidated (1,3). Previous studies have demonstrated that the malignant proliferation of B-ALL cells was closely associated with a low level of anti-tumor immunity (1-3). However, the molecular mechanism of antitumor immune dysfunction remains unclear (1). It may

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be associated with the emergence and accumulation of immune regulatory cells, including regulatory T regulatory cells (Tregs) suppressing anti-cancer immunity (6-8).

CD4<sup>+</sup>CD25<sup>+</sup> Tregs have been discovered recently as a subpopulation of T cells, characterized by low reactive, immune suppression and expression of forkhead box P3 (FoxP3) (9,10). Tregs are produced in the thymus during T-cell maturation and are generated in the peripheral blood from naïve CD4<sup>+</sup> T cells (11). Previously studies revealed that numerous cancers induced the generation of Tregs from naïve T cells and promoted their proliferation, resulting in the accumulation of these cells in the tumor microenvironment and peripheral blood, leading to the suppression of tumor-specific T cells and regulation of antitumor responses (6-8).

To gain insight into this potential mechanism of B-ALL pathogenesis, the present study investigated the changes of Treg cells in pediatric patients and the possible mechanism of differentiation and regulation, with the objective to further elucidate the tumorigenesis of B-ALL.

## Materials and methods

**Patients.** A total of 18 newly diagnosed pediatric patients with B-ALL and admitted to Shenzhen Children's Hospital (Shenzhen, China) between July 2012 and February 2013 were enrolled in the current study. The cohort consisted of 13 males and 5 females, aged 2.3-11.5 years, with a mean age of 5.1 years. All patients with B-ALL were examined and diagnosis was confirmed using clinical examination, bone marrow cell morphology and immunophenotyping by flow cytometry (12). The diagnosis was confirmed in accordance with the Recommendation of Diagnosis of Pediatric Acute Lymphoblastic Leukemia (3rd Amendment Draft) (13). Inclusion criteria of patients with B-ALL: Age >12 months and <13 years; confirmed new diagnosis of B-ALL with ≥25% blasts in the bone marrow; no prior therapy. Exclusion criteria: Age ≥13 years at the time of consent; relapsed or refractory B-ALL; prior therapy; known HIV positive. Blood samples were collected prior to chemotherapy. A total of 15 outpatients (10 males, 5 females; age range, 3.2-9.6 years; mean age, 4.8 years) recruited between June 2012 and March 2013, received a physical examination in the Department of Pediatrics in Shenzhen Children's Hospital and served as the healthy control group. No significant differences in age or gender were identified between the two groups. Blood samples of patients with B-ALL and control group were analyzed immediately and without mitogen stimulation to avoid interference of the activation of immunocompetent cells. Informed consent was obtained from family members of all subjects and the study was approved by the Biomedical Ethics Committee of Shenzhen Children's Hospital.

**Isolation of peripheral blood cluster of differentiation (CD)4<sup>+</sup> T cells.** Anti-coagulant EDTA was used to collect 3 ml sterile venous blood from each patient in the current study. Peripheral blood mononuclear cells were isolated using density gradient centrifugation at 500 x g for 20 min at 4°C with fructose-diatrizoate (P=1.077; GE Healthcare Life Sciences, Little Chalfont, UK). Peripheral CD4<sup>+</sup> T cells were isolated using immunomagnetic beads according to manufacturer's instructions

