Immune reconstitution dynamics after unrelated allogeneic transplantation with post-transplant cyclophosphamide compared to classical immunosuppression with anti-thymocyte globulin: a prospective cohort study

Mariana Nassif Kerbauy,[1] Fernanda Agostini Rocha,[2] Leonardo Javier Arcuri,[1] Priscila Silva Cunegundes,[2] Lucila Nassif Kerbauy,[1] Clarisse Martins Machado,[2,3] Andreza Alice Feitosa Ribeiro,[1] Pinaki P. Banerjee,[4] Luciana Cavalheiro Marti[2#] and Nelson Hamerschlak[1#]

[1]Department of Hematology and Bone Marrow Transplantation, Hospital Israelita Albert Einstein, São Paulo, Brazil; [2]Instituto Israelita de Ensino e Pesquisa (IIEP), Hospital Israelita Albert Einstein, São Paulo, Brazil; [3]Virology Laboratory, Institute of Tropical Medicine, University of São Paulo, São Paulo, Brazil and [4]The University of Texas MD Anderson Cancer Center, Houston, TX, USA

[#]LCM and NH contributed equally as senior authors.

Correspondence: M. N. Kerbauy
mariana.kerbauy@einstein.br

Received: May 21, 2024.
Accepted: September 3, 2024.
Early view: September 12, 2024.

https://doi.org/10.3324/haematol.2024.285921

Abstract

Post-transplant cyclophosphamide (PTCy) has contributed to the success of haploidentical hematopoietic stem cell transplantation (HSCT) and is also used in transplantation from matched donors. However, limited data on the immune reconstitution after this type of immunosuppression is available. We aimed to evaluate immune reconstitution after HSCT from unrelated donors, comparing anti-thymocyte globulin (ATG) and PTCy. Consecutive patients undergoing HSCT from unrelated donors and receiving either ATG or PTCy were prospectively included. Immune reconstitution analyses were performed by flow cytometry pre-transplant and on days 30, 60, 90, and 180 post-transplant. We included 36 patients, 20 in the ATG group and 16 in the PTCy group. In the early post-transplant period (day [d]+30), the ATG group showed a higher number of total lymphocytes, T, B, and natural killer (NK) cells compared to the PTCy group. However, at d+180, the PTCy group exhibited a higher number of B cells. On d+60 and d+90, the ATG group displayed higher number of NK cells $CD56^{dim}$ compared to the PTCy group, while on d+180, the PTCy group showed higher number of $CD56^{-}$, $CD16^{+}$, and, $NKG2D^{+}$ NK cells. Naive $CD4^{+}$, transition $CD4^{+}$, and naive $CD8^{+}$ T cells on d+60 were identified as risk factors for acute graft-*versus*-host disease grade 2-4, and a higher count of $CD4^{+}$ memory cells on d+180 was identified as a risk factor for chronic graft-*versus*-host disease. In the context of unrelated allogeneic transplantation, immunosuppression with PTCy was associated with later B-, T- and NK-cell reconstitution compared to ATG.

Introduction

Different immunosuppression protocols are employed in allogeneic hematopoietic stem cell transplantation (HSCT) to mitigate the acute graft-*versus*-host disease (aGVHD) and chronic GVHD (cGVHD) risk. These protocols are especially used in the scenario of transplants from unrelated donors, in which classical immunosuppression with calcineurin inhibitor and methotrexate are associated with high GVHD risk.[1] Some studies have shown that the association of anti-thymocyte globulin (ATG) in transplant protocols results in reduction in grade 2-4 aGVHD and moderate to severe cGVHD.[2-5]

The use of post-transplant cyclophosphamide (PTCy) has revolutionized haploidentical HSCT, with impressive outcomes related do GVHD, while it is relatively simple, cost-effective, easily reproducible, and eliminates the need for expensive T-cell depletion techniques. The PTCy strategy, when compared with haploidentical transplantation using depleted *in vitro* T cells, yields better outcomes related to infectious complications.[6-8] Given the encouraging outcomes observed in haploidentical HSCT, especially concerning GVHD, and the inherent assumption that PTCy could mitigate GVHD following

a matched transplant, some trials are being conducted to investigate the value of PTCy in HLA-matched transplants,[9-14] with encouraging results in the context of myeloablative conditioning regimens,[15,16] or reduced-intensity conditioning.[17] Poor immune reconstitution post-allogeneic HSCT is directly related to significant post-transplant morbidity and mortality, including viral, bacterial, and fungal infections, as well as relapse of the underlying disease. T-specific immunity is affected by a combination of factors, including the conditioning regimen used, thymic involution, patient age, graft type, type of T-cell depletion (*in vivo* or *in vitro*), HLA mismatches between donor and patient, immunosuppression used, and GVHD.[18] In general, innate immunity recovers early after transplant, initially with the recovery of monocytes, followed by neutrophils. Natural killer (NK) are the first lymphoid cells to reconstitute and remain the predominant lymphocytes in circulation in the first 3 months post-transplant[19] and its early reconstitution may be associated with transplant outcomes.[20] The recovery of adaptive immunity, especially T lymphocytes and B lymphocytes, is slower, potentially longer than 1 year.[21] The reconstitution of T lymphocytes occurs through two main mechanisms: i) survival and peripheral expansion of donor-infused memory T lymphocytes, or ii) generation of donor T lymphocytes via the thymus from donor hematopoietic precursors.

