

T Regulatory Cells in Donor Grafts May Predict the Severity of Acute Graft Versus Host Disease After Matched Sibling Donor Allogeneic Peripheral Blood Stem Cell Transplantation

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Abstract Acute graft-versus-host disease (aGVHD) and relapse are major issues for patients undergoing allogeneic hematopoietic stem cell transplant (allo-HSCT). T-regulatory (Treg) cells in the donor graft are negatively correlated with the incidence of aGVHD without any impact on relapse. In this study to determine the association of Treg cells with aGVHD in allo-HSCT patients. Thirty-two patients with hematological disorders, who underwent allo-HSCT. Twenty-nine patients who achieved engraftment were enrolled in the study. Treg cells were quantified in donor graft by flowcytometry and were assessed for their association with aGVHD and other clinical outcomes. Fifteen of 29 patients developed aGVHD. According to the occurrence and severity of aGVHD, patients were divided into two groups: 20 (68.9%) patients with grade 0–I aGVHD and 9 (31.1%) patients with grade II–IV aGVHD. Treg cells/CD4 ratio was significantly higher in the grade 0–I aGVHD group than in grade II–IV aGVHD group, ($p = 0.0002$). We could not find the association of CD34 dose ($p = 0.55$) or CD3 dose ($p = 0.57$) with the severity of aGVHD. Higher Treg cells/CD4 ratio in donor graft was associated with less severe aGVHD. Though more studies

are needed, Treg cells/CD4 ratio may be used as a predictive marker for severity of aGVHD in post allo-HSCT.

Keywords T-regulatory cells · Acute GVHD · Allo-HSCT

Introduction

Allogeneic Hematopoietic Stem Cell Transplantation (allo-HSCT) is a curative therapy for many malignant as well as non-malignant hematological disorders. However; acute graft versus host disease (aGVHD) and relapse are major complications for patients undergoing allo-HSCT [1]. HLA disparity is one of the main reasons for GVHD. Other factors such as age gender mismatch, cytomegalovirus (CMV) infection, conditioning regimen and donor immune cells component may also be important factors affecting the occurrence of aGVHD [2]. A subpopulation of T lymphocytes, the regulatory T cells (Treg cells) have an important role in the maintenance of peripheral tolerance [3]. Treg cells can suppress aGVHD without any impact on the Graft Versus Leukemia (GVL) effect [4]. These cells are characterized by the expression of CD4, CD25 (IL-2receptor α -chain), FoxP3, CD45RB and low expression of CD127 [5].

It was also shown that CD4+ CD25+ and low expression of CD127 can be used as a specific marker to identify Treg cell [6]. Sakaguchi et al. [7] demonstrated the role of CD4+ CD25+ T cells in immune suppression and prevention of autoimmunity. Taylor et al. [8] found that CD4+ CD25+ Treg cells depleted donor grafts were associated with an increase in the incidence of severe aGVHD, while infusion of donor Treg cells significantly reduced GVHD.

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In contrast to the previous approach, recently the number of Treg cells in the donor graft has been assessed to determine the severity of aGVHD in post allo-HSCT. In this regard, Rezvani et al. [9] and Wolf et al. [10] determined that increased numbers of CD4+ Foxp3+ Treg cells in the donor graft negatively correlated with the incidence of GVHD without any difference in the incidence of relapse. Recently, in a cohort of 50 children with hematological malignancies, Fang et al. [11], reported that lesser number of Treg cells in donor grafts were associated with grade II–IV aGVHD compared to those with grade 0–I aGVHD. In this study, we enumerated Treg cells by flowcytometry and studied its correlation with the severity of aGVHD in post allo-HSCT patients. In addition, its utility as a potential biomarker for aGVHD was also ascertained.

