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Kinetics of Recovery of Naïve and Memory T Cells in Acute Leukemia Patients after Allogeneic Stem Cell Transplantation Depending on Different GVHD Prophylaxis Regimens

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ABSTRACT

Background: Memory T cells are a heterogeneous population of immune cells that provide adaptive immunity. Its full recovery seems essential for graft-versus-tumor reactions that provide an opportunity for biological cure in patients with acute leukemia. The use of mismatched or haploidentical donors has increased, which has become possible because of modifications in graft versus host disease (GVHD) prophylaxis.

Materials and Methods: Sixty-five leukemia patients (acute myeloid leukemia – 40, acute lymphoblastic leukemia – 25), median age 33 (17–61) years, underwent allo-HSCT from 2016 to 2019 in the National Research Centre for Hematology. Patients were divided into three groups based on the impact of GVHD prophylaxis on T cell recovery: horse antithymocyte globulin (ATG)-based regimen (n=32), horse ATG combined with posttransplant cyclophosphamide (PT-Cy) (n=18), and *ex vivo* T cell depletion (n=15).

Results: The early period after transplantation (before day +100) was characterized by significantly lower absolute numbers of T naïve, memory stem and T central memory cells in peripheral blood in patients after ATG+PT-Cy-regimen or ex vivo T cell depletion than after ATG-based prophylaxis ($p<0.05$). Moreover, strong depletion of naïve T and memory stem cells prevents the development of GVHD, and determining the absolute number of $CD8^+$ naïve T and memory stem cells with a cutoff of 1.31 cells per microliter seems to be a perspective in assessing the risks of developing acute GVHD ($p=0.008$). The dynamics of T cell recovery showed the involvement of either circulating or bone marrow resident T effector cells shortly after allogeneic transplantation in all patients, but the use of manipulated grafts with *ex vivo* T cell depletion requires the involvement of naïve and memory stem cells. There was no significant effect of T cell recovery on leukemia relapse after allogeneic transplantation.

Conclusion: These experimental outcomes contribute to providing the best understanding of immunological events that occur early after transplantation and help in the rational choice of GVHD prophylaxis in patients who will undergo allogeneic transplantation. Our study demonstrated the comparable immunological effects of posttransplant cyclophosphamide and *ex vivo* T cell depletion and immunological inefficiency of horse ATG for GVHD prevention**.**

Keywords: GVHD prophylaxis; Immune reconstitution; T memory cells; Post-transplant cyclophosphamide; T cell depletion; Horse ATG

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a method to achieve durable remission and potentially full recovery in adults with acute leukemia. Its effect is based on the transfer of the immune system from the donor to the recipient,

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which triggers a graft-versus-leukemia response (GVL). However, prolonged immunodeficiency after allo-HSCT is associated with various infections, and the delays or inadequacy in immune reconstitution determine posttransplant complications such as graft versus host disease (GVHD), leukemia relapse, or secondary tumors[1-3].

Different immune cells recover differently after allo-HSCT. The reconstitution of innate immunity occurs rapidly during the first month after allo-HSCT, but the full recovery of adaptive immunity that provides anti-infectious and antitumor protection takes several years[3]. Moreover, conditioning regimens and GVHD prophylaxis influence immune cells and their recovery kinetics. The choice of therapy is significant and determines the course of the posttransplant period [4-6]. The recovery of T cell immunity takes approximately 1 year after allo-HSCT; humoral immunity requires more than 2 years[3]. In this research, we focused on T cell reconstitution after allo-HSCT.

The early posttransplant period is characterized by the thymus-independent pathway of T cell recovery involving the proliferation of mature donor T cells that were transplanted to the patient together with hematopoietic stem cells[7]. A distinctive feature of thymus-dependent reconstitution is the generation of de novo naïve T cells and the subsequent formation of a memory T cell pool [7, 8]. Immunological memory is characterized by a prompt reaction to foreign antigens and appears to be essential for GVL[9,10].

Immune reconstitution depends on many factors. Some factors are invariable, such as patient age, and others can be modified. For example, myeloablative conditioning (MAC) is associated with prolonged immune restoration [11, 12]. The literature has reported that total body irradiation or high-dose busulfan causes irreversible death of epithelial cortical thymic cells, leading to the impossibility of forming new lymphoid populations[13,14]. Reduced intensity conditioning (RIC) is significantly less damaging to thymic cells than MAC is [4, 15]. This was confirmed by the detection of recent thymic emigrants on day +90, whereas after MAC, these cells were not observed even on day +365 after allo-HSCT [16, 17].

The number of haploidentical transplantations (haplo-HSCT) has been increasing, which has facilitated the modification of GVHD prophylaxis protocols. Posttransplant cyclophosphamide (Post-Cy) on days +3 and +4 has been highly effective in many clinical studies [18, 19], and today, it has almost replaced other preventive regimens. Competitive GVHD prophylaxis based on *in vivo* T cell depletion is the regimen with antithymocyte globulin (ATG)[20,21]. Many studies have demonstrated the impact of Post-Cy and ATG on immune recovery and the advantages of Post-Cy over ATG[22-24]. Another platform for GVHD prevention is *ex vivo* T cell depletion[25]. However, the use of this approach is limited to adults, and the results are controversial.

In this study, we compared the recovery of memory T cells as the main population providing adaptive immunity in patients with acute leukemia after allo-HSCT with different GVHD prophylaxis regimens (ATG-based versus ATG + Post-Cy versus *ex vivo* T cell depletion). We analyzed the impact of these GVHD prophylaxis regimens on T memory cell subsets in the short term after allo-HSCT and found an immunological basis for the rational choice of immunosuppressive therapy.

MTERIALS AND METHODS

Patient characteristics

This study assessed 65 patients with acute leukemia who underwent allo-HSCT between September 2016 and February 2019 at the National Research Center for Hematology, Moscow, Russia. The median patient age was 33 years (range, 17–61 years). Acute myeloid leukemia (AML) was diagnosed in 40 patients, and acute lymphoblastic leukemia (ALL) in 25 patients. Most patients (n=63) were confirmed to achieve complete remission (CR). Molecular relapse before the start of pretransplant conditioning was detected in two patients. Patient characteristics are presented in Table 1.

