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# High Level of CD4+CD25+CD127- Treg Cells in Donor Graft is Associated with a Low Risk of aGVHD after allo-HSCT for Children with Hematologic Malignancies

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**Abstract**

Acute Graft-Versus-Host Disease (aGVHD) is the major problem for patient undergoing allogeneic Hematopoietic Stem Cell Transplantation (allo-HSCT). Previous study showed the significant role of CD4+CD25+ Treg cells in inhibiting aGVHD. This retrospective study of 50 children with hematological malignancies undergoing allo-HSCT investigated the influence of donor CD4+CD25+CD127- Treg cells on aGVHD. The proportion of Treg cells in graft is significantly higher in patient with grade 0-I aGVHD than in patients with grade II-IV aGVHD (3.08 ± 0.72% vs. 2.52 ± 0.86%, P=0.016). There was no significant difference on Treg cells proportion in graft between relapsed and non relapsed patients (3.20 ± 0.80% vs. 2.80 ± 0.81% P=0.549). CD4+CD25+CD127- Treg cells in donor graft can reduce the incidence of aGVHD after children received allo-HSCT without increasing the risk of relapse. Graft CD4+CD25+CD127- Treg cells level is a valuable biomarker to predict aGVHD.

**Keywords:** Treg cells; Children; Allogeneic hematopoietic stem cell transplantation; Acute graft versus host disease; Leukemia relapse

## Introduction

Allogeneic Hematopoietic Stem Cell Transplantation (allo-HSCT) is a curative therapy for malignant hematological disorders. Acute Graft-Versus-Host Disease (aGVHD) and relapse are major problems for patients undergoing allo-HSCT. Due to the lack of reliable laboratory measurement for clinical observation and unsatisfactory treatment effect of severe aGVHD, searching for new biomarkers to predict aGVHD is one of the research focuses in the field of HSCT.

As a subpopulation of T lymphocytes, Treg cell has gained increasing concerns in recent years. It plays a pivotal role in maintaining self-tolerance and controlling adaptive immune responses [1]. They can suppress aGVHD yet retain the Graft Versus Leukemia (GVL) effect [2]. There were clinical trials that proved the importance of Tregs after HSCT [3-5] while some studies provided negative results of correlation between reduced Treg frequency and GVHD severity [6,7].

Several markers were used to identify Treg cells. CD4 and CD25 are most commonly used, however, they are difficult to discriminate between Treg and activated T effector cells [8]. Foxp3 is the most definitive marker for identification of Treg whereas identification of this marker requires permeabilization which can totally kill the cells [9]. CD127 is an excellent biomarker of human Treg cells, especially when combined with CD25. The combination of CD25 and CD127 identifies Treg cells that account for up to 7-8% of CD4+ T cells, a significantly greater percentage than identified by previous approaches. Moreover, these cells suppress the proliferative response of alloreactive T cells in mixed lymphocyte response and are themselves anergic to the same stimuli [10]. Cell surface marker CD127 could enrich human Treg Cells selectively for in vitro functional studies and has the potential in vivo therapy [11]. Therefore, the CD4+CD25+CD127- population has recently been suggested in preclinical studies to be most suitable for human adoptive transfer studies and represent accurately the level of human Treg cells [11-14].

Since rare use of CD4+CD25+CD127- markers for Tregs detection and the controversy on roles of Treg cells in clinical observations, we conducted this retrospective study to observe the role of CD4+CD25+CD127- Treg cells in aGVHD after allo-HSCT for children with malignant hematological disorders.

## Material and Methods

### Patients and transplantation characteristics

Fifty consecutive children with malignant hematological diseases who underwent allo-HSCT at a single institution between July 2012 and August 2013 and achieved engraftment were enrolled in the study. The treatment protocol was approved by the local Ethics Committee. Informed consent was obtained from all guardians. The median age of patients was 8 years (range from 1 to 14 years), with 29 males and 21 females. Primary diseases included Acute Myeloid Leukemia (AML) (n=19), Acute Lymphoblastic Leukemia (ALL) (n=18), Chronic Myeloid Leukemia (CML) (n=3), Myelodysplastic Syndrome (MDS) (n=3), Juvenile Myelomonocytic Leukemia (JMML) (n=5), Non-Hodgkin's Lymphoma (NHL) (n=1) and Langerhans cell histiocytosis (LCH) (n=1). 6 patients received bone marrow transplantation, 42 patients received G-CSF-mobilized peripheral blood stem cell transplantation and 2 patients received cord blood transplantation. 12 patients received graft from Matched Sibling Donors (MSD), 8 from Mismatched Related Donors (MMRD) and 30 from Matched Unrelated Donor (MUD). Myeloablative conditioning regimen was adopted for all patients. The median follow-up time of all live patients was 288 days, ranging from 89 to 496 days.