(DynalBeads CD4 kit; cat. no. 111.45D; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cell activity was determined by light microscopic examination (BX41; Olympus Corporation, Tokyo, Japan), following staining with 4% trypan blue at room temperature for 5 sec. Cell purity was determined using flow cytometry and Diva V6.1.3 software (BD Biosciences, Franklin Lakes, NJ, USA) following staining with CD4-fluorescein isothiocyanate (FITC) antibody (10 µg/ml; cat. no. 11-0049-80; Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h at 4°C. Cells were then prepared completely and immediately used in further experiments.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from isolated peripheral CD4<sup>+</sup> T cells using the RNAqueous kit (cat. no. AM1912; Ambion; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RNA was quantified using a UV spectrophotometer. cDNA was synthesized by reverse transcription using a RevertAid H Minus First Strand cDNA Synthesis kit (cat. no. K1632; Thermo Fisher Scientific, Inc., Waltham, MA, USA) following the manufacturer's protocol. cDNA (1 µl) was used as a template and PCR amplification was conducted as follows: First cycle at 95°C for 15 min, followed by 35-50 cycles at 95°C for 15 sec, 54 to 62°C for 15 sec and 72°C for 25 sec. Primers were designed using the mRNA sequence of target genes from Genebank (<https://www.ncbi.nlm.nih.gov/genbank/>), as presented in Table I. All primers were synthesized by Shanghai Yingjun Biotechnology Co., Ltd. (Shanghai, China). The amplification products (each 10 µl) of forkhead box 3 (Foxp3), cytotoxic T lymphocyte-associated antigen 4 (CTLA4), glucocorticoid-induced tumor necrosis factor receptor (GITR), lymphocyte activation gene 3 (LAG3), interleukin (IL)-2 receptor (R)β/γ, IL-6Rα/β, mothers against decapentaplegic homolog (Smad)3/4, runt-related transcription factor (RUNX)1/3 and β-actin were loaded into 2% agarose gel. Electrophoresis was conducted at 90 v for 30 min. The gel was then recovered and purified for sequencing at Shanghai Yingjun Biotechnology Co., Ltd. The sequencing results were compared with the mRNA sequence of the target genes obtained from Genebank. All products of amplification, including Foxp3, CTLA4, GITR, LAG3, IL-2Rβ/γ, IL-6Rα/β, Smad3/4, RUNX1/3 and β-actin were identical to the mRNA sequences presented in Genebank. The cDNA synthesized in RT-qPCR was detected using a SYBR Green kit (cat. no. DRR820S; Takara Biotechnology Co., Ltd., Dalian, China) and an RT-PCR cycler (LightCycle 480II; Roche Applied Science, Penzberg, Germany). Results were analyzed using the 2<sup>-ΔΔCq</sup> method with LightCycler Software V1.5 (Roche Applied Science) (14). The results were expressed as the ratio of tested gene to β-actin. This procedure was performed following the manufacturer's protocol.

**Cytometric bead array.** A total of 2 ml peripheral blood was collected from patients with B-ALL and healthy control subjects, following 6 h of fasting. Heparin was added for anti-coagulation. Samples were centrifuged at 500 x g for 10 min at room temperature and plasma from the upper layer was separated. The plasma concentrations of IL-6 and transforming growth factor (TGF)-β were measured using a cytometric bead array (eBioscience; Thermo Fisher Scientific, Inc.). The procedure was performed

Table I. Primers utilized in reverse transcription-quantitative polymerase chain reaction.

Gene	Primer sequence	Renaturation temperature (°C)	Number of cycles	Amplification position	Length of products (bp)
Foxp3	Sense: 5'-GTGGCATCATCCGACAAG G-3' Antisense: 5'-TGTGGAGGAAGCTCTGGGAAT-3'	58	50	1,002-1,167	166
CTLA4	Sense: 5'-GTCCGGGTGACAGTGCTTCG-3' Antisense: 5'-CCAGGTAGTATGGCGGTGGG-3'	58	59	360-579	220
GITR	Sense: 5'-ACACGCACTTCACCTGGGTCG-3' Antisense: 5'-TGTGCCATGCTCGGGTTTCA-3'	58	50	18-146	129
LAG3	Sense: 5'-CCTCACTGTTCTGGGTCTGG-3' Antisense: 5'-GGATATGGCAGGTGTAGGTC -3'	56	48	1,117-1,355	239
IL-2R $\beta$	Sense: 5'-AAGCCCTTTGAGAAC CTTCG-3' Antisense: 5'-GATTTCCAGCTTATGTTGC-3'	56	42	504-593	90
IL-2R $\gamma$	Sense: 5'-ATTGGAAGCCGTGGTTATCT-3' Antisense: 5'-AAAGTTCCCGTGGTATTCAG-3'	56	42	869-1013	145
IL-6R $\alpha$	Sense: 5'-TAGTGTCTGGGAGCAAGTTCAG-3' Antisense: 5'-CGGCAGTGACTGTGATGTTGG-3'	58	40	1,025-1,116	92
IL-6R $\beta$	Sense: 5'-AGTCGTGCCTGTTTGCTTAG-3' Antisense: 5'-ATTGTGCCTTGGAGGAGTGT-3'	56	40	2,176-2,338	163
TGF- $\beta$ RI	Sense: 5'-AATGGGCTTAGTATTCTGGG-3' Antisense: 5'-ATATTGGCCTTAACCTCTG-3'	55	35	1,285-1,437	153
Smad3	Sense: 5'-GGGCTTTGAGGCTGTCTACC-3' Antisense: 5'-TGTCTCCTGTACTCCGCTCC-3'	56	45	1,372-1,458	87
Smad4	Sense: 5'-GGATACGTGGACCCTTCTGG-3' Antisense: 5'-CAATGGCTTCTGTCTGTGG-3'	55	45	1,592-1,667	76
RUNX1	Sense: 5'-CATCGCTTTCAAGGTGGTGG-3' Antisense: 5'-TGGCTGCGGTAGCATTTCTC-3'	58	50	1,836-1,948	113
RUNX3	Sense: 5'-CCAGGAAAGCACCTACAGAC-3' Antisense: 5'-AATGATCCCTCACCTCAA TG -3'	56	50	2,447-2,644	198
$\beta$ -actin	Sense: 5'-GAGCTACGAGCTGCCTGACG-3' Antisense: 5'-GTAGTTTCGTGGATGCCACAG-3'	56-61	50	787-906	120