However, immune reconstitution in the context of unrelated transplant, using PTCy, has still been little studied, with limited data comparing immune reconstitution with this immunosuppression strategy to the classical approach with methotrexate, calcineurin inhibitor, and ATG. The assessment of immunological profiles may allow preventive measures after transplant. Thus, our objectives were to evaluate the kinetics of immune reconstitution post-allogeneic HSCT from unrelated donors with two types of immunosuppression, ATG or PTCy. In addition, we aimed to correlate the impact of the immune reconstitution with complications in HSCT.

Methods

Study design, setting, and ethics

In this prospective cohort study, we included patients undergoing allogeneic HSCT from unrelated donors using immunosuppression with ATG or PTCy in the years 2019 to 2021 in a single private tertiary hospital (Hospital Israelita Albert Einstein). The study protocol was approved by the local ethics committee (CAAE 00527018.6.0000.0071). Informed consent for inclusion in this study was obtained from all research participants.

Participants' eligibility and recruitment for this study

We included patients of any age undergoing their first allogeneic HSCT for hematological diseases with an unrelated donor, using an immunosuppression protocol with ATG or PTCy. The choice for one or another approach was based on the allocation in a concurrent study at the same institution, a clinical trial in which patients were randomized between ATG and PTCy (*clinicaltrials gov. Identifier: NCT03818334*) or the clinical choice of the bone marrow transplant team. We excluded cases in which both patient and donor had negative pre-transplant cytomegalovirus (CMV) serology. Clinical evaluations and treatments may be found in the *Online Supplementary Appendix*.

Immune reconstitution monitoring

Patients' peripheral blood samples were collected pre-transplant and on day (d)+30, d+60, d+100 and d+180 post-transplant. Flow cytometric immunophenotyping for immune recovery was performed prospectively. For the analysis of mononuclear cells, an initial gate on CD45 was used. For T cells, CD3 and subsets CD3CD4 and CD3CD8 were used. For NK cells, $CD3^-$, $CD14^-$, $CD11b^-$, $CD19^-$, $CD56^+$ or $CD56^-$, $CD16^+$ and $NKG2D^+$ were used, and for B cells, $CD3^-$, $CD14^-$, $CD11b^-$, $CD19^+$. Monocytes included $CD3^-$, $CD14^+$ and $CD11b^+$. A subset analysis of $CD4^+$ and $CD8^+$ naive ($CD45RA^+$), memory ($CD45RO^+$) and transitional T cells ($CD45RA^{+/-}CD45RO^{+/-}$) was also performed. We also analyzed some patients data using the Cytobank platform to support and illustrate the findings of flow cytometry.[22]

Outcomes

The primary endpoint in this study was B-, T-, and NK-cell immune recovery in patients undergoing unrelated allogeneic transplantation using PTCy or ATG. The secondary endpoints studied included overall survival, cumulative incidence of treatment-related mortality (TRM), 100-day mortality, aGVHD, CMV reactivation, and disease.

Statistical analysis

Categorical variables were summarized using frequencies and percentages, and continuous variables using means and standard deviations, or medians and ranges where appropriate, as well as the number of observations.

To compare categorical variables, the χ^2 or the Fisher's exact tests were used, as appropriate. To compare continuous variables, the parametric Student's *t* test was used, if applicable, or the non-parametric Mann-Whitney test.

Overall survival was defined from the date of HSCT to the patient's death, and was estimated using the Kaplan-Meier method, with patients alive at the time of data collection being censored. The log rank test was used for comparison of survival curves. Cumulative incidence curves (non-relapse mortality and relapse) were built with the Gray method. For relapse, non-relapse mortality was the competing event, and *vice versa*. For uni and multivariable analyses for survival outcomes, we used the Cox model.

Statistically significant values were defined as $P \leq 0.05$. Statistical tests were performed using the R (Vienna, Austria) statistical software, version 4.3.2.

Results

Clinical outcomes

The demographic characteristics and clinical outcomes of the included population and their donors are reported in Table 1. At 180 days post-transplant, aGVHD grade 2-4 occurred in 40% of the ATG group and 25% of the PTCy group (*P*=0.26). cGVHD at 3 years was 31% for ATG and 19% for PTCy (*P*=0.19) (*Online Supplementary Figures S1, S2*). In a follow-up of 3.25 years, overall survival was 59% for ATG and 74% for PTCy (*P*=0.4) (*Online Supplementary Figure S3*). CMV reactivation rates were 70% in the ATG group and 75% in the PTCy group within 180 days (*P*=0.98) (*Online Supplementary Figure S4*), with similar median viral loads (median 959 Ui/mL in ATG and 932 Ui/mL in PTCy group), *P*=0.81. Second CMV reactivation occurred in 20% of ATG patients and 6% of PTCy patients, with no CMV-related disease in the ATG group compared to three cases in the PTCy group. Other viral infections are described in the *Online Supplementary Appendix*.

Table1. Patient characteristics and clinical outcomes.