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## GVHD prophylaxis

The diagnosis and grading of aGVHD was defined according to the published criteria [15]. Cyclosporine (with serum valley drug levels 150-200 μg/l) and methotrexate (15 mg/m$^2$ on day +1, 10 mg/m2 on day +3 and day +6) were used for GVHD prophylaxis. Mycophenolate mofetil was added in 8 patients who underwent MMRD-HSCT. Cyclosporine was slowly tapered beginning day+60 and discontinued between day+120 and day+427 according to the clinical manifestation.

## Graft cells population Detection

Fifty samples (2ml each) were extracted from donor graft (bone marrow, peripheral blood or cord blood) before transplantation. Red blood cells were lysed at room temperature for 10min by ACK solution (150 mM NH4CL, 1 mM KHCO3, 0.1 mM EDTA, reagents from Sigma), followed by cell counting and washing. Antibody staining was performed at 4°C for 30 min in dark: CD34-PE and CD45-V500 for stem/progenitor cells detection, CD3-perCP and CD45-V500 for T lymphocytes, CD19-PE and CD45-V500 for B lymphocytes, CD56-PE and CD45-V500 for natural killer cells. CD25-FITC, CD127-PE, CD4-APC, CD3-perCP and CD45-V500 markers were used for Treg cells detection (Figure 1). Stained cells were analyzed by flow cytometer (Canton II, BD). CD127-PE was from Beckman and all the rest from BD Pharmingen.

## Statistical analysis

Descriptive statistical analysis was performed to evaluate the variables related to patients and transplantation characteristics. The counting variables of two groups were compared by the Chi-square test (Fisher's exact test when demanded). Nonparametric test (Mann-Whitney test) was applied to compare the abnormally distributed measurement variables from the two groups. The impact of Treg cells and other related factors on aGVHD was evaluated using binary logistic regression. Statistical software, SPSS 14.0 (SPSS Inc., Chicago, IL) was used and all the tests were set at the 5% significance level.

# Results

## Patient characteristics

According to the occurrence and severity of aGVHD, patients were divided into 2 groups: 31 with grade 0-I aGVHD and 19 with grade II-IV aGVHD. No significant differences were found between the two groups on primary diseases, graft type and sources, conditioning regimen and GVHD prophylaxis (Table 1).

## Influence factors of aGVHD

In logistic regression analysis, the proportion of Treg cells in graft was found significantly decrease the risk of aGVHD (RR=0.273, 95%CI