Materials and Methods

Thirty-two consecutive patients with malignant and non-malignant hematological disorders were underwent allogeneic hematopoietic stem cell transplantation (allo-HSCT) from April 2015 and June 2016. Three patients died of regimen-related toxicities before engraftment, so these cases were excluded from the study. Twenty-nine patients who achieved engraftment were enrolled in the study. The research protocol was approved by the institute's ethics committee. Informed consents were obtained from patients and donors. The median age of the patients was 26 years (2–51 years). There was male preponderance with 20 males and 9 females. The baseline characteristics of patients underwent allo-HSCT were given in Table 1. Patient and donor were HLA-matched using typing at HLA-A/B/C/DRB1/DQB1. All patients received the graft from matched sibling donors. All patients received granulocyte colony-stimulating factor (G-CSF) mobilized peripheral blood stem cells as a source of donor graft. The median duration from diagnosis to transplant was 16 months (3–74 months). The median follow-up time for all patients was 6 months, (one and half months to 14 month).

Conditioning Regimen

Patients of malignant diseases (AML, CML-BC, CMML, JMML, MPAL, MDS), received myeloablative regimen [Busulfan 3.2 mg/kg/day for 4 days (Day-7 to Day-4) and Cyclophosphamide 60 mg/kg/day for 2 days (Day-3 to Day-2)]. Thalassemia major patients received myeloablative regimen in form of Thiotepea 8 mg/kg for 1 day (Day-6), Treosulfan 14 g/m²/day for 3 days (Day-5 to Day-3) and Fludarabine: 40 mg/m² for 4 days (Day-5 to Day-2).

Patients of AA received Fludarabine 30 mg/m² for 6 days (Day-10 to -5), Cyclophosphamide 60 mg/kg/day for 2 days (Day-6 to -5) and Horse ATG 30 mg/kg/day for 4 days (Day-4 to -1) based non myeloablative regimen.

GVHD Prophylaxis

All patients received cyclosporine A and methotrexate for the GVHD prophylaxis. All patients received Cyclosporine A with serum trough drug levels 150–300 ng/ml. Patients with acute leukemia and thalassemia received Inj methotrexate @ 15 mg/m² on day 1 and 10 mg/m² on day 3, 6, 11, along with leucovorin prophylaxis @ 15 mg/m² 6 hourly on day 2, 4, 7, 12 for 6 doses. Patients with aplastic anemia received Inj methotrexate 10 mg/m² on day 1 and 7 mg/m² on day 3, 6, 11 along with leucovorin prophylaxis. Dose modification has been done for patient with liver or renal dysfunction.

Processing of sample for T Reg Cell Analysis

Two milliliter of ethylene diamine tetra acetic acid (EDTA) anticoagulated samples (n = 32) were obtained from donor graft (peripheral blood stem cell) before transplantation. Samples were prepared using Stain–Lyse–Wash protocol. Antibody staining was performed in a single tube five color combination which included CD45-PE Cy5.5, CD3-PE Cy7, CD4-FITC, CD25-APC, and CD127-PE. Briefly, the samples were incubated with antibody cocktail in dark for 30 min, followed by RBC lysis using commercial FACS Lyse solution followed by washing and a final suspension in sheath fluid. Data acquisition was performed immediately after completion of sample staining, using a BD FACS-Canto flowcytometer and the BD FACSDiva software. For each sample, a minimum of 2,00,000 events was acquired (Fig. 1).

Outcomes

Neutrophil and platelet engraftment were defined as the first of three consecutive days with a count > 0.5 × 10⁹/L and > 20 × 10⁹/L, respectively and the counts being maintained, without any secondary graft failure. Acute GVHD was graded using the Glucksberg criteria [12]. CMV reactivation was defined when the detectable number of CMV DNA copy numbers (500/μL) was noted by PCR. Relapse was defined as recurrence of disease, while death without relapse was considered as nonrelapse mortality. Disease-related mortality was defined as death related to disease progression or recurrence. Overall survival was from the day of transplantation to death or last follow-up.