Table 1: Patient characteristics

Characteristics	Diagnosis (n=65)	
	AML (n=40)	ALL (n=25)
Median age	35 (18-60)	30 (17-61)
Sex (m/f), n	19/21	13/12
CR 1, n	31 (77.5%)	13 (52%)
Risk factors for CR 1:		
MRD before allo-HSCT	7/31 (22.6%)	6/13 (46.2%)
Primary-resistant disease	5/31 (16.1%)	1/13 (7.7%)
t (9;22)	–	8/13 (61.5%)
$FLT3^+$	4 (12.9%)	–
t (8;21), MRD-persistent	3 (9.7%)	–
Complex karyotype	3 (9.7%)	–
CR >1, n	8 (20%)	11 (44%)
Molecular relapse, n	1 (2.5%)	1 (4%)

Conditioning regimens, type of allo-HSCT, and graft source

All patients underwent pretransplant conditioning. MAC was administered to 20 young patients, median age, 25 years (range, 17–41 years), who had no comorbidities. Classical MAC based on busulfan (12 mg/kg) and cyclophosphamide (120 mg/kg) (BuCy) was used in 12 of the 20 patients. Eight of the 20 patients underwent haplo-HSCT with *ex vivo* TCR αβ and CD 19 depletion and received thiotepa 10 mg/kg combined with treosulfan 42 g/m^2 and fludarabine 150 mg/m^2 (TreoThiotepaFlu). RIC was selected for 45 patients. The classical RIC protocol was fludarabine 180 mg/m^2 and busulfan 8 mg/kg (FluBu) and administered to 38 patients, and the regimen with treosulfan 42 g/m^2, melphalan 140 mg/m^2, and fludarabine 150 mg/m^2 (TreoMelFlu) was applied in case of haplo-HSCT with *ex vivo* TCR αβ and CD 19 depletion in 7 patients.

The detailed conditioning regimens are presented in Table 2.

Table 2. Conditioning regimens

Conditioning	Drug	Dose	Days
BuCy	Busulfan	4 mg/kg - oral in 4 doses (1 mg/kg) every 6 hours	-6 to -4
	Cyclophosphamide	60 mg/kg i.v. over an hour	-3 to -2
TreoThiotepaFlu	Thiotepa	5 mg/kg i.v. over 2 hours	-6 to -5
	Treosulfan	14 g/m^2 i.v. over 2 hours	-5 to -3
	Fludarabine	30 mg/m^2 i.v. over 30 min	-6 to -2
FluBu	Busulfan	4 mg/kg - oral in 4 doses (1 mg/kg) every 6 hours	-6 to -5
	Fludarabine	30 mg/m^2 i.v. over 2 hours	-10 to -5
TreoMelFlu	Treosulfan	14 g/m^2 i.v. over 2 hours	-5 to -3
	Melphalan	70 mg/m^2 i.v. over an hour	-3 to -2
	Fludarabine	30 mg/m^2 i.v. over 30 min	-6 to -2

Thirteen patients were transplanted from 10/10 matched donors (MRDs), 21 from haploidentical donors, 19 from matched 10/10 unrelated donors (MUDs), and 12 from mismatched 9/10 or 8/10 unrelated donors (MMUDs).

Bone marrow (BM) was used in 22 patients, and peripheral blood stem cells (PBSC) in 43 as the graft source.

GVHD prophylaxis

All patients were subdivided into three groups based on the GVHD prophylaxis regimen. Thirty-two patients (median age, 33 years; range, 20–61 years) received an ATG-based regimen including horse ATG, and the dosage was 10 mg/kg per day on days -4, -3, -2, and -1, combined with cyclosporin A (CsA), methotrexate (MTX), and mycophenolate mofetil (MMF). This regimen has been applied in cases of MRDs or MUDs transplantation.

Post-Cy at a dosage of 50 mg/kg per day on days +3 and +4 combined with horse ATG, CsA, and MMF was administered to patients who underwent allo-HSCT from unrelated mismatched donors or related haploidentical donors (n=18, median age of 36, range 23–58).

Fifteen patients with a median age of 20 (17-57) underwent haplo-HSCT with manipulated grafts. *Ex vivo* TCRαβ and CD19 depletion were performed to prevent GVHD. Rituximab 100 mg/m^2 on day -1, bortezomib 1.3 mg/m^2 per day on days -5, -2, +2, +5; Tocilizumab 8 mg/kg on day -; and Abatacept 10 mg/kg per day on days -1, +7, +14, +28 were used as additional immunosuppressive therapy in this group. Detailed GVHD prophylaxis and types of allo-HACT are presented in Table 3.

Table 3: Choice of GVHD prophylaxis and types of allo-HSCT

Type of allo-HSCT	GVHD prophylaxis	Drug	Dose	Days
MRD or MUD	ATG-based (n=32)	Horse ATG	10 mg/kg in 2 doses, i.v. over 6 hours	-4 to -1
		CsA	3 mg/kg in 2 doses, i.v. over 5 hours	-1 to +90
		MTX	15 mg/m^2 i.v. 10 mg/m^2 i.v.	+1 +3, +6, +11
		MMF	2 g per day, oral (MRD) 3 g per day, oral (MUD)	+1 to +90
MMUD or Haplo-HSCT	Post-Cy-based (n=18)	Horse ATG	10 mg/kg in 2 doses, i.v. over 6 hours	-4 to -1
		Cy	50 mg/kg i.v. over an hour	+3, +4
		CsA	3 mg/kg in 2 doses, i.v. over 5 hours	+5 to +180
		MMF	3 g per day, oral	+5 to +90
Haplo-HSCT	*Ex vivo* TCRαβ and CD19 depletion (n=15)	transplant processing	-	-1
		Rituximab	100 mg/m^2 i.v. over 4 hours	-1
		Bortezomib	1.3 mg/m^2 subcutaneously	-5, -2, +2, +5
		Tocilizumab	8 mg/kg i.v. over an hour	-1
		Abatacept	10 mg/kg i.v. over an hour	-1, +7, +14, +28