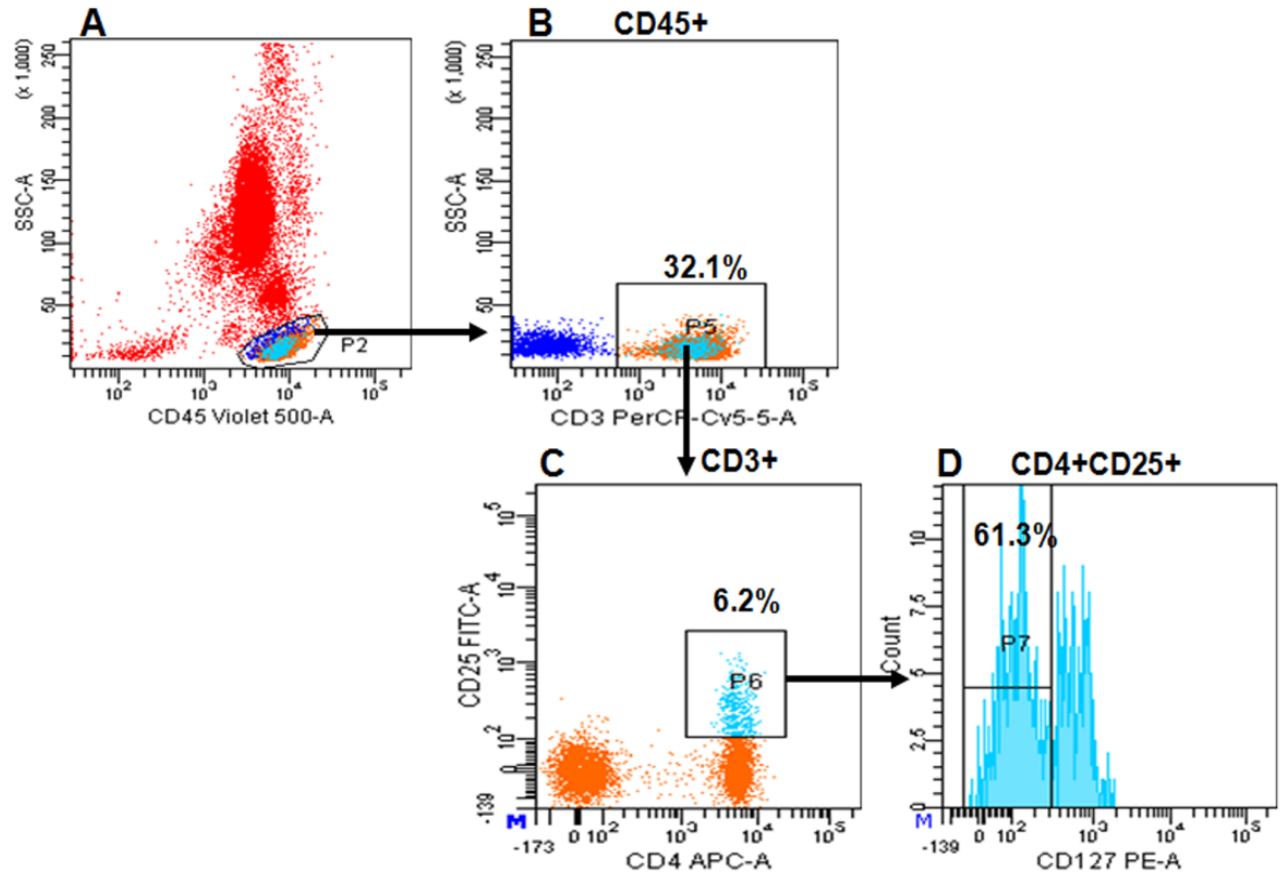


**Figure 1:** Phenotypic characterization of Treg cells: Treg cells in donor graft were detected by flow cytometry in a five-color panel, surface labeling of CD45, CD3, CD4, CD25 and CD127 (A) Gate shows CD45+ Mononuclear cells (B) Gate shows CD45+CD3+ T lymphocytes (C) Bivariate dot plot illustrates the CD4+CD25+ phenotype pattern of Treg cells (D) Bivariate dot plot illustrates the CD4+CD25+CD127- phenotype pattern of Treg cells.

0.095-0.787, p=0.016). Stem cell transplantation from alternative donors was the risk factor of aGVHD occurrence (RR=10.574, 95%CI 1.163-96.131, p=0.036) (Table 2).

### Impact of graft cells subpopulation on aGVHD and hematological malignancies relapse

The proportion of CD4+CD25+CD127- Treg cells was significantly higher in the grade 0-I aGVHD group than in grade II-IV aGVHD group (3.08 ± 0.72% vs. 2.52 ± 0.86%, P=0.016). There was no statistical difference on the proportions of CD34+ stem/progenitor cells, CD3+ T lymphocytes, CD19+ B lymphocytes and CD56+ natural killer cells between the two patient groups (Figure 2).

Eight patients suffered from relapse after allo-HSCT. No statistical difference on proportion of CD4+CD25+CD127- Treg cells was found between the patients with diseases relapse and those without relapse (3.20 ± 0.80% vs. 2.80 ± 0.81% P=0.549).

| | Patients with grade 0-I aGVHD (n=31) | Patients with grade II-IV aGVHD (n=19) | P value |
|---|---|---|---|
| Male/Female, n/n | 19/12 | 10/9 | 0.547 |
| Age, year (mean ± std) | 8.2 ± 4.0 | 8.5 ± 4.0 | 0.841 |
| Primary diseases, n (%) | | | 0.139 |
| ALL | 9 (29.0) | 9 (47.4) | |
| AML | 14 (45.2) | 5 (26.3) | |
| CML | 2 (6.4) | 1 (5.3) | |
| MDS | 0 (0.0) | 3 (15.8) | |
| JMML | 4 (12.9) | 1( 5.3) | |
| NHL | 1 (3.2) | 0 | |
| LCH | 1 (3.2) | 0 | |
| HSC donor, n (%) | | | 0.404 |
| MSD | 10 (41.9) | 2 (10.5) | |
| MMRD | 4 (12.9) | 4 (21.1) | |
| MUD | 17 (54.8) | 13 (68.4) | |
| Stem cell source, n (%) | | | 0.836 |
| BM | 3 (9.7) | 3 (10.5) | |
| PBSC | 27 (87.1) | 15 (84.2) | |
| CB | 1 (3.2) | 1 (5.3) | |
| GVHD prophylaxis, n (%) n(%)n(%) prophylaxis,n(%) | | | 0.693 |
| CsA+MTX | 27 (87.1) | 15 (78.9) | |
| CsA+MTX+MMF | 4 (12.9) | 4 (21.1) | |