Table 1 Baseline characteristics of patients according to grade of acute GVHD

	Grade 0–I aGVHD (n = 20)	Grade II–IV aGVHD (n = 9)	<i>p</i> value
Male: Female	14:6	6:3	1.0
Age, year (median, range)	26 (2–51)	30 (7–48)	0.37
Duration from diagnosis to transplant (months, range)	14 (3–70)	22 (4–74)	0.46
Primary hematological disorders 0.56			
Thalassemia major	1	1	
Severe AA	7	4	
JMML	1	0	
CMML-2	1	0	
CML-BC	2	1	
Myelofibrosis	1	0	
MDS	0	2	
AML	6	1	
MPAL	1	0	
Stem cell source			
PBSC	20	9	
Conditioning regimen 0.58			
Flu + CY + ATG	7	4	
Bu + CY	12	4	
Thio + Treo + Flu	1	1	
aGVHD prophylaxis			
MTX + CSA	20	9	
Donor characteristics			
Age	19 (2–56)	28 (13–45)	0.36
Gender mismatched	7	4	0.62
ABO-mismatched	6	6	0.106

JMML, juvenile myelomonocytic leukemia; CMML, chronic myelomonocytic leukemia; CML-BC, chronic myeloid leukemia-blast crisis; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; MPAL, mixed phenotypic acute leukemia; PBSC, peripheral blood stem cells; Flu + CY + ATG, fludarabine + cyclophosphamide + horse ATG; Bu + CY, busulfan + cyclophosphamide; Thio + Treo + Flu, Thiotepa + Treosulphan + Fludarabine; MTX, methotrexate; CSA, cyclosporin A; BM, bone marrow; PBSC, peripheral blood stem cells

Statistical Analysis

Descriptive statistical analysis of main characteristics of patients was performed. Categorical and continuous data were presented in frequency (%), mean \pm SD/median (minimum–maximum). The association between two categorical variables was seen by Chi square/Fisher's exact test. Comparison of continuous variables between two independent groups was done by using Wilcoxon rank sum test. The receiver operating characteristic (ROC) curve analysis was performed to find an appropriate cut off of continuous variable. All the *p* value < 0.05 were taken as statistically significant. All the statistical analysis was done using Stata 12.1 software.