Variable	ATG N=20	PTCY N=16	*P*
Age in years, median (range)	45 (2-76)	37.8 (3-66)	0.319
Performance status, N (%) Karnofsky <90 % Karnofsky 90-100 %	 6 (30) 14 (70)	 2(12.5) 14 (87.5)	0.257
Diagnosis, N (%) AML MDS ALL CML Non-Hodgkin lymphoma Others	 7 (35) 4 (20) 2 (10) 2 (10) 1 (5) 1 (5)	 5 (31) 4 (25) 4 (25) 3 (18.7) 2 (12.5) 2 (2.5)	0.479
Status of response, N (%) 1st CR 2nd CR Active disease	 13 (65) 2 (10) 5 (25)	 12 (75) 1 (6.2) 3 (18.8)	0.872
CMV sorology, N (%) Patient IgG+ Donor IgG+	 19 (95) 18 (90)	 14 (87.5) 14 (87.5)	1.000
Conditioning regimen, N (%) Myeloablative RIC	 12 (60) 8 (40)	 14 (87.5) 2 (12.5)	0.133
Graft source, N (%) BM PBSC	 9 (45) 11 (55)	 10 (62.5) 6 (37.5)	0.296
Stem cell infused CD34 x10^6/kg, median (range)	5.4 (1.54-9)	4.6 (1.81-9.15)	0.286
Donor age in years, median (range)	33.1 (19-55)	33.7 (22-49)	0.833
Donor sex Male, N (%)	 17 (85)	 10 (62.5)	0.146
Neutrophil engraftment in days, median (range)	12 (11-19)	16 (12-21)	0.0001
Length of hospitalization in days, median (range)	33 (17-103)	33 (28-67)	0.26
Calcineurin inhibitor use in days, median (range)	355 (10-1,053)	279 (59-550)	0.3

ATG: anti-thymocyte globulin; PTCy: post-transplant cyclophosphamide; AML: acute myeloid leukemia; MDS: myelodysplastic syndrome; ALL: acute lymphocytic leukemia; CML: chronic myeloid leukemia; CR: complete remission; CMV: cytomegalovirus; RIC: reduced intensity conditioning; BM: bone marrow; PBSC: peripheral blood stem cells.

Immune reconstitution

There was no difference between the ATG and PTCy groups in terms of pre-transplant lymphomononuclear cell profile (represented in Table 2; Figures 1, 2; *Online Supplementary Figure S5*). On d+30, in the ATG group, there was a signifi-

Table 2. Lymphomononuclear profile.

Variable	ATG cells/µL, median (IQR)	PTCy cells/µL, median (IQR)	*P*
Pre Monocytes Lymphocytes T lymphocytes B lymphocytes NK cells	 156 (45-294) 1,177 (565-4,396) 648 (417-971) 42 (16-183) 126 (90-242)	 202 (136-303) 1,372 (68-6,486) 717 (314-1,141) 166 (3-372) 146 (60-294)	 0.466 0.459 0.890 0.835 0.972
d+30 Monocytes Lymphocytes T lymphocytes B lymphocytes NK cells	 379 (212-477) 924 (282-3,278) 238 (111-801) 17 (4-27) 385 (241-718)	 892 (469-1,362) 473 (176-864) 82 (47-160) 2 (1-7) 96 (59-196)	 0.037 0.0004 0.022 0.004 <0.001
d+60 Monocytes Lymphocytes T lymphocytes B lymphocytes NK cells	 204 (74-386) 946 (504-3,268) 406 (174-717) 50 (13-60) 319 (184-597)	 299 (132-422) 706 (261-1,407) 190 (110-420) 16 (4-98) 203 (128-278)	 0.488 0.020 0.100 0.338 0.074
d+90 Monocytes Lymphocytes T lymphocytes B lymphocytes NK cells	 192 (127-387) 1,193 (342-2,464) 368 (263-839) 37 (14-81) 282 (185-440)	 289 (117-588) 874 (211-3,141) 359 (140-886) 50 (22-130) 221 (146-311)	 0.428 0.415 0.597 0.571 0.082
d+180 Monocytes Lymphocytes T lymphocytes B lymphocytes NK cells	 195 (83-361) 976 (161-3,821) 513 (304-602) 54 (13-87) 294 (198-517)	 144 (77-557) 1,688 (485-3,909) 925 (431-1,416) 282 (138-553) 257 (155-245)	 0.790 0.085 0.344 0.003 0.790

Pre: pre-transplant; d: day; ATG: anti-thymocyte globulin; PTCy: post-transplant cyclophosphamide; NK: natural killer cells.

cantly higher number of total lymphocytes (924 cells/μL *vs*. 473 cells/μL; *P*=0.0004), T lymphocytes (238 cells/μL *vs*. 82 cells/μL; *P*=0.022), B lymphocytes (17 cells/μL *vs*. 2 cells/μL; *P*=0.004), and NK cells (385 cells/μL *vs*. 96 cells/uL; *P*<0.001) compared with the PTCy group (2 illustrative cases of patients from the ATG and PTCy groups, using the Cytobank platform, are represented in Figure 3 and the *Online Supplementary Appendix*). On d+180, there was a higher count of B cells in the PTCy group (54 cells/μL *vs*. 282 cells/uL; *P*=0.003).

The subtypes of T cells are represented in Table 3. A predominance of CD8 T cells was observed compared to CD4 T cells from the early stages of transplantation, persisting until d+180 in both analyzed groups. We observed a significantly higher population of double-positive $CD4^+$ and $CD8^+$ T cells in the PTCy group compared to the ATG group on d+180 (21 cells/μL *vs*. 98 cells/μL; *P*=0.003) (*Online Supplementary Figure S6*).