Laboratory tests and flow cytometry

Samples of peripheral blood and BM were collected in EDTA tubes on days +30, +60, +90, and +180 after allo-HSCT. Flow cytometry was performed on a BD FACS Canto II (Becton Dickinson, USA) to define the CD4$^+$ and CD8$^+$ T memory subsets. Anti- CD4 PerCP-Cy5.5, anti- CD8 APC-Cy7, anti- CD197 PE-Cy7, anti-PD-1 PE, anti-CD28 APC, and anti-CD45RO FITC (Becton Dickinson, USA) were used to analyze the expression of surface markers. We identified T naïve and T stem cell memory (Tnv+Tscm) as CD45R0-CCR7+CD28+, T central memory (Tcm) as CD45R0+CCR7+CD28+, T transitional memory (Ttm) as CD45R0+CCR7-CD28+, T effector memory (Tem) as CD45R0+CCR7-CD28-, and T terminal effector (Tte) as CD45R0-CCR7-CD28-. The absolute and relative cell counts were obtained for analysis. Values for the control group were used as references. The control group comprised 10 healthy individuals with a median age of 29 years (range, 18–40 years; five males and five females).

Statistical analysis

All data analyses were conducted using R ver. 4.1 (R Core Team, Vienna, Austria). The Shapiro-Wilk criterion was used to check the normality of the distribution of variables. The Kruskal-Wallis test was used for nonparametric data analysis for three or more independent groups and the Mann-Whitney U-test for two independent samples. The Friedman criterion was used to analyze repeated measurements (dynamics). The chi-square test was performed to analyze contingency tables, and the exact Fischer test was used for 2 × 2 tables. Analysis of overall survival and relapse-free survival was performed using the Kaplan-Meyer method, and a log-rank test was used to compare survival curves.

Data are presented as median and interquartile intervals (the difference between the 1st and 3rd quartiles). Box-and-whisker diagrams indicate the median, 25^{th} and 75^{th} percentiles. Line graphs show the dynamics. Statistical significance was set at $P<0.05$.

RESULTS

Clinical results

All patients (n=65) achieved neutrophil engraftment (a sustained absolute neutrophil count of ≥ 500 cells/μL for 3 consecutive days) on day +21 (range, 9–47). Platelet engraftment (platelet count of ≥20000 cells per microliter without transfusions for at least 7 consecutive days) was registered in 59 patients. The median time for platelet engraftment was 19 days (range: 9–46 days).

CR and full donor chimerism were confirmed in all patients on day +30. Acute GVHD grades II–IV were diagnosed in 18/65 (27.7%) patients. The median time of acute GVHD onset was 87 days (range, 22-178). Steroid-refractory GVHD was diagnosed in 7/18 (38.9%) patients. Notably, in 5 (38.5%) patients who previously received ATG-based prophylaxis, steroid-refractory GVHD developed after donor lymphocyte transfusions due to mixed chimerism that appeared on days +65 to +157. No cases of mixed chimerism were observed in the Post-Cy group or the ex vivo T cell depletion group. However, there were no statistically significant differences in the groups' acute GVHD rates. The rate of acute GVHD was 40.6% for horse ATG prophylaxis, 11.1% after horse ATG with PT-Cy, and 20% after ex vivo T cell depletion.

Leukemia relapse after day +30 was diagnosed in 14/65 (21.5%) of patients. Relapse-free survival during 30 months was 41.6%. The relapse rates did not differ significantly after different GVHD prophylaxis regimens.

Nineteen patients died of relapse (n=7), steroid-refractory acute GVHD (n=4), infectious complications (n=4), and secondary graft failure (n=4). Thirty-month overall survival was 51.8%, and no significant differences were observed among the three groups.

Reconstitution of CD4+ and CD8+ T cells, different memory T cells

We compared the absolute cell counts of different T cells in peripheral blood on days +30, +60, +90, and +180 in patients after the ATG-based and ATG+Post-Cy regimens and *ex vivo* T cell depletion. The data are summarized in Table 4.