Foxp3, forkhead box p3; CTLA4, cytotoxic T lymphocyte-associated antigen 4; GITR, glucocorticoid-induced tumor necrosis factor receptor; LAG3, lymphocyte activation gene 3; IL, interleukin; R, receptor; TGF- $\beta$ RI transforming growth factor  $\beta$  receptor I; RUNX, runt-related transcription factor.

according to the manufacturer's protocol, using FlowCytomix Pro v3.0 (eBioscience; Thermo Fisher Scientific, Inc.).

**Flow cytometry.** The percentage of CD4<sup>+</sup>CD25<sup>high</sup>FoxP3<sup>+</sup> T cells was detected using a whole blood counting method. According the instruction from the Foxp3 Staining Buffer set (cat. no. 00-5523-00; Invitrogen; Thermo Fisher Scientific, Inc.), cells were gated with CD4-FITC (10  $\mu$ g/ml; cat. no. 11-0049-80; Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min at 4°C, fixed and permeabilized with Foxp3/Transcription Factor Staining Buffer set (cat. no. 00-5523-00; Invitrogen; Thermo Fisher Scientific, Inc.) for 60 min at 4°C. Samples were blocked with normal mouse serum (cat. no. 24-5524-94; Invitrogen; Thermo Fisher Scientific, Inc.) for 15 min at 4°C and incubated with anti-CD25-phycoerythrin (PE) (1.25  $\mu$ g/ml; cat. no. 12-0259-41; Invitrogen; Thermo Fisher Scientific, Inc.) and anti-Foxp3-allophycocyanin (APC) (1.25  $\mu$ g/ml; cat.

no. 17-4777-42; Invitrogen; Thermo Fisher Scientific, Inc.) antibodies for 30 min at 4°C. Cells were also incubated with anti-phosphorylated (p)-signal transducer and activator of transcription factor (STAT)3-PerCP-Cy5.5 (as supplied; cat. no. 560114; BD Bioscience, Inc., San Jose, CA, USA) and anti-pSTAT5-Alexa Fluor647 (as supplied; cat. no. 612599; BD Bioscience, Inc.) for 30 min at 4°C, to detect the protein mean fluorescence intensity (MFI) of pSTAT3 and pSTAT5 in CD4<sup>+</sup>T cells. To detect the MFI of TGF- $\beta$ RII and IL-2R $\alpha$  on CD4<sup>+</sup>T, peripheral blood samples were stained and gated with CD4-eFlour450 (2.5  $\mu$ g/ml; cat. no. 48-0049-42; Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min at 4°C, fixed and permeabilized with intracellular fixation and permeabilization buffer set (cat. no. 88-8824-00; Invitrogen; Thermo Fisher Scientific, Inc.) for 20 min at room temperature and stained with anti-TGF- $\beta$ RII-FITC (as supplied; cat. no. FAB241F-100; R&D System, Inc., Minneapolis, MN, USA) and anti-IL-2R $\alpha$ -PE

Table II. Comparison of associated factors between patients with B-ALL and controls.

Variables	Group		T-value	P-value
	B-ALL (n=18)	Controls (n=15)		
CD25 <sup>high</sup> FOXP3 <sup>+</sup> /CD4 <sup>+</sup>	9.62±4.35%	4.87±2.61% <sup>a</sup>	3.71	P<0.001
IL-2Rα/CD4 <sup>+</sup>	120.89±37.93	79.62±20.22 <sup>a</sup>	9.79	P<0.05
pSTAT3/CD4 <sup>+</sup>	29.61±6.85	41.92±17.12 <sup>a</sup>	2.79	P<0.05
pSTAT5/CD4 <sup>+</sup>	45.83±14.17	34.01±9.04	2.90	P<0.05
TGF-βRII/CD4 <sup>+</sup>	50.78±18.87	31.39±9.02 <sup>a</sup>	3.65	P<0.05
RUNX1	2.38±1.44	3.07±1.17 <sup>a</sup>	2.87	P<0.05
Smad3	6.58±4.41	4.77±2.38 <sup>a</sup>	6.81	P<0.05
IL-6 (pg/ml)	27.32±8.12	16.39±5.78 <sup>a</sup>	4.51	P<0.05
TGF-β (ng/ml)	23.53±13.28	8.61±6.10 <sup>a</sup>	4.01	P<0.05

Data are presented as the mean ± standard deviation. <sup>a</sup>P<0.05 vs. healthy control group. B-ALL, B cell acute lymphocytic leukemia; CD, cluster of differentiation; Foxp3, forkhead box p3; IL, interleukin; pSTAT, phosphorylated signal transducer and activator of transcription; TGF, transforming growth factor; RUNX1, runt-related transcription factor 1; Smad, mothers against decapentaplegic homolog.