Among the subsets of T cells, we found no difference between the groups regarding the profile of $CD4^+$ and $CD8^+$ T cells. Pre-transplant, there was a predominance of $CD4^+$ memory T lymphocytes and naive $CD8^+$ T lymphocytes in both groups. On d+30, in both groups, there was a predominance of $CD4^+$ and $CD8^+$ memory T lymphocytes. On d+60 and d+90 in both groups, we observed a persistence of predominance of $CD4^+$ memory T cells, but with an

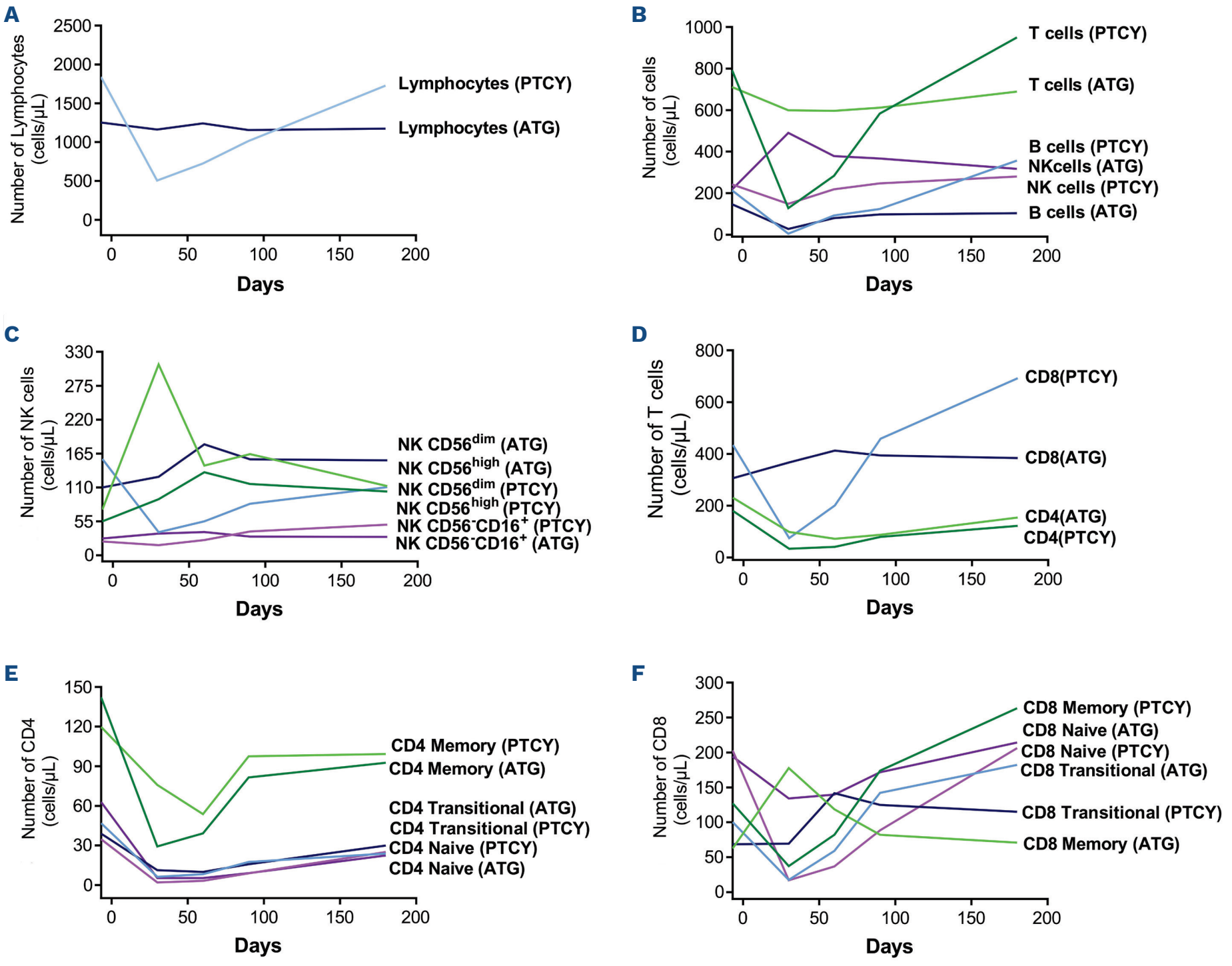

Figure 1. Total lymphocytes, T-, B-, and natural killer-lymphocyte reconstitution. (A) Earlier lymphocyte reconstitution was observed in the anti-thymocyte globulin (ATG) group; however, there was a greater increase in lymphocytes in the post-transplant cyclophosphamide (PTCy) group after day (d)+100. (B) There was a significant early reduction in T, B, and natural killer (NK) lymphocytes in the PTCy group on d+30, while B-lymphocyte recovery was observed on d+180. (C) In NK-cell subsets, $CD56^{dim}$, CD56 ^{high} and $CD56^-CD16^+$ was higher in the ATG group and there was higher, and higher counts of $CD56^{dim}$ on d+60 and d+90 in ATG group compared with PTCy. (D) There was an inversion of the CD4/CD8 ratio in both groups. (E) $CD4^+$ T cells were predominantly represented by memory $CD4^+$ cells in both groups. (F) Compared do $CD4^+$ cells, there was earlier recovery of transitonal and naive $CD8^+$ cells in both groups.

increase in naive and transitional CD8⁺ T cells, while on d+180, naive CD8⁺ T cells were predominant in both groups (*Online Supplementary Figures S7, S8*; *Online Supplementary Table S1*).