Table 4: Absolute count of different T cells in peripheral blood after allo-HSCT

Day after allo-HSCT	T cell subsets	Absolute cell count per microliter, median (percentile 25–percentile 75)			
		ATG-based regimen	ATG+PT-Cy	*ex vivo* TCR αβ depletion	P
+30	Total of $CD4^+$	111.88 (56.29-194.45)	22.89 (13.45-4764)	14.29 (5.34-16.70)	0.0001
	$CD4^+$ Tnv+scm	19.19 (5.47-55.00)	0.73 (0.25-2.87)	0.26 (0.08-0.34)	0.0001
	$CD4^+$ Tcm	23.27 (6.67-50.99)	5.43 (1.81-11.62)	5.03 (1.01-6.00)	0.002
	$CD4^+$ Ttm	46.65 (28.81-76.17)	10.46 (6.30-26.62)	7.36 (3.71-10.54)	0.0001
	$CD4^+$ Tem	1.23 (0.91-2.25)	0.8 (015-4.28)	0.08 (0.03-0.14)	0.0001
	$CD4^+$ Tte	1.12 (0.37-1.94)	0.26 (0.04-0.60)	0.13 (0.03-0.38)	0.001
	Total of $CD8^+$	51.20 (15.38-126.67)	26.76 (8.23-44.21)	3.12 (1.78-16.10)	0.001
	$CD8^+$ Tnv+scm	4.45 (1.62-7.00)	0.35 (0.11-0.57)	0.06 (0.04-0.15)	0.0001
	$CD8^+$ Tcm	0.76 (0.27-1.75)	0.22 (0.17-0.30)	0.12 (0.01-0.34)	0.001
	$CD8^+$ Ttm	14.25 (5.4-32.99)	8.73 (2.88-14.06)	1.0 (0.56-8.23)	0.001
	$CD8^+$ Tem	7.96 (2.03-39.58)	5.84 (0.77-14.58)	0.73 (0.41-5.23)	0.025
	$CD8^+$ Tte	9.46 (3.86-37.4)	3.62 (0.52-14.1)	0.71 (0.25-0.9)	0.0001
+60	Total of $CD4^+$	82.84 (57.58-227.82)	54.85 (45.74-84.92)	37.05 (15.92-197.18)	0.111
	$CD4^+$ Tnv+scm	16.24 (3.59-30.13)	2.92 (0.68-9.43)	0.73 (0.27-1.29)	0.001
	$CD4^+$ Tcm	17.38 (9.18-36.81)	17.16 (9.90-24.63)	8.17 (4.95-15.53)	0.178
	$CD4^+$ Ttm	44.55 (29.86-81.89)	31.61 (23.86-41.14)	22.74 (8.69-53.42)	0.039
	$CD4^+$ Tem	1.62 (0.86-8.33)	0.99 (0.27-3.38)	0.82 (0.18-55.71)	0.555
	$CD4^+$ Tte	0.46 (0.21-0.77)	0.13 (0.05-0.72)	0.23 (0.08-1.62)	0.329
	Total of $CD8^+$	99.54 (31.55-331.97)	71.56 (27.36-206.61)	27.15 (5.42-169.43)	0.153
	$CD8^+$ Tnv+scm	3.06 (1.86-5.73)	1.7 (0.84-3.83)	0.12 (0.03-0.22)	0.0001
	$CD8^+$ Tcm	0.99 (0.36-2.04)	0.78 (0.42-1.57)	0.11 (0.05-0.35)	0.001
	$CD8^+$ Ttm	20.87 (9.05-50.79)	13.52 (9.75-83.61)	4.77 (1.55-8.87)	0.006
	$CD8^+$ Tem	40.62 (9.51-133.23)	16.98 (3.4-88.29)	14.12 (1.43-135.30)	0.499
	$CD8^+$ Tte	23.96 (5.09-117.75)	15.52 (5.13-28.78)	7.37 (2.07-18.36)	0.122
+90	Total of $CD4^+$	184.64 (101.37-290.21)	55.51 (37.53-125.59)	62.44 (41.46-146.75)	0.019
	$CD4^+$ Tnv+scm	18.68 (6.95-45.89)	1.83 (1.25-3.37)	0.70 (0.32-4.76)	0.002
	$CD4^+$ Tcm	39.26 (18.92-52.98)	12.09 (6.21-36.04)	10.54 (1.05-16.82)	0.007
	$CD4^+$ Ttm	76.13 (47.28-159.32)	30.15 (26.39-62.52)	26.24 (19.51-49.66)	0.004
	$CD4^+$ Tem	7.57 (1.82-15.91)	2.49 (0.86-4.93)	3.8 (1.22-20.00)	0.230
	$CD4^+$ Tte	0.47 (0.22-1.59)	0.21 (0.08-0.45)	0.66 (0.06-5.35)	0.153
	Total of $CD8^+$	258.03 (185.71-684.00)	127.36 (25.47-507.58)	33.53 (23.20-104.10)	0.002
	$CD8^+$ Tnv+scm	4.56 (2.99-12.04)	2.11 (1.27-3.7)	0.34 (0.04-0.7)	0.0001
	$CD8^+$ Tcm	2.36 (0.72-5.74)	0.34 (0.15-4.36)	0.17 (0.05-0.81)	0.002
	$CD8^+$ Ttm	51.60 (36.32-115.87)	22.25 (6.88-179.53)	3.97 (0.44-8.78)	0.0001
	$CD8^+$ Tem	143.83 (33.19-275.20)	25.20 (4.64-215.07)	18.67 (5.36-65.04)	0.055
	$CD8^+$ Tte	78.10 (28.92-219.92)	48.93 (7.46-74.60)	8.66 (4.08-27.68)	0.002
+180	Total of $CD4^+$	255.19 (166.41-319.97)	141.49 (102.91-182.60)	183.80 (73.62-266.40)	0.105
	$CD4^+$ Tnv+scm	23.56 (6.76-45.14)	4.42 (2.58-9.31)	13.53 (4.78-75.13)	0.060
	$CD4^+$ Tcm	46.99 (24.23-62.66)	18.55 (14.80-27.00)	37.18 (17.15-44.94)	0.082
	$CD4^+$ Ttm	121.24 (97.19-164.90)	87.96 (69.43-148.19)	82.43 (45.24-87.57)	0.022
	$CD4^+$ Tem	11.38 (3.85-42.73)	3.46 (1.20-17.57)	4.86 (1.97-27.80)	0.455
	$CD4^+$ Tte	1.14 (0.38-4.04)	0.66 (0.17-1.35)	1.13 (0.29-5.31)	0.433
	Total of $CD8^+$	586.27 (360.56-922.28)	237.38 (99.90-484.39)	88.26 (67.23-198.27)	0.001
	$CD8^+$ Tnv+scm	10.76 (4.5-19.12)	6.73 (1.88-8.11)	4.79 (1.58-33.52)	0.273
	$CD8^+$ Tcm	3.07 (1.16-5.73)	0.74 (0.62-1.63)	1.41 (0.27-2.49)	0.129
	$CD8^+$ Ttm	83.85 (49.58-115.97)	37.40 (22.58-153.61)	21.01 (6.43-25.86)	0.0001
	$CD8^+$ Tem	264.67 (160.78-369.76)	81.59 (31.46-108.40)	47.66 (13.07-105.16)	0.008
	$CD8^+$ Tte	152.27 (55.59-307.35)	50.98 (25.35-158.40)	14.32 (10.62-42.93)	0.005

According to the obtained data, the short-term period after allo-HSCT (days +30, +60, +90) is characterized by significantly lower absolute numbers of Tnv+scm and Tcm in the peripheral blood of patients after ATG+Post-Cy-regimen or *ex vivo* T cell depletion than with ATG-based prophylaxis ($p<0.05$). The absolute numbers of Tnv+scm and Tcm did not differ on day +180 in the groups.

The recovery of the effector pool consisting of Ttm, Tem, and Tte differed among the groups. The absolute counts of Ttm, Tem, and Tte were lower on day +30 for the ATG+Post-Cy-regimen or *ex vivo* T cell depletion than for the ATG-based regimen ($p<0.05$). There were significant differences in the absolute cell counts of $CD4^+$ and $CD8^+$ Ttm during the follow-up period, and the counts of $CD8^+$ Tem and $CD8^+$ Tte were distinguished on day +180 and on days +90 – +180, respectively.

The recovery of T memory cell subsets is presented separately in the graphs for $CD4^+$ and $CD8^+$ cells (Figures 1 and 2, respectively).