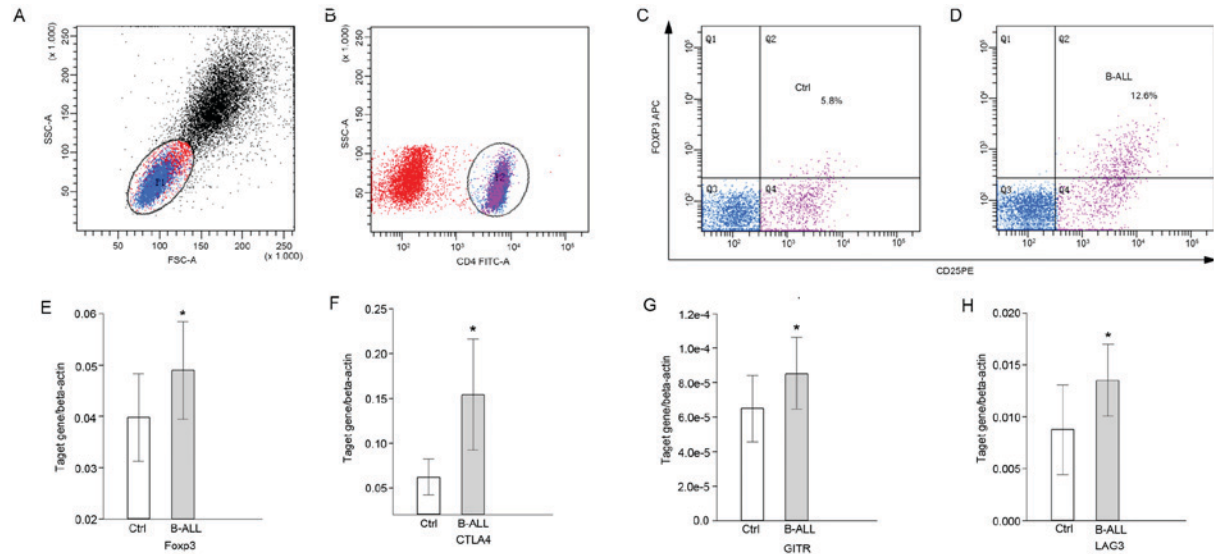


Figure 1. Proportions of Treg cells and expression of molecules associated with the suppressor function of Tregs in pediatric patients with B-ALL, using flow cytometry and reverse-transcription quantitative polymerase chain reaction. (A) Dot-plot representing the lymphocytes gated by FSC and SSC; (B) dot-plot representing CD4<sup>+</sup> T cells gated by CD4-FITC antibody. Dot-plots representing the CD4<sup>+</sup>CD25<sup>high</sup>FOXP3<sup>high</sup> Treg gated by (C) CD25-PE and (D) FOXP3-APC antibodies. Transcription levels of (E) Foxp3, (F) CTLA4, (G) GITR and (H) LAG3 relative to β-actin. \*P<0.05 vs. control. P1, lymphocytes gated by FSC and SSC; P2, CD4<sup>+</sup> T cells gated by CD4-FITC antibody; Q1-4, quadrants representing cells of single positive for the antibody representing the x-axis, double positive, double negative and single positive for the antibody representing the y-axis; Ctrl, control; B-ALL, B-cell acute lymphocytic leukemia; SSC-A, side scatter area; FSC-A, forward scatter area; CD, cluster of differentiation; FITC, fluorescein isothiocyanate; Foxp3, forkhead box protein 3; APC, allophycocyanin; Ctrl, control; PE, phycoerythrin; CTLA4, cytotoxic T-lymphocyte associated protein 4; GITR, glucocorticoid-induced tumor necrosis factor receptor; LAG3, lymphocyte activation gene 3.

(1.25 μg/ml; cat. no. 12-0259-41; Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min at 4°C. Cell counting was conducted using a CantoII flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Data were obtained and analyzed using Diva V6.1.3 software.

**Statistical analysis.** SPSS v19.0 statistical software (IBM Corp., Armonk, NY, USA) was used for all statistical analyses. Continuous variables were represented as the mean ± standard deviation. A two-tailed t-test was used for the comparison of continuous variables between two groups.

P<0.05 was considered to indicate a statistically significant result. Associations between the expression of pSTAT3 and CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> Treg in children with B-ALL were analyzed using a Pearson's correlation test.