An analysis of NK-cell subsets was conducted based on the expression of $CD56^{high}$ or $CD56^{dim}$ and the expression of CD16 (Table 4). On d+30, there was a significantly higher number of all NK-cell subtypes in the ATG group. On d+60 and d+90, there was a higher number of $CD56^{dim}$ cells in the ATG group. On d+180, there was a significantly higher number of $CD56^{-}$ NK cells ($CD3^{-}$, $CD11b^{-}$, $CD14^{-}$, $CD19^{-}$, $CD56^{-}$, $CD16^{+}$, $NKG2D^{+}$) in the PTCy group (Figure 4).

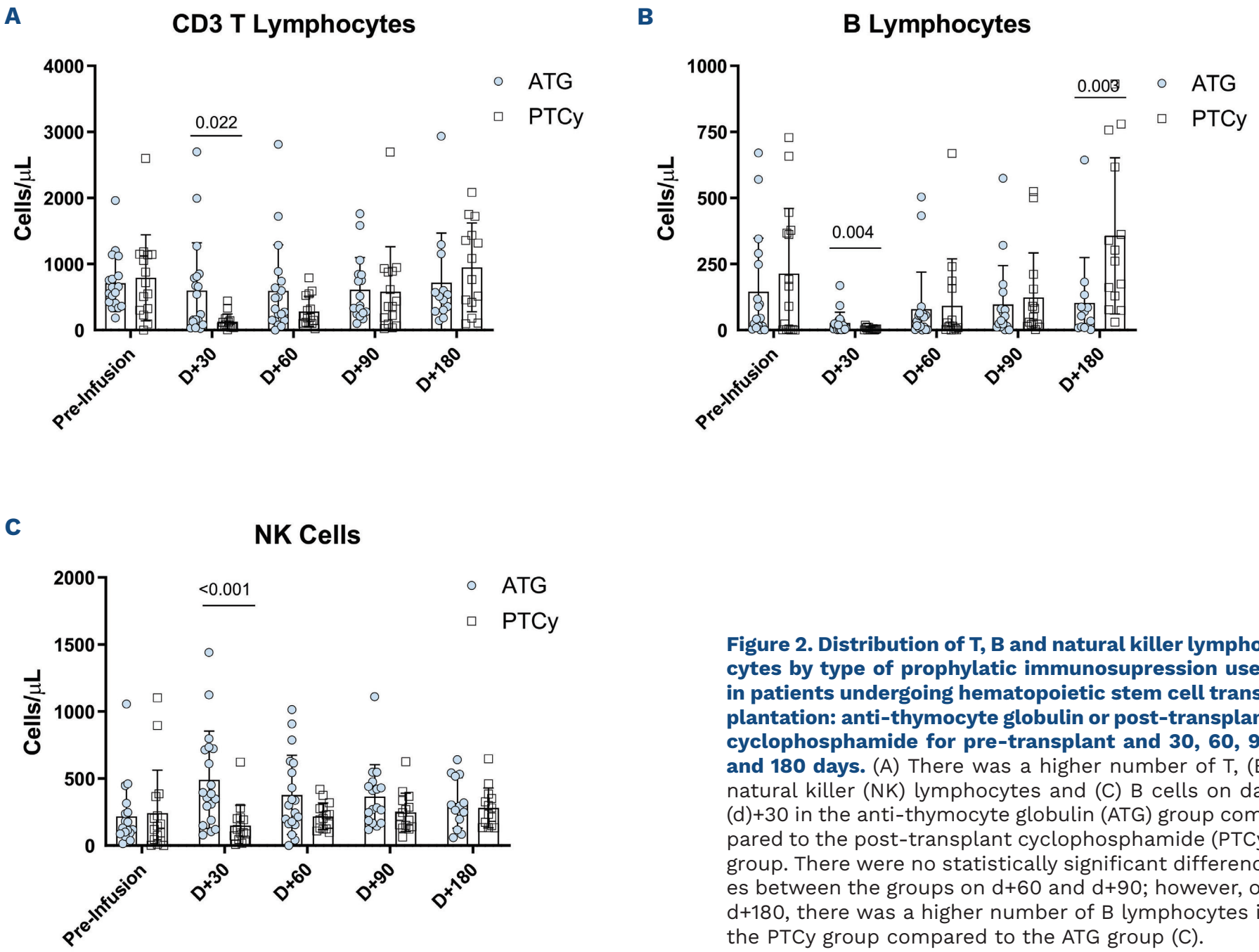

Figure 2. Distribution of T, B and natural killer lymphocytes by type of prophylatic immunosupression used in patients undergoing hematopoietic stem cell transplantation: anti-thymocyte globulin or post-transplant cyclophosphamide for pre-transplant and 30, 60, 90 and 180 days. (A) There was a higher number of T, (B) natural killer (NK) lymphocytes and (C) B cells on day (d)+30 in the anti-thymocyte globulin (ATG) group compared to the post-transplant cyclophosphamide (PTCy) group. There were no statistically significant differences between the groups on d+60 and d+90; however, on d+180, there was a higher number of B lymphocytes in the PTCy group compared to the ATG group (C).