aGVHD: acute Graft Versus Host Disease; ALL: Acute Lymphoblastic Leukemia; AML: Acute Myeloid Leukemia; CML: Chronic Myeloid Leukemia; MDS: Myelodysplastic Syndrome; JMML: Juvenile Myelomonocytic Leukemia; NHL: Non-Hodgkin's Lymphoma; LCH: Langerhans Cell Histiocytosis; HSC: Hematopoietic Stem Cell; MSD: Matched Sibling Donor; MMRD: Mismatched Related Donor; MUD: Matched Unrelated Donor; BM: Bone Marrow; PBSC: Peripheral Blood Stem Cells; CB: Cord Blood; CsA: Cyclosporine A; MTX: Methotrexate; MMF: Mycophenolate Mofetil.

**Table 1:** Characteristics of patients and transplantation.

| Factors | aGVHD | |
|---|---|---|
| | RR (95.0%CI) | P value |
| Age | 1.118 (0.906-1.380) | 0.296 |
| Stem cell donor (MSD/AD) | 10.574 (1.163-96.131) | 0.036 |
| GVHD prophylaxis (CsA+MTX/ CsA+MTX+MMF) | 2.575 (0.285-23.229) | 0.399 |
| Proportion of CD3 cells in graft | 1.042 (0.967-1.124) | 0.276 |
| Proportion of Treg cells in graft | 0.273 (0.095-0.787) | 0.016 |

aGVHD: acute Graft Versus Host Disease; MSD: Matched Sibling Donor; AD: Alternative Donor (mismatched related donor and matched unrelated donor included); CsA: Cyclosporine A; MTX: Methotrexate; MMF: Mycophenolate Mofetil.

**Table 2:** Influencing factors of aGVHD: logistic regression.

## Discussion

Acute GVHD is an important reason for the failure of allo-HSCT. Severe, refractory aGVHD has plagued extensive application of allo-HSCT. HLA disparity is the main reason inducing GVHD while graft components may also be an important factor to affect the occurrence of aGVHD. In 1995, Sakaguchi first reported the CD4+CD25+ Treg cells with immune suppressive function which can control GVHD [16]. Liu also reported the expression of donor CD4+CD25+ Treg cells in the group of patients with aGVHD at lower levels than the non aGVHD group [17]. Taylor found that removing the CD4+CD25+Treg cells from donor grafts can make the patients with severe aGVHD while infusion of separated purified donor Treg cells can significantly reduce GVHD [18]. Therefore, in 2007 Minnesota University began the first clinical trial by using cord blood Treg cells to control GVHD. Brunstein reported preliminary results of umbilical cord blood Treg cells against GVHD [19]. Till now, researches were mostly based on adults or focus on CD4+CD25+ Treg cells. There were very few reports of children with allo-HSCT or reports of CD4+CD25+CD127- Treg cells in clinical. In our present study on 50 children with hematological malignancies, graft CD4+CD25+CD127- Treg cells expression was found significantly lower in children with grade II-IV aGVHD than in those with grade 0-I aGVHD, which proved that donor CD4+CD25+CD127- Treg cells can reduce aGVHD for children with allo-HSCT. Shin et al found that rapamycin, interleukin-2 can reduce GVHD as they promote Treg cells proliferation [20]. Lim proved that the combined application of mesenchymal cells and Treg cells have anti-GVHD effect [21]. Veerapathran mechanically verified the anti-GVHD function of Treg cells, which achieve the effect by inhibiting immunogenicity of minor histocompatibility antigen [22].