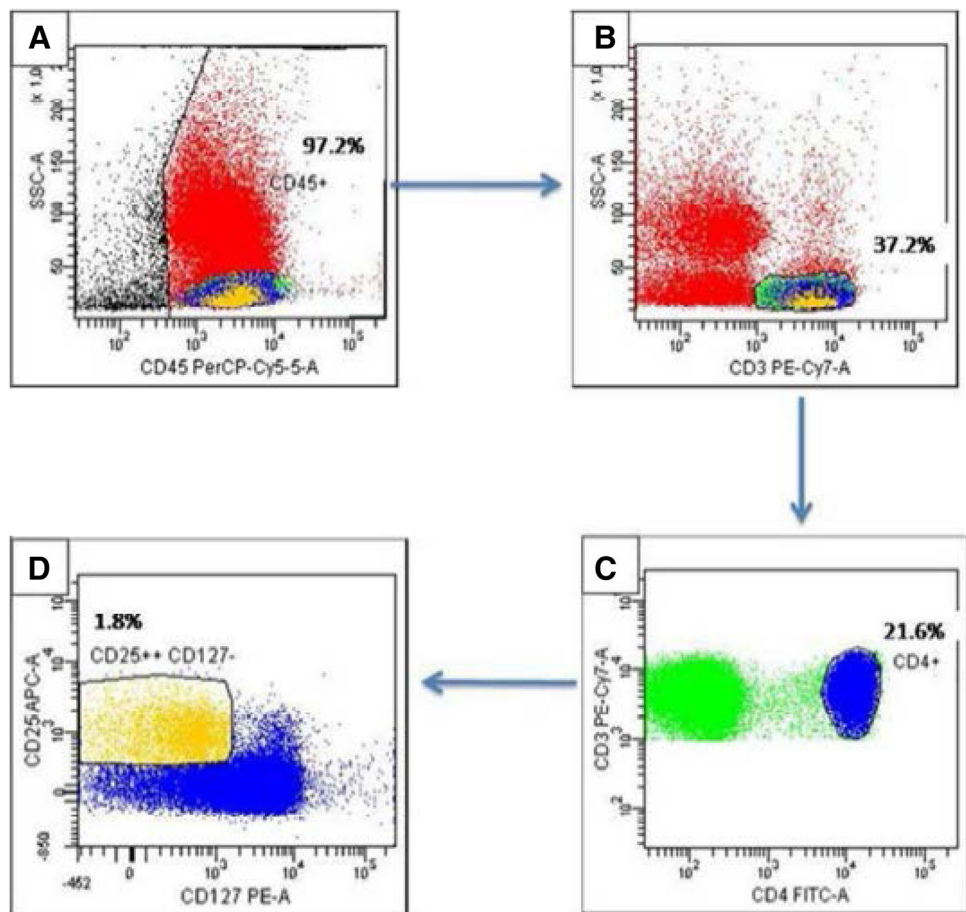
Results

We analyzed the relationship between Treg cells, CD34, CD3 and total nucleated cell dose in allo-HSCT with regard to the severity of aGVHD and clinical outcome.

Graft Characteristics

The median total nucleated cell (TNC), CD3+ cells, CD4+ cells and CD34+ cells, dose transfused were $1088 \times 10^6/\text{kg}$ (215–2251.98), $92.23 \times 10^6/\text{kg}$ (12.87–430.76), $42.51 \times 10^6/\text{kg}$ (8.45–242.71) and $6 \times 10^6/\text{kg}$ (2.44–8.35) respectively. Treg cells dose accounted for a median of $2.84 \times 10^6/\text{kg}$ (0.8–16.76). The median Treg/CD4 ratio was 0.074 (0.023–0.163). There was no difference in Treg cells proportion or Treg/CD4 ratio with donor age, gender or ABO blood group.

Fig. 1 Detection and quantification of Treg cells: Immunophenotyping by flowcytometry was performed to detect Tregs in donor graft, using a five color panel, surface CD45, CD3, CD4, CD25 and CD127. **a** Gate shows all nucleated cells, **b** gate shows CD3+ T-lymphocytes, **c** gate shows CD3+ CD4+ T-lymphocytes and **d** CD4+ CD25+ CD127-Treg cells



According to the occurrence and severity of aGVHD, patients were divided into two groups, where 20 (68.9%) patients had grade 0–I aGVHD and 9 (31.1%) patients had grade II–IV aGVHD. Fourteen patients did not develop any acute GVHD (grade 0). The basic characteristics of patients and transplantation of both the groups were given in Table 1. There was no difference between the two groups for age ($p = 0.37$), gender ($p = 1$), duration of diagnosis to transplant ($p = 0.46$), type of disease ($p = 0.56$), conditioning regimen (myeloablative vs non-myeloablative) ($p = 0.58$) and aGVHD prophylaxis ($p = 1$).

Acute GVHD

Fifteen out of 29 patients developed aGVHD post allo HSCT. The median onset of aGVHD was 36 days (12–86 days). Six patients developed grade-I and 9 patients developed grade II–IV aGVHD. Skin and gut were commonly involved organs; however, the liver was also involved in 3 cases. The proportion of Treg cells dose was higher in patients with grade 0–I aGVHD [3.53 (1.26–16.76)] as compared to those with grade II–IV aGVHD [2.86 (0.8–4.0)]; however, it fell short of statistical significance ($p = 0.13$). The Treg/CD4 ratio was

significantly higher in the grade 0–I aGVHD group [0.088 (0.056–0.163)] as compared to grade II–IV aGVHD group [0.042 (0.023–0.072)], ($p = 0.0002$) (Table 2). There was no correlation between CD34 ($p = 0.55$) dose or CD3 dose ($p = 0.57$) with the severity of aGVHD. Only one patient of aplastic anemia developed chronic GVHD during the study period.