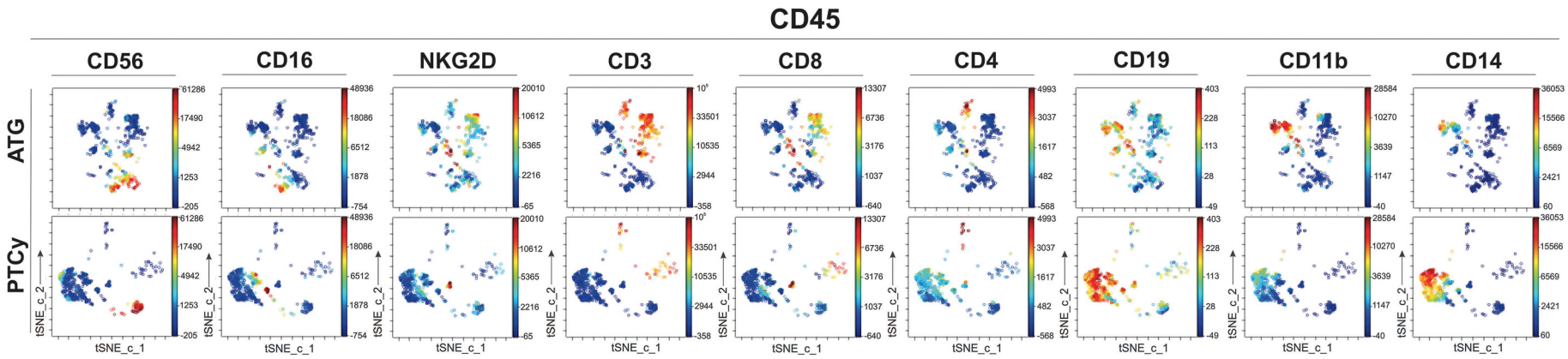

Figure 3. Cell clusters from the Cytobank platform showing differences between two patients, one who received anti-thymocyte globulin and another who received post-transplant cyclophosphamide regarding markers of B, T, natural killer cells and monocytes on the day +30. In the patient receiving anti-thymocyte globulin (ATG), there is a greater aggregate of $CD56^{+}$ natural killer (NK) cells, a higher aggregate of $CD3^{+}$ T cells, mainly represented by $CD8^{+}$ cells, while in the patient who received post-transplant cyclophosphamide (PTCy), a higher population of $CD14^{+}$ and $CD11b^{+}$ monocytes can be visualized.

Impact of immune reconstitution on clinical outcomes

Performance status of 90 or 100 in the Karnofsky scale was a protective factor for overall survival (hazard ratio [HR]=0.3, 95% confidence interval [CI]: 0.1-0.96; *P*=0.042), and a higher absolute number of transitional CD4$^+$ cells was associated with worse survival (HR=1.01, 95% CI: 1.01-1.04; *P*=0.0083).

Higher naive CD4$^+$ cells (HR=1.04, 95%: CI 1-1.09; *P*=0.047), CD4$^+$ cells transitional stage (HR=1.04, 95% CI: 1.01-1.07; *P*=0.012), and naive CD8$^+$ cells (HR=1, 95% CI: 1-1.01; *P*=0.026) on d+60 were risk factors associated with aGVHD grade 2-4. On d+180 post-transplant, a higher quantity of memory CD4$^+$ cells (HR=1.01, 95% CI: 1-1.02; *P*=0.014) was a risk factor for cGVHD. We also analyzed the impact of aGVHD grade 2-4 in immune reconstitution and found that patients who had GVHD had lower lymphocytes count on d+60 (939 cells/μL *vs*. 476 cells/μL; *P*=0.009), lower B cells on d+90 (78.8 cells/uL *vs*. 19.8 cells/μL; *P*=0.042) and lower CD4$^+$ memory T cells on d+90 (76.5 cells/μL *vs*. 36.8 cells/uL; *P*=0.025) (*Online Supplementary Table S2*).

Regarding graft source, peripheral blood, compared to bone marrow, was associated with higher counts of naïve CD4$^+$ T cells on d+30 (2.24 cells/μL *vs*. 0.44 cells/μL; *P*=0.03), higher counts of T cells on d+60 (523 cells/μL vs. 188 cells/μL; *P*=0.03), higher CD4$^+$ T cells on d+60 (39 cells/μL *vs*. 22 cells/μL; *P*=0.04), higher CD8$^+$ T cells on d+60 (89 cells/μL *vs*. 30 cells/μL; *P*=0.01) and higher counts of NK cells CD56^{dim} on d+60 (105 cells/μL *vs*. 47 cells/μL; *P*=0.04). We found no strong or median correlation with CD34 product counts and immune reconstitution on Spearman correlation test.

We also analyzed the impact of conditioning regimen on immune reconstitution and found that reduced intensity conditioning, compared with myeloablative, was associated with higher counts of CD4$^+$ naïve T cells on d+30 (3.86 cells/μL *vs*. 0.53 cells/μL; *P*=0.011), higher NK CD56^{high} on d+30 (287.8 cells/μL *vs*. 79.4 cells/μL; *P*=0.01) and NK CD56^{dim} on d+60 (137 cells/μL *vs*. 52.9 cells/μL; *P*=0.03) while myeloablative conditioning was associated with higher NK cells CD56$^-$, CD16$^+$ on d+90 (20.7 cells/μL *vs*. 9.21 cells/μL; *P*=0,03) and higher double-positive CD4 and CD8 T cells on d+90 (34 cells/μL *vs*. 7.4, cells/μL; *P*=0.005).