In addition to the Treg cells, other cell components of donor graft have also been reported to be relevant to aGVHD. High proportion of T lymphocytes was reported to be a predictive factor of severe aGVHD [23]. CD34+ cells and CD19+CD5+ B cells may increase the incidence of aGVHD and cGVHD [24,25]. CD3-CD16+CD56+ NK cell has been generally considered to reduce the aGVHD effect [26,27], whereas there were reports with different results [28]. The present study did not find any influence of graft CD3, CD34, CD19 and CD56 cells on aGVHD; maybe sample size is not large enough to provide objective assessment.

Clinical, GVHD and GVL cannot be separated, it has been a great concern that whether Treg cells could reduce GVL effects as well as reduce GVHD. Considerable researches clarified that Treg cells can alleviate GVHD without abating GVL effect [29,30]. In the present research, no significant difference on the proportion of Treg cells was found between the 8 patients with their primary hematologic malignancies relapse and another 42 patients without relapse. However, a short follow-up period and limited number of cases disenable us to draw an objective and reliable conclusion on the effect of Treg cells on GVL.

In conclusion, CD4+CD25+CD127- Treg cells in donor graft can reduce the incidence of aGVHD after allo-HSCT for children with hematological malignancies. It can be a predictive biomarker for monitoring aGVHD clinically and provide important information for GVHD prophylaxis.

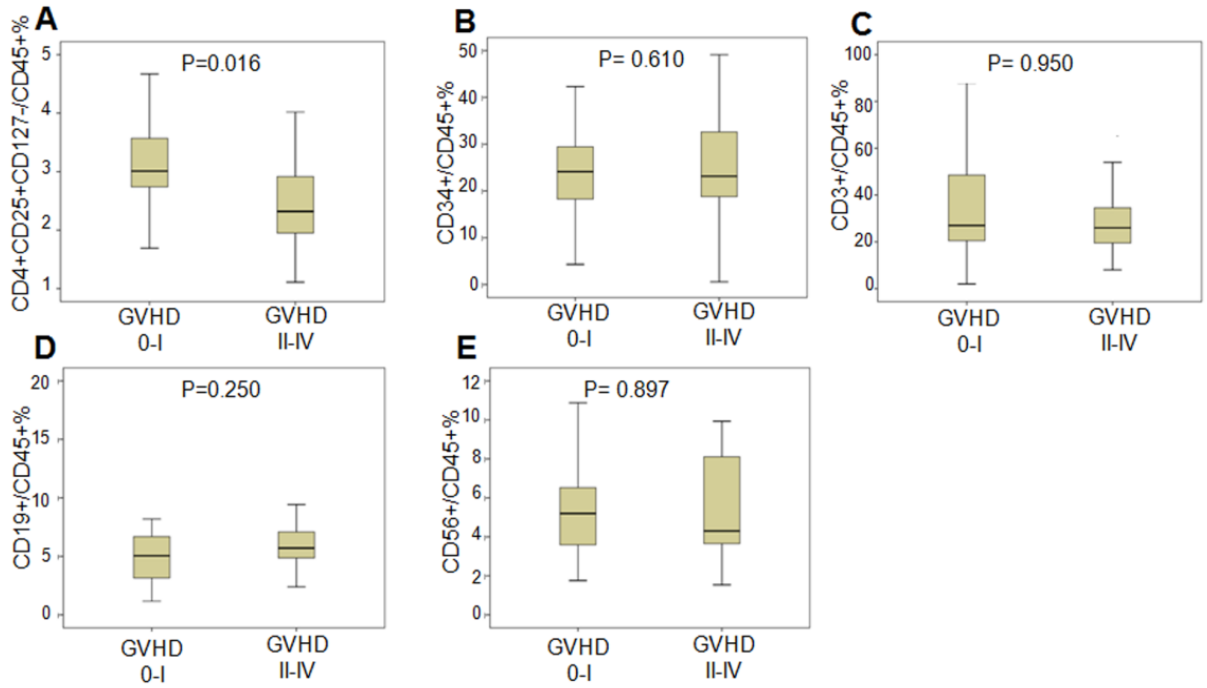


**Figure 2:** The relationship between donor graft cell components and aGVHD: The proportion of CD4+CD25+CD127+ Treg cells in donor graft was significantly higher in patients with grade 0-I aGVHD than in patients with grade II-IV aGVHD (p=0.016, A). No statistical difference was found between the two group of patients on the proportion of CD34+ stem/progenitor cells (p=0.610, B), CD3+ T lymphocytes (p=0.950, C), CD19+ B lymphocytes (p=0.250, D) and CD56+ natural killer cells (p=0.897, E).

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