Neutrophil and Platelet Engraftment

The median time to achieved neutrophil and platelet recovery was 11 days (9–35 days) and 12 days (7–76 days), respectively. There was no correlation between neutrophil or platelet recovery with Treg cells dose and Treg/CD4 ratio.

CMV Reactivation and Relapse

CMV reactivation after allo HSCT was observed in 9 patients at a median of 42 days (26–208). CMV reactivation was more in grade aGVHD II–IV group in comparison to grade 0–I aGVHD group ($p = 0.01$). However, there was no correlation between CMV reactivation and Treg cells dose ($p = 0.55$) or Treg/CD4 ratio ($p = 0.1$). Two patients

Table 2 Donor graft cells subpopulation and effect on acute GVHD

	Grade 0–I aGVHD (n = 20)	Grade II–IV aGVHD (n = 9)	<i>p</i> value
Transfused TNC	1103.93 (215–2251.98)	1086.59 (753.25–1615.19)	0.77
Transfused CD34	5.67 (2.44–8.35)	6 (2.5–7.73)	0.55
Transfused CD3	73.33 (12.87–430)	113.31 (32.16–184.47)	0.57
Transfused CD4	34.71 (8.45–242.71)	56.61 (19.96–93.48)	0.29
Transfused Treg cells	3.53 (1.26–16.76)	2.86 (0.8–4)	0.13
Treg/CD4 ratio	0.088 (0.056–0.16)	0.042 (0.023–0.072)	0.0002

relapsed in grade 0–I aGVHD group. As the number of the patients with relapse of the disease was small, the association between the graft composition i.e. Treg cell dose and relapse can not be concluded.

Relapse and Non-relapse Mortality

Seven of 29 patients died post transplantation with a median duration of 2 months (one and half to 14 months). Mortality was higher in grade II–IV aGVHD group as compared to grade 0–I aGVHD group [(55.56% vs 10%), ($p = 0.01$)]. On analyzing overall survival; a higher Treg/CD4 ratio was found to have a better survival outcome ($p = 0.01$); however, we did not find any correlation with the total Treg cells dose ($p = 0.68$). In non-relapse mortality, 4 patients died due to aGVHD, one patient due to secondary graft failure, and one due to infection. One patient died of disease relapse.

Discussion

Despite advancement in molecular HLA typing and matched sibling donors, aGVHD related mortality occurs in 26% of the allo-HSCT patients [13]. However; it is difficult to identify patients who are at a risk of aGVHD. Therefore, there is a need to identify other factors to predict aGVHD in post allo-HSCT.

A large number of studies in murine models have suggested that CD4+ CD25+ Treg cells play an important role in T cell responses and autoimmunity [13, 14]. These cells also play an important role in controlling graft rejection and aGVHD after allo-HSCT [15, 16]. Given the impressive potential of donor Treg cells to control aGVHD in hematological malignancies, we sought to determine whether Treg cells in donor graft could influence the severity of aGVHD in allo-HSCT recipients of malignant and benign hematological disorders.

We studied 29 patients, who underwent allo-HSCT for malignant and non-malignant hematological disorders. It was noted that the patients who received a higher dose of Treg cells in donor graft developed less severe aGVHD as compared to those who received low Treg cell dose. The