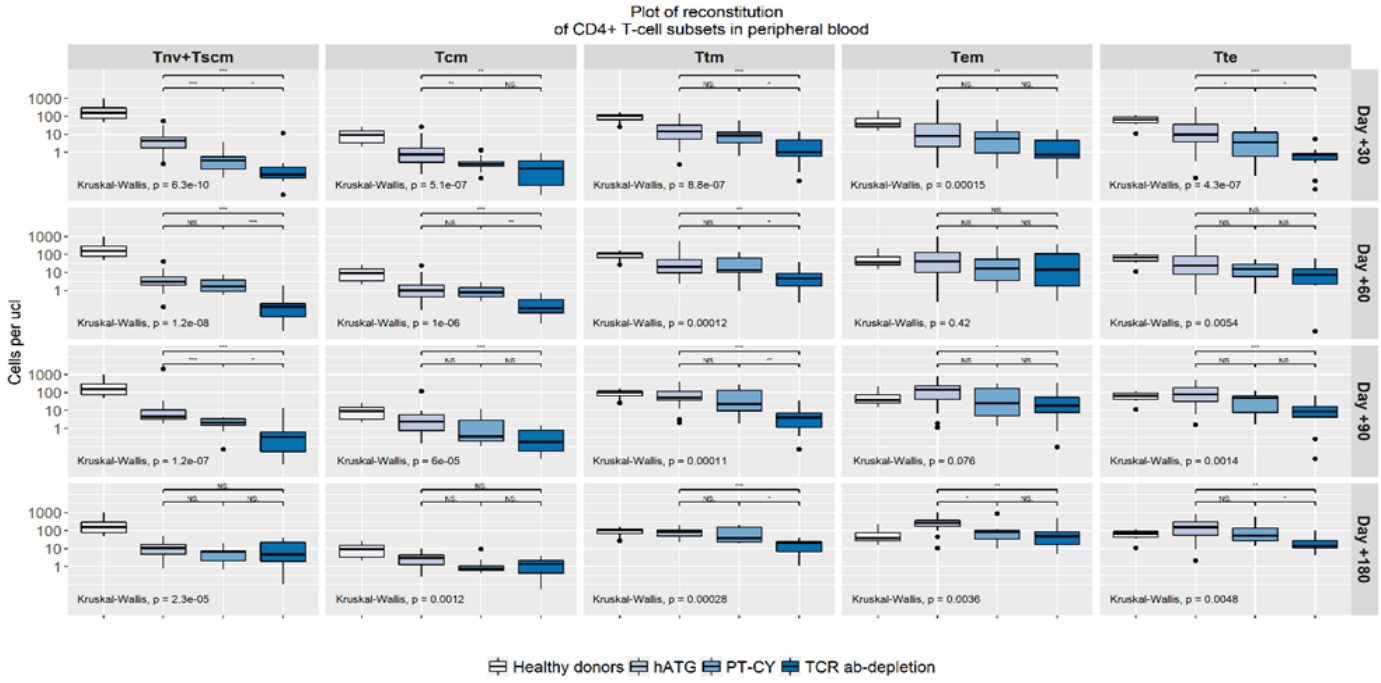

Figure 1. – Reconstitution of $CD4^+$ T memory cell subsets in peripheral blood in acute leukemia patients after allo-HSCT. A star denotes the significance level between two groups, * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$. Kruskal-Wallis exact p value for the significance for 3 and more independent groups.

Tnv+scm, Tcm, Ttm, Tem, and Tte belonged to $CD4^+$ and were measured quantified at the posttransplant time points (+30, +60, +90, and +180 days) among the patients after horse ATG, horse ATG combined with PT-Cy, and *ex vivo* T cell depletion. Absolute cell counts in healthy donors were used as a reference. Significant differences between groups are indicated by *.

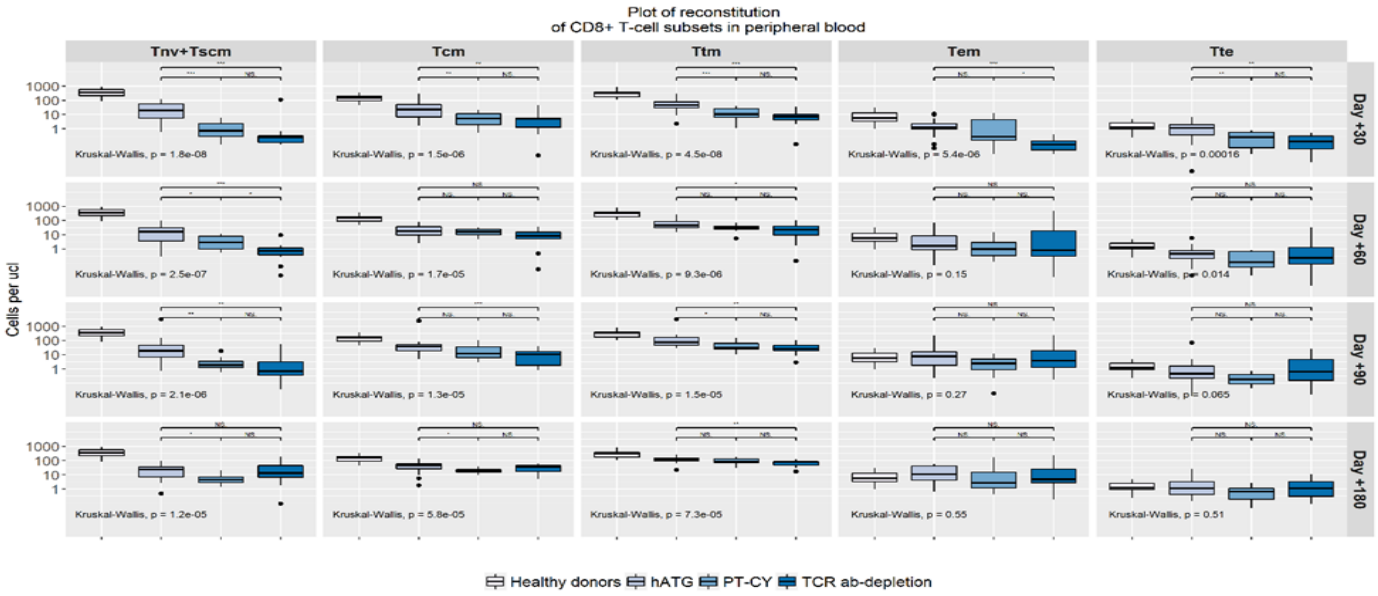

Figure 2. Reconstitution of CD8$^+$ T memory cell subsets in peripheral blood in acute leukemia patients after allo-HSCT. A star was used for the significance level between two groups, * - p<0.05, ** - p<0.01, *** - p<0.001. Kruskal-Wallis exact p value for the significance for three and more independent groups.