**Results**

**Detection of CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> cells.** The percentage of Tregs and the expression of Treg associated molecules were detected using flow cytometry (Fig. 1A-D) and RT-qPCR (Fig. 1E-H). The percentage of CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> cells



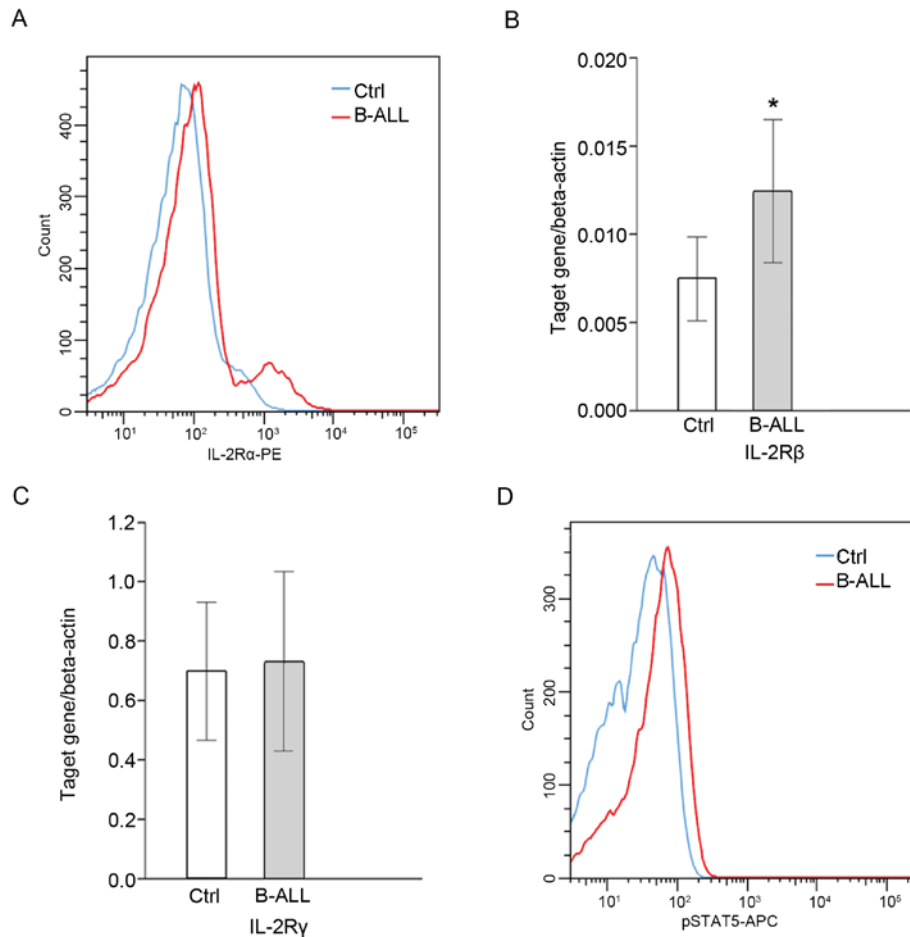


Figure 2. Expression levels of molecules associated with IL-2 signaling in pediatric patients with B-ALL determined using flow cytometry and reverse-transcription quantitative polymerase chain reaction. (A) Histogram representing the protein level of IL-2Rα on the surface of CD4<sup>+</sup> T cells. Transcription levels of (B) IL-2Rβ and (C) IL-2Rγ relative to β-actin. (D) Histogram representing the protein levels p-STAT5 in CD4<sup>+</sup> T cells. \*P<0.05 vs. control. Ctrl, control; B-ALL, B-cell acute lymphocytic leukemia; PE, phycoerythrin; IL, interleukin; R, receptor; p, phosphorylated; STAT, signal transducer and activator of transcription factor; APC, allophycocyanin.

(P<0.0001; Table II and Fig. 1A-D) and the expression of Foxp3 were significantly increased (P<0.05; Fig. 1E) in peripheral blood samples of pediatric patients with B-ALL compared with the healthy controls. The expression of inhibitory signaling molecules CTLA4, GITR and LAG3 was also significantly higher in pediatric patients with B-ALL compared with the control group (P<0.05; Fig. 1F-H).

**Detection of IL-2 signaling molecules.** The expression of IL-2Rα/β in CD4<sup>+</sup> T cells in patients with B-ALL was significantly upregulated when compared with healthy controls (P<0.05; Fig. 2A and B); however, no significant difference in IL-2Rγ was identified (Fig. 2C). Further investigation into the activation of downstream molecules associated with the IL-2 signal transduction pathway and the effect of IL-2 signaling on Treg cell differentiation in B-ALL patients revealed that the expression of pSTAT5 was significantly higher in patients with B-ALL compared with healthy controls (P<0.05; Table III; Fig. 2D). Furthermore, pSTAT5 expression was positively correlated with the percentage of CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> cells (r=0.17; P<0.05; Table III).