Discussion

In the present study, we prospectively analyzed patients with onco-hematological diagnoses who underwent allogeneic transplantation with unrelated donors and compared two different post-transplant immunosuppression approaches (classical immunosuppression with ATG or PTCy) according to post-transplant immune reconstitution. Early after the transplant, at d+30, the ATG group exhibited a higher number of total lymphocytes including T, B, and NK lymphocytes, as well as CD56^{high}, CD56^{dim} and CD56$^-$, CD16$^+$,

Table 3. T-cell subtypes.

Variable	ATG	PTCy	*P*
	cells/μL, median (IQR)	cells/μL, median (IQR)	
Pre			
CD4$^+$CD8$^-$	285 (113-312)	107 (50-257)	0.127
CD8$^+$CD4$^-$	219 (144-242)	325 (130-559)	0.466
CD4$^+$CD8$^+$	34 (10-47)	91 (29-167)	0.048
d+30			
CD4$^+$CD8$^-$	28 (12-125)	13 (6-24)	0.083
CD8$^+$CD4$^-$	82 (21-435)	47 (22-83)	0.270
CD4$^+$CD8$^+$	14 (3-39)	8 (5-16)	0.551
d+60			
CD4$^+$CD8$^-$	44 (22-92)	23 (20-32)	0.116
CD8$^+$CD4$^-$	316 (103-550)	95 (48-324)	0.174
CD4$^+$CD8$^+$	17 (7-36)	20 (13-28)	0.970
d+90			
CD4$^+$CD8$^-$	56 (47-132)	49 (20-125)	0.521
CD8$^+$CD4$^-$	265 (159-550)	255 (68-505)	0.514
CD4$^+$CD8$^+$	17 (6-35)	33 (10-47)	0.260
d+180			
CD4$^+$CD8$^-$	125 (43-173)	91 (54-135)	0.827
CD8$^+$CD4$^-$	240 (202-353)	678 (170-1,035)	0.320
CD4$^+$CD8$^+$	21 (14-30)	98 (57-134)	0.003

Pre: pre-transplant; d: day; ATG: anti-thymocyte globulin; PTCy: post-transplant cyclophosphamide.

Table 4. Subsets of natural killer cells.

Variable	ATG	PTCy	*P*
	cells/μL, median (IQR)	cells/μL, median (IQR)	
Pre			
CD56^{dim}	58 (29-105)	60 (14-139)	0.890
CD56^{high}	65 (33-126)	44 (24-70)	0.367
CD56$^-$CD16$^+$NKG2D$^+$	7 (2-14)	27 (8-36)	0.061
d+30			
CD56^{dim}	108 (72-176)	25 (9-48)	<0.001
CD56^{high}	177 (123-464)	62 (10-121)	0.003
CD56$^-$CD16$^+$NKG2D$^+$	24 (4-47)	3 (2-23)	0.041
d+60			
CD56^{dim}	158 (55-232)	50 (39-59)	0.009
CD56^{high}	116 (86-155)	136 (67-183)	0.986
CD56$^-$CD16$^+$NKG2D$^+$	21 (8-51)	10 (4-24)	0.164
d+90			
CD56^{dim}	148 (81-234)	68 (41-88)	0.009
CD56^{high}	119 (76-188)	90 (68-164)	0.450
CD56$^-$CD16$^+$NKG2D$^+$	19 (9-37)	21 (15-50)	0.326
d+180			
CD56d^{im}	120 (55-228)	87 (52-127)	0.320
CD56h^{igh}	133 (63-157)	89 (64-131)	0.790
CD56$^-$CD16$^+$NKG2D$^+$	10 (4-19)	24 (19-37)	0.027

Pre: pre-transplant; d: day; ATG: anti-thymocyte globulin; PTCy: post-transplant cyclophosphamide.

NKG2D$^+$ NK cells, compared with the PTCy group. However, we observed a greater increase in the total lymphocyte count in the PTCy group compared to the ATG group after d+90, mainly represented by CD8$^+$ T lymphocytes. Additionally, there was a statistically significant higher number of B lymphocytes and CD56$^-$, CD16$^+$, NKG2D$^+$ NK lymphocytes in the PTCy group at d+180.

Rambaldi *et al.*[23] also assessed immune reconstitution in patients undergoing haploidentical transplantation with PTCy or matched transplantation with classic prophylaxis. Their study described a delayed reconstitution of T cells, with a significant reduction in naive T lymphocytes, and an early decrease in NK cells following haploidentical HSCT with PTCy. Furthermore, the authors observed a preferential expansion of immature CD56^{bright}CD16 NK cells in the haploidentical group, compared to classic immunosuppression prophylaxis. The later reconstitution of total lymphocytes, B, T, and NK cells in the PTCy group may explain the higher grade 2 infection rate observed in the PTCy group in the phase III study comparing PTCy and classical immunosuppression in matched and mismatched transplants with a reduced-intensity conditioning regimen[17] or the higher incidence of CMV reactivation in haploidentical and matched sibling transplants with PTCy compared to classical immunosuppression in sibling transplants in the retrospective CIBMTR analysis.[24]