Tnv+scm, Tcm, Ttm, Tem, and Tte belonged to CD8$^+$ and were quantified at the posttransplant time points (+30, +60, +90, and +180 days) among the patients after horse ATG, horse ATG combined with PT-Cy, and *ex vivo* T cell depletion. Absolute cell counts in healthy donors were used as a reference. Significant differences between groups are indicated by *.

Dynamics of different memory T cells' recovery depended on GVHD prophylaxis

We also considered the influence of time on the dynamics of T cell recovery. All patients were subdivided into three groups by the immunosuppressive protocol, and analysis was conducted within each patient group, separately for the ATG group, ATG+PT-Cy, and *ex vivo* T cell depletion.

The dynamics of recovery of all T subsets (Tnv+scm, Tcm, and effector T cells) was different in the case of *ex vivo* TCR αβ depletion (p<0.05), whereas prophylaxis with ATG or ATG+Post-Cy was accompanied by significant changes in the pool of effector T cells (Figure 3).

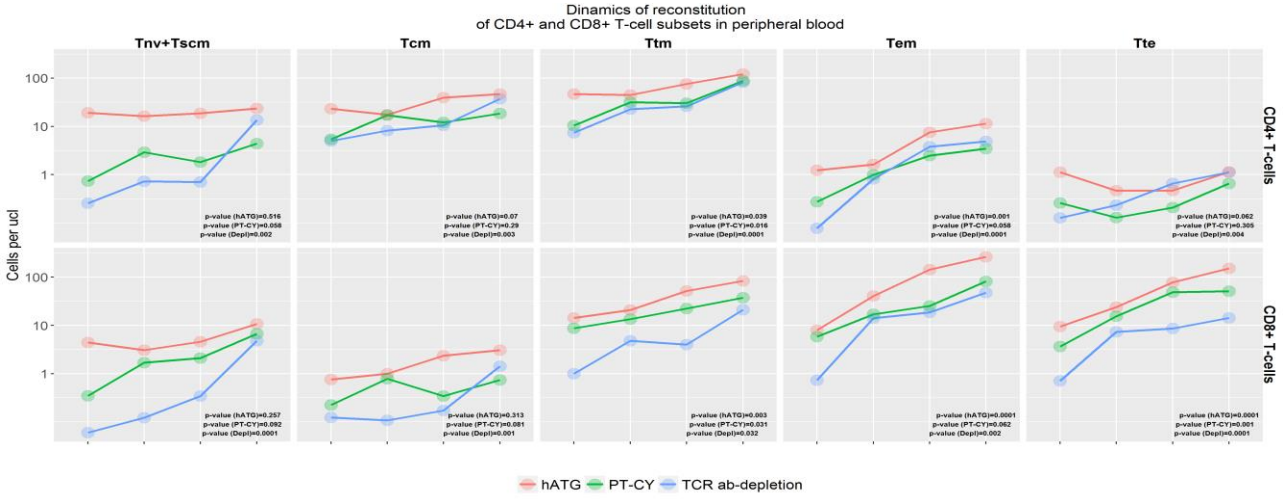

Figure 3. Dynamics of T memory cell subsets recovery in patients after different GVHD prophylaxis.

Each line on the graph reflects the quantitative change in cells, separately for Tnv+scm, Tcm, Ttm, Tem, and Tte, over time for each patient group – separately for patients after horse ATG only (red), horse ATG combined with PT-Cy (green), and *ex vivo* T cell depletion (blue).

Reconstitution of BM resident T memory cell subsets in patients after allo-HSCT

We compared the relative cell counts in the BM of patients after different GVHD prophylaxis regimens on days +30, +60, +90, and +180. The relative counts of Tnv+scm, Tcm, Ttm, Tem, and Tte in the BM of the control group were used as a reference.

Reconstitution of Tnv+scm, Tcm, Ttm, Tem, and Tte did not differ after ATG, ATG+Post-Cy, and *ex vivo* T cell depletion during the follow-up period. However, in patients after allo-HSCT, the distribution of cellular subpopulations of BM is shifted toward the effector pool, whereas in healthy individuals, the BM is heterogeneous with the greatest number of naïve and stem memory cells. The distribution of BM T memory cell subsets, separately for $CD4^+$ and $CD8^+$, in patients after allo-HSCT compared that of healthy individuals is presented in Figures 4 and 5, respectively.