**Detection of IL-6/TGF-β signaling molecules.** The peripheral concentration of TGF-β and the expression of

Table III. Correlation between the expression of differentiation associated factors and CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> T regulatory cells in pediatric patients with B-cell acute lymphocytic leukemia.

Variables	CD4 <sup>+</sup> CD25 <sup>high</sup> Foxp3 <sup>+</sup>	
	R-value	P-value
pSTAT3/CD4 <sup>+</sup>	-0.39	6.73x10 <sup>-3</sup>
pSTAT5/CD4 <sup>+</sup>	0.17	9.62x10 <sup>-4</sup>
RUNX1	0.60	7.24x10 <sup>-3</sup>
Smad3	0.87	9.59x10 <sup>-5</sup>

CD, cluster of differentiation; Foxp3, forkhead box protein 3; pSTAT, phosphorylated signal transducer and activator of transcription; TGF, tumor growth factor; RUNX1, runt-related transcription factor 1; Smad, mothers against decapentaplegic homolog.

TGF-βRI/II in CD4<sup>+</sup> T cells were significantly upregulated in patients with B-ALL compared with healthy controls (P<0.05; Fig. 3A and B). The expression of the downstream signaling molecules Smad3/4 was also significantly increased

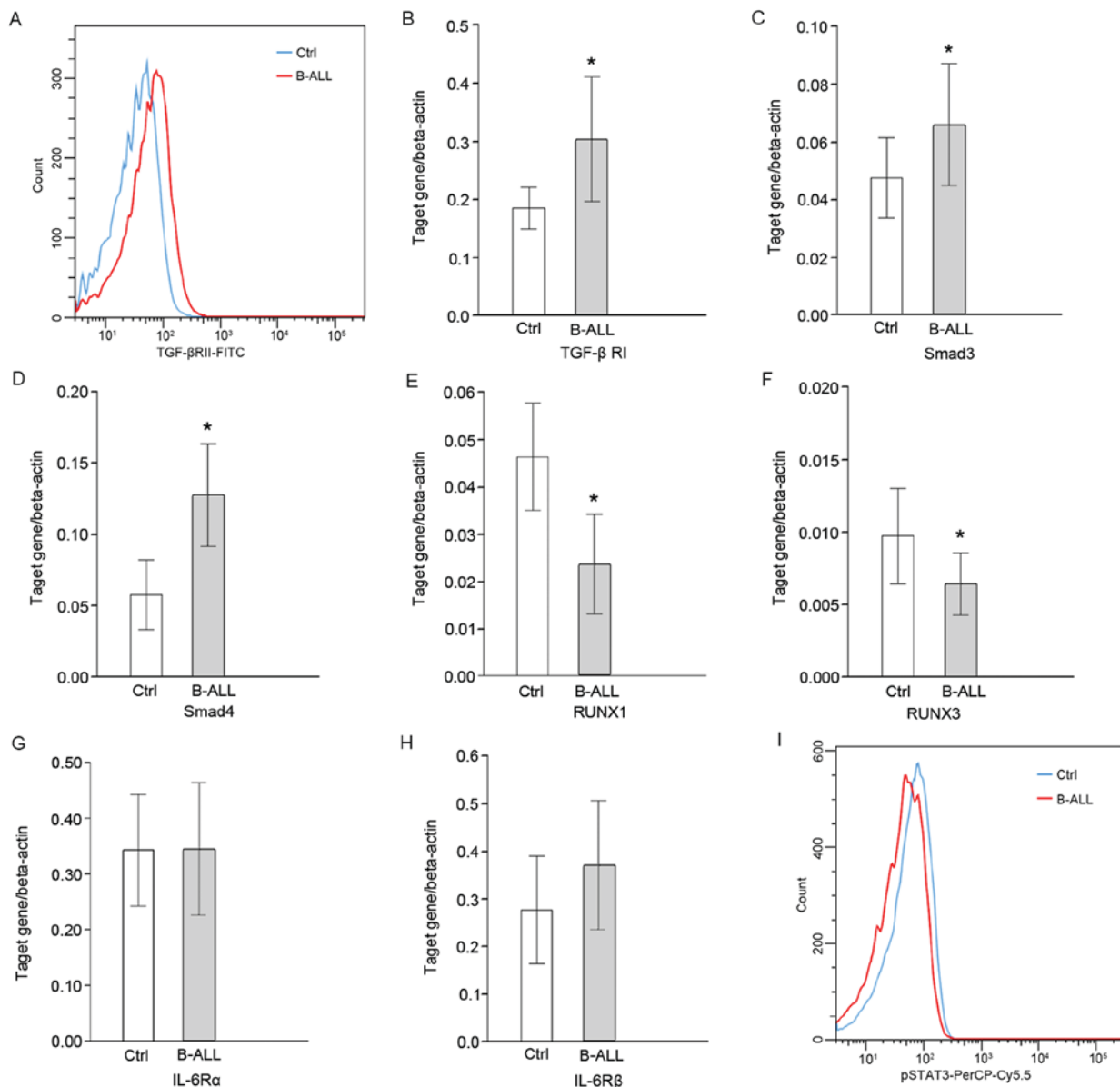


Figure 3. Protein and mRNA levels of molecules associated with IL-6/TGF- $\beta$  signaling in patients with B-ALL determined using flow cytometry and reverse-transcription quantitative polymerase chain reaction. (A) Histogram representing the protein levels of TGF- $\beta$ RII on the surface of CD4<sup>+</sup> T cells. Transcription levels of (B) TGF- $\beta$ RI, (C) Smad3, (D) Smad4, (E) RUNX1, (F) RUNX3, (G) IL-6R $\alpha$  and (H) IL-6R $\beta$  relative to  $\beta$ -actin. (I) Histogram representing the protein level of p-STAT3 on CD4<sup>+</sup> T cells. \* $P$ <0.05 vs. control. Ctrl, control; B-ALL, B-cell acute lymphocytic leukemia; TGF- $\beta$ R, transforming growth factor- $\beta$  receptor; RUNX, runt-related transcription factor; IL, interleukin; R, receptor; p, phosphorylated; STAT, signal transducer and activator of transcription factor; FITC, fluorescein isothiocyanate; PerCP-Cy5.5, peridinin chlorophyll protein complex with cyanine-5.5.

( $P$ <0.05; Fig. 3C and D, respectively). However, the expression of RUNX1/3 was significantly lower than the control group ( $P$ <0.05; Fig. 3E and F, respectively). Expression levels of Smad3 and RUNX1 were positively correlated with CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> cell percentage ( $r$ =0.87 and 0.60, respectively;  $P$ <0.05; Table III). The changes of IL-6 signaling were further analyzed using flow cytometry and RT-qPCR. The concentration of IL-6 in the peripheral blood of patients with B-ALL was significantly higher than the control group, but no significant difference was observed in the expression of IL-6R $\alpha$ / $\beta$  in CD4<sup>+</sup>T cells (Fig. 3G and H, respectively). In addition, the expression of downstream pSTAT3 was significantly decreased ( $P$ <0.05; Fig. 3I) and the expression of pSTAT3 was negatively correlated with CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> cell percentage ( $r$ =−0.39,  $P$ <0.05; Table III).