inability of this value being statistically significant ($p = 0.13$) may be attributed to a lesser number of samples. However, the Treg/CD4 ratio was found to be significant ($p = 0.0002$) between the two groups. Thus, we can suggest that there was a trend towards lesser incidence of aGVHD in patients receiving graft with higher Treg cells and Treg/CD4 ratio. Similar results were observed by Danby et al., in their study. However; their initial results also fell short of statistical significance when analyzing Treg cells dose and Treg/CD4 ratio. But on longer follow-up of their cohort, they found that higher proportion of Treg cell in donor graft predicted an improved overall survival after allo-HSCT [17]. Several other studies also demonstrate the association between higher counts of Treg cells in donor graft and reduced incidence of grade II–IV aGVHD in allo-HSCT [9, 10, 18]. Recently, in a study on a cohort of 50 children with hematological malignancies undergoing allo-HSCT, Fang et al. [11], found that graft Treg cells were significantly lower in children with grade II–IV aGVHD as compared to those with grade 0–I aGVHD. In contrast to above studies, Stanzani et al. [19] found that CD4+ CD25+ T cells were associated with increased risk of GVHD in patients following allogeneic stem cell transplantation.

We also analyzed the effect of patient's age, gender, diagnosis, duration from diagnosis to transplant, conditioning regimen and gender mismatch on the severity of aGVHD. There was no statistical difference for above parameters between the two groups and these observations were similar to the studies by Denby et al. [17] and Fang et al. [11].

Additionally, Danby et al. [17] observed no correlation between Treg cell number or Treg/CD4 ratio with donor's age, gender or ABO blood group which also corroborated with our findings.

CD34 and CD3 cells form an important component of donor graft. These cells have been extensively studied for their effect on aGVHD post allo-HSCT. Zaucha et al. and Pabst et al. [18, 20] observed no impact of CD34 dose on aGVHD. Our data also did not show the association between CD34 cell dose and a probability of acute GVHD ($p = 0.55$). However, Zaucha et al. [20] demonstrate the correlation between CD34 cell dose in grafts and chronic

GVHD in HLA-identical sibling recipients without any impact on acute GVHD. One patient in our study developed chronic GVHD; however, the short follow-up period in our patients heralded the analysis of the effect of CD34 on chronic GVHD. The above observations were in contrast to the results reported by Przepiorka et al. and Czerw et al. [21, 22]. Recently Czerw et al. demonstrated that high CD3+ and CD34+ cell dose content in the grafts was an independent prognostic factor associated with higher probability of severe acute GVHD grades II–IV and III–IV. At the same time, they did not observe the beneficial effect of such high cell doses on AML control, relapse rate, and the other transplant-related outcomes [22].

The role of CD3 cells in the development of acute and chronic GVHD is still a matter of debate because of the lack of unequivocal conclusions. Gaziev et al. [23] and Urbano-Ispizua et al. [24] found in their studies that grade II–IV aGVHD was associated with high CD3+ cell doses in the graft. In the current study, we found no statistically significant association between CD3 cell dose and the probability of acute GVHD. Our results are consistent with observations suggesting that the risk of acute GVHD is not influenced by CD3 cells after allogeneic PBSC transplant [18, 25].

In our study, we did not observe the difference in the incidence of relapse, when analyzing either Treg cells dose or Treg/CD4 ratio in two groups. Similar observations were made by Fang et al., Danby et al. and Pabst et al. [11, 17, 18].

CMV reactivation and relapse are commonly observed in post-transplant patients. We did not find any significant association of Treg cell dose or Treg/CD4 ratio with CMV reactivation and relapse. Concurrent result regarding Treg cell dose and CMV reactivation or relapse were observed in several other studies [11, 17, 18].

The major limitations of our study were small sample size, heterogeneity in sample and short follow-up period. A larger sample size with a longer follow-up would allow clarification about the influence of Treg cells dose in donor graft on the severity of aGVHD.

Conclusion

Our findings suggested a relationship between Treg/CD4 ratio in donor graft and severity of aGVHD. Higher Treg/CD4 ratio in donor graft was found to be associated with less severe grade of aGVHD. However, additional studies with more number of the patients are needed. The Treg/CD4 ratio may be used as a predictive marker for severity of aGVHD in post allo-HSCT patients.

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Compliance with Ethical Standards

Conflict of interest Authors have no conflict of interest to declare.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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