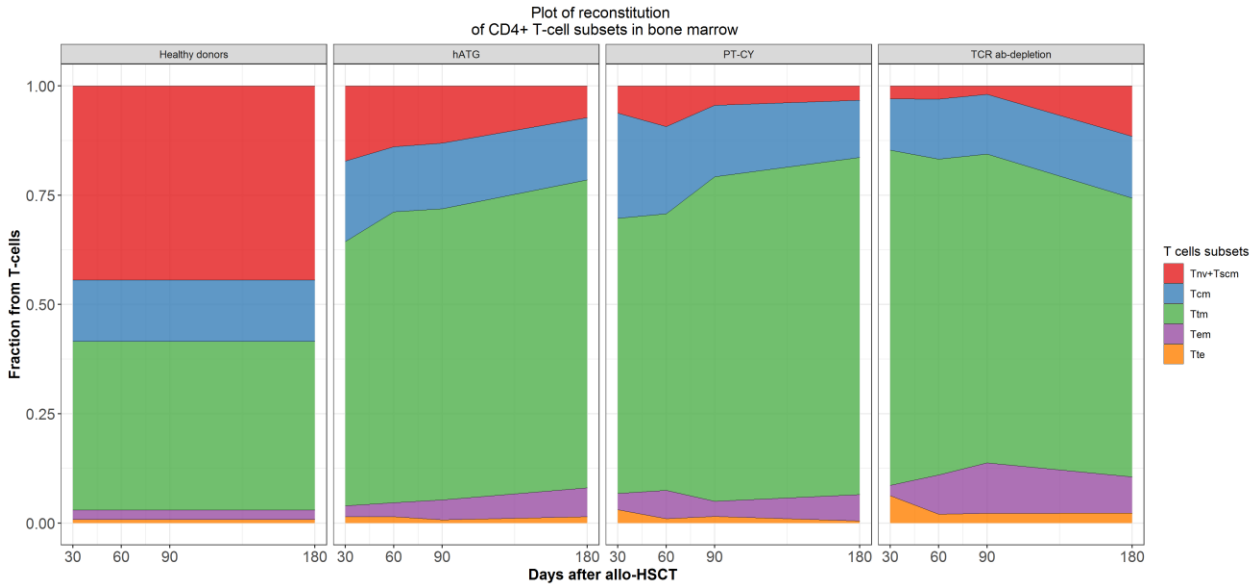

Figure 4. Distribution of BM $CD4^+$ T memory cell subsets in patients after allo-HSCT and healthy individuals.

The graph shows the percentage of cell populations among $CD4^+$ T cells for patients after allo-HSCT, separately for the horse ATG group, horse ATG combined with PT-Cy, *ex vivo* T cell depletion, and healthy controls.

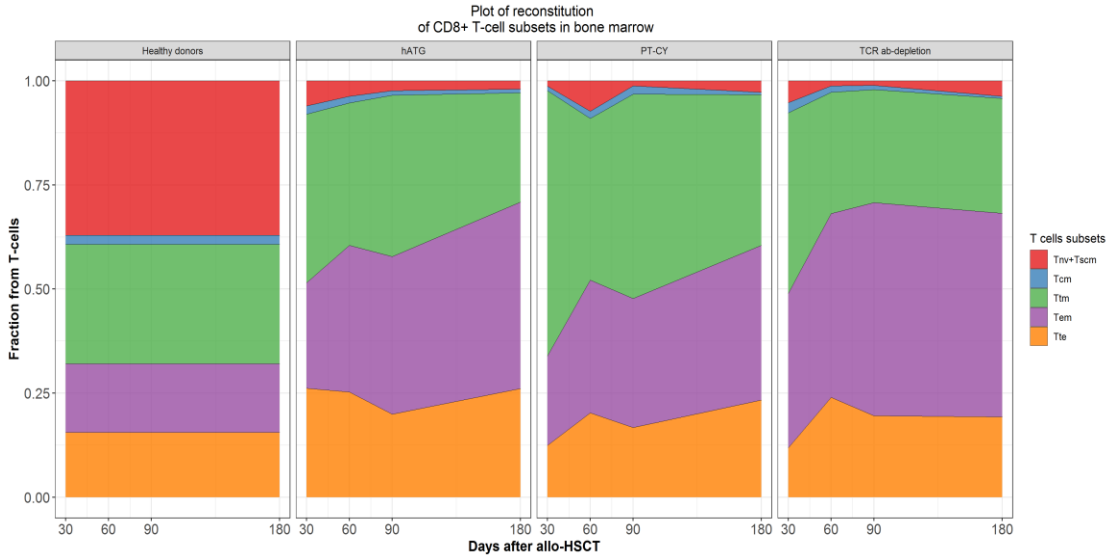

Figure 5. Distribution of BM CD8⁺ T memory cell subsets in patients after allo-HSCT and the healthy individuals

The graph shows the percentage of cell populations among CD8⁺ T cells for patients after allo-HSCT, separately for the horse ATG group, horse ATG combined with PT-Cy, *ex vivo* T cell depletion, and healthy controls.

Impact of types of T memory cell recovery on the development of leukemia relapse after allo-HSCT

We compared the absolute numbers of Tnv+scm, Tcm, Ttm, Tem, and Tte in peripheral blood and the relative counts of BM resident T cells on day +30 in two patient groups. The first group comprised patients with durable CR after allo-HSCT (n=51), and the second group comprised patients who relapsed after day +30 (n=14). According to our data, reconstitution of memory T cell subsets in the peripheral blood and BM had no impact on the development of leukemia relapse after allo-HSCT (Table 5).

Impact of different T memory cells' recovery on development of acute GVHD

We compared the absolute number of T cells on day +30 between two patient groups. The first group comprised patients without acute GVHD (n=47), and the second group comprised patients with acute GVHD after day +30 (n=17). One patient was excluded from the analysis because of the development of acute GVHD on day +22.

The absolute number of CD8⁺ Tnv+scm cells in peripheral blood on day +30 was significantly higher in the group with acute GVHD after day +30 (p=0.008), and the total count of CD8⁺ T cells did not differ between the two groups. Therefore, ROC analysis was conducted to determine the cutoff for CD8⁺ Tnv+scm in the peripheral blood on day +30. If the CD8⁺ Tnv+scm count did not exceed 1.31 cells per, the probability of acute GVHD was 12.9% versus 46.2%, p=0.008.

No statistical differences were observed in the absolute counts of Tcm, Ttm, Tem, and Tte in the peripheral blood on day +30 between the two groups.

Table 5. Impact of different T memory cell counts on developing leukemia relapse after allo-HSCT