## Discussion

ALL is a heterogeneous disease, primarily caused by primitive and immature lymphocytic malignant clones, which exhibit increased cell proliferation, extensive infiltration and the inhibition of normal hematopoiesis (1). However, the etiology and immune pathogenesis of ALL remains unclear (1-3). A healthy immune system effectively identifies and removes abnormal cells to maintain tumor immune tolerance. Through cell contact or cytokine secretion, Tregs inhibit the development and activation of anti-tumor effects, which may lead to tumor immune escape (10,11). The transcription factor Foxp3 is primarily expressed in Tregs, serving key roles in differentiation, maturation and cell function maintenance; thus, Foxp3 is considered to be a specific marker of Tregs (7). Previous

studies have demonstrated that abnormal Treg cell number and percentage occurs in certain tumors, including non-small cell lung cancer and ovarian cancer (15-18), which indicates that the immunosuppressive effect of Tregs is closely associated with tumorigenesis. However, the function and status of Tregs in patients with ALL is yet to be fully elucidated (19,20). This may be due to the disease exhibiting various subtypes and durations, as well as the different methods used for detection. In the present study, under conditions that represented the *in vivo* active status of immunocompetent cells without mitogen stimulation, it was determined that CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> cell percentage and Foxp3 expression were higher in patients with B-ALL compared with healthy controls. In addition, the expression of inhibitory molecules, including CTLA4, GITR and LAG3 were elevated, suggesting that overactivation of Tregs may be a factor contributing to tumor immune escape in B-ALL.

The induction of Treg cell differentiation still remains unclear. Previous studies have demonstrated that IL-2, IL-6 and TGF- $\beta$  signaling serve important roles in Treg differentiation, proliferation and function (21-26). However, interaction of IL-2 with IL-2R, may trigger various signal conduction pathways of IL-2; the fast-conducting janus tyrosine kinase (JAK)/STAT pathway remaining the most predominant (27). IL-2 and STAT5 signals may ensure the consistent expression of Foxp3 in induced Tregs and may further its suppressive function (28). The IL-2R signaling conduction pathway modulates Treg function by activating STAT5 to upregulate the expression of Foxp3 (29). IL-2 facilitates Treg cell development and maintenance in peripheral blood, and its proliferation (29). The knockout of IL-2 signaling may lead to significant Treg cell deficiency, which may result in autoimmune disease (22,23). The present study determined that the expression of IL-2R $\alpha/\beta$  on the surface of CD4<sup>+</sup>T cells and the downstream signaling molecule pSTAT5 were upregulated when compared with controls. Furthermore, pSTAT5 expression was positively correlated with CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> percentage, indicating that the overactivation of Treg cells in patients with B-ALL may be associated with an abnormal IL-2 signal.

Naïve CD4<sup>+</sup> T cells can be induced by cytokines into different subpopulations of T helper cells, which mutually transform to each other with various cytokine concentrations (6). The TGF- $\beta$  cytokine inhibits cellular mitosis, proliferation and migration (30,31). In early stage tumors, TGF- $\beta$  exerts an inhibitory function; however various changes occur within certain components of the TGF- $\beta$  signaling pathway, leading to the loss of TGF- $\beta$  inhibitory function, resulting in uncontrollable cell proliferation and tumor progression (32,33). The transcription factor RUNX is one of the primary targets of TGF- $\beta$ , including RUNX 1, 2 and 3. RUNX1 and RUNX3 have important implications to T lymphocyte differentiation; any functional changes that occur within RUNX impacts the transduction of the TGF- $\beta$  signaling pathway. A previous study has demonstrated that RUNX and Foxp3 form a feedback loop, such that RUNX proteins facilitate Foxp3 expression and are associated with the co-modulation of downstream target gene expression with Foxp3 proteins (34). TGF- $\beta$  binds to the cell surface receptor TGF- $\beta$ RI/II, and triggers Smad and RUNX signaling (24-26). The former induces the demethylation of

the Foxp3 promoter and initiates its expression (24,25); the latter interacts with the Smad signal, upregulates the Foxp3 expression and facilitates the differentiation of primary CD4<sup>+</sup> T cells into Tregs (25,26). IL-6 suppresses the TGF- $\beta$  induced expression of Foxp3 by methylating the Foxp3 upstream enhancer through STAT3 signaling (21). In the present study, peripheral concentrations of TGF- $\beta$ , TGF- $\beta$ I/II and Smad3/4 in CD4<sup>+</sup> T cells were upregulated in patients with B-ALL when compared with healthy controls, whereas the expression of RUNX1/3 was decreased. Correlation analysis also revealed that Smad3 and RUNX1 expression were positively correlated with CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> cells. In addition, it was determined that although the concentration of IL-6 in the peripheral blood of patients with B-ALL was higher than that of the control group, the expression of downstream pSTAT3 was decreased and negatively correlated with CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> cell percentage. These results suggested that TGF- $\beta$ /Smad signal overactivation and the lack of pSTAT3 expression may lead to an abnormal increase of Tregs in pediatric patients with B-ALL. However, other factors may be associated with the regulation of TGF- $\beta$ /RUNX and IL-6/pSTAT3 signaling. The interaction of IL-6 with IL-6R activates various signal transduction pathways, including JAK/STAT (35). The activation of IL-6 enables the phosphorylation of JAK to activate STAT3 transcription factors (36). Phosphorylated STAT3 then forms dimers that are transduced into nucleus, further activating or modulating the transcription capacity of genome (36). Thus, IL-6 leads to the methylation of Foxp3 gene enhancers through STAT3 signaling (21). The present study revealed that although pediatric patients with acute B-ALL exhibit higher IL-6 concentrations than the healthy control group, the expression of their downstream signaling factor, pSTAT3, is significantly lower. Considering that variations in IL-6, its receptors and the signaling factors are inconsistent, it is speculated that the reason for insufficiency of pSTAT3 and abnormal proliferation of Treg cell in patients with acute B-ALL may involve other factors, which may further participate in modulating IL-6/pSTAT3 signal. Therefore, further study into the mechanism of modulation is required.

In conclusion, the overactivation of Tregs in patients with B-ALL may be associated with the overactivation or insufficient expression of a variety of regulatory cytokine signaling molecules. The overactivation of IL-2/pSTAT5 and TGF- $\beta$ /Smad, and the insufficient expression of pSTAT3 served an important role in the regulation of Tregs in pediatric patients with B-ALL. However, further investigation into the molecular mechanism of aforementioned abnormal signaling, as well as the factors participating in the regulation of IL-6 and TGF- $\beta$  signaling is required.

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

FW and CL contributed to the conception of the study. HX, GW and XC contributed significantly to the analysis and manuscript preparation; HM, XY and GL contributed to the clinical diagnosis and data management, and obtained the informed consents. SL performed the data analyses and wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

### Ethics approval and consent to participate

Informed consent was collected from family members of all subjects and the study was approved by the Biomedical Ethics Committee of Shenzhen Children's Hospital (Shenzhen, China).

### Patient consent for publication

Informed consent was collected from family members of all subjects.

### Competing interests

The authors declare that they have no competing interests.

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