Subsets	Cell count on day +30 after allo-HSCT, median (percentile 25–percentile 75)		P
	Relapse after day +30	Durable CR after allo-HSCT	
	$CD4^+$ T cells of peripheral blood, absolute count		
Total of $CD4^+$	57.62 (16.70-149.22)	40.26 (14.29-87.84)	0.549
$CD4^+$ Tnv+scm	8.92 (0.58-16.10)	2.49 (0.32-21.22)	0.442
$CD4^+$ Tcm	8.22 (5.03-30.52)	6.67 (3.99-20.78)	0.807
$CD4^+$ Ttm	26.06 (10.42-30.81)	25.03 (7.29-46.65)	1.000
$CD4^+$ Tem	0.40 (0.08-2.31)	0.56 (0.13-1.35)	0.813
$CD4^+$ Tte	0.38 (0.20-0.55)	0.37 (0.06-1.12)	0.864
	$CD8^+$ T cells of peripheral blood, absolute count		
Total of $CD8^+$	23.13 (4.99-36.85)	29.02 (10.58-80.25)	0.332
$CD8^+$ Tnv+scm	0.55 (0.24-3.34)	1.13 (0.16-5.38)	0.661
$CD8^+$ Tcm	0.17 (0.10-0.77)	0.33 (0.19-1.25)	0.108
$CD8^+$ Ttm	5.64 (1.85-12.18)	9.59 (3.17-23.43)	0.323
$CD8^+$ Tem	4.55 (0.54-10.27)	5.43 (1.20-20.97)	0.273
$CD8^+$ Tte	3.08 (0.71-13.34)	4.32 (0.90-19.89)	0.558
	$CD4^+$ T cells of BM, %		
$CD4^+$ Tnv+scm	7.17 (3.34-21.58)	8.52 (3.08-14.96)	0.988
$CD4^+$ Tcm	15.15 (11.15-19.13)	14.10 (8.02-24.71)	0.876
$CD4^+$ Ttm	62.31 (53.59-72.34)	52.98 (46.81-64.74)	0.379
$CD4^+$ Tem	3.17 (1.06-7.77)	2.22 (1.52-4.77)	0.957
$CD4^+$ Tte	1.14 (0.92-5.49)	1.85 (0.62-6.32)	0.869
	$CD8^+$ T cells of BM,%		
$CD8^+$ Tnv+scm	2.29 (1.24-4.78)	4.47 (1.96-10.75)	0.073
$CD8^+$ Tcm	1.13 (0.82-2.18)	1.66 (0.75-3.31)	0.514

DISCUSSION

The immunological aspects of allo-HSCT are based on the formation of GVL without GVHD. Studies have described the phenomenon of GVL [26-29]. The role of T cells, especially $CD4^+$ T cells, in the mechanism of GVL was discussed in 1997 by Barrett [41]. However, much has been clarified since then, and the role of other immune cells such as dendritic cells or NK has been shown. The key aspect of developing GVL still belongs to T cells because of their ability to implement pathogen-specific immunologic effects, including anti-leukemic effects[42,43]. This phenomenon is known as adaptive immunity, in which the main participants are memory cells [44].

A distinctive feature of true memory cells (Tscm and Tcm) is their ability to proliferate in the BM without the need for permanent antigenic stimulation and a prompt reaction against the antigen[30,31]. We hypothesized that donor memory T cells, mostly Tcm, could be a key subset responsible for implementing the anti-leukemic effect shortly after allo-HSCT. The same effect is observed after chimeric antigen receptor (CAR) T cell therapy when long-lasting circulating Tcm provides durable remission[32]. However, our data demonstrate that low numbers of Tcm are in the peripheral blood of patients on days +30 to +90 after allo-HSCT, particularly in the case of the addition of PT-Cy for GVHD prophylaxis or when using a manipulated graft with *ex vivo* T cell depletion. There was also no difference in the absolute count of circulating Tcm in patients in CR or those who relapsed after allo-HSCT, which questioned the ability of GVL formation only with circulating donor Tcm. Moreover, a shift toward the prevalence of the effectors and reduced number of naïve and central memory cells in the BM of patients after allo-HSCT could reflect the phenomenon of homeostatic proliferation rather than the formation of antitumor immunity [33, 34].

Tcm is considered the only pool providing immune recovery without the risk of inducing GVHD[35]. This phenomenon is possible because of the ability of Tcm to proliferate and generate a pool of Tem and Tte that subsequently react against the antigen. However, transplantation of selective T effector cells fails because of the inability of Tem and Tte to proliferate and self-regenerate[36,37]. Some studies have demonstrated that naïve T cells and memory T stem cells are the only subpopulations that provide full immune recovery, including generation of effectors and formation of immunological memory[38]. Therefore, we propose that a higher count of Tnv+scm early after allo-HSCT and its prompt recovery, together with the generation of Tcm, provide essential anti-leukemia effects.

However, other studies have shown that alloreactive T cells that cause severe GVHD are naïve T cells, due to the ability of these cells to migrate and proliferate in secondary lymphoid tissues[39,40]. The maximum count of these naïve-like alloreactive T cells is observed on days +3 to +6 after allo-HSCT, explaining the use of PT-Cy to prevent GVHD[18,39,40]. The mechanism of ATG is less selective than that of PT-Cy and shows the possibility of total lymphodepletion[20,21]. Our data demonstrate that ATG alone failed to deplete naïve T cell compartment compared with PT-Cy or *ex vivo* T cell depletion, which seems to be an immunological reason for mixed chimerism and the next requirement of donor lymphocyte infusion in this patient cohort. This finding can explain the high frequency of GVHD in patients after fully matched allo-HSCT with ATG-based prophylaxis (40.6% vs. 11.1% after ATG+PT-Cy and 20% after *ex vivo* T cell depletion) and 38.5% steroid-refractoriness.

The role of naïve T cells as GVHD triggers has been discussed[39,40], and together with our results, and considering the depletion of these cells as an effective way to prevent GVHD seems appropriate. According to our data, depletion of Tnv+scm by PT-Cy is comparable in quantitative cellular assessment and the rate of acute GVHD to the effect of *ex vivo* T cell depletion. However, regardless of GVHD risk, the reconstitution of this cell population is a key aspect for the recovery of whole T cell immunity and the formation of immunological memory [38]. Despite the similar immunoablative effects in the study groups with PT-Cy and *ex vivo* T depletion, the dynamics of T cell recovery significantly differed. The reconstitution involving Tnv+scm, Tcm, and the effector pool (Ttm, Tem, Tte) occurs after *ex vivo* TCR αβ depletion, and the effect of PT-Cy seems to be more selective than that and affects only the effector pool. However, differences in the dynamics of T cell

recovery in the study groups had no influence on clinical outcomes. The rates of acute GVHD and leukemia relapse were comparable after ATG+PT-Cy and *ex vivo* T cell depletion.

In summary, we can conclude that the reconstitution of T cell immunity depends on many factors. In this research, we analyzed the impact of GVHD prophylaxis and demonstrated that its choice might determine future events such as acute GVHD.

CONFLICT OF INTEREST

The authors have no relevant conflicts of interest to disclose.

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