In the present study, at d+60 and d+90, the ATG group showed a higher number of mature NK cells (CD56^{dim}) compared to the PTCy group. Early-reconstituted NK cells exhibit an immature CD56^{bright} phenotype, representing 40-50% of NK cells in the initial months, acquiring a predominantly CD56^{dim} phenotype after several months, whereas, in healthy adults, CD56^{bright} phenotype constitute approximately 5-10%.[25] It is believed that NK-cell reconstitution primarily derives from the differentiation and maturation of progenitor cells rather than the expansion of mature NK cells within the graft. This idea is supported by the finding that NK-cell reconstitution is mainly independent of graft type and its NK-cell content.[26] The difference found in the NK maturation profile between the groups may be explained

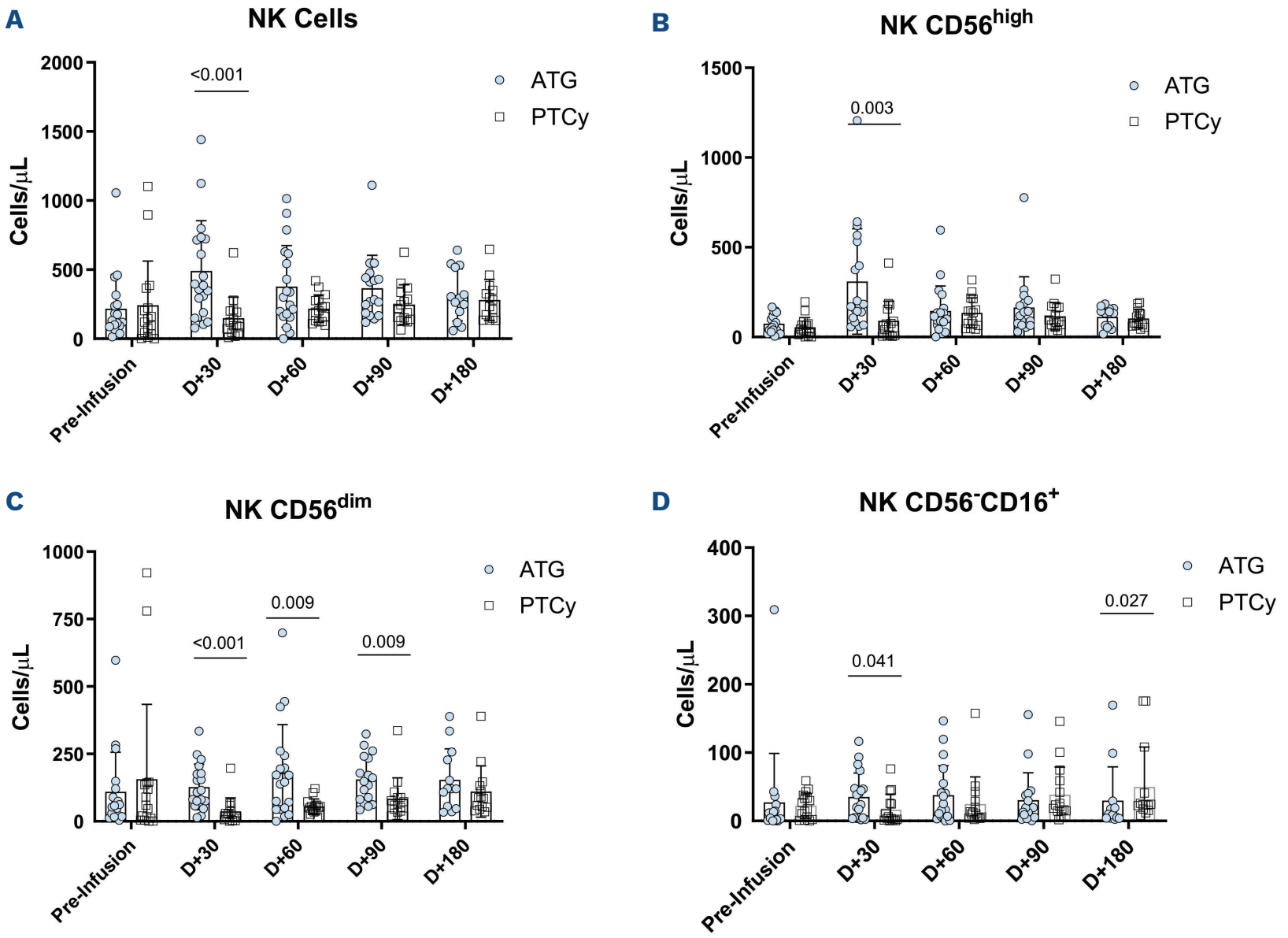

Figure 4. Distribution of natural killer cells and subtypes of CD56^{high}, CD56^{dim} and CD56$^-$CD16$^+$ natural killer cells by type of prophylatic immunosupression used in patients undergoing hematopoietic stem cell transplantation: anti-thymocyte globulin or post-transplant cyclophosphamide for pre-transplant and 30, 60, 90 or 180 days. (A) Natural killer (Nk) cells, (B) CD56^{high}, (C) CD56^{dim}, (D) CD56$^-$CD16$^+$ NK cells. There is a higher number of total NK cells and CD56^{dim}, CD56^{high}, and CD56$^-$CD16$^+$ NK cells in the anti-thymocyte globulin (ATG) group on day (d)+30, a higher number of CD56^{dim} cells in the ATG group on d+60 and d+90, and a higher number of CD56$^-$CD16$^+$ cells in the post-transplant cyclophosphamide (PTCy) group on d+180.

by the selective purging of dividing cells by PTCy, which has already been demonstrated by Russo *et al*. and may be supported by the fact that proliferating NK cells after HSCT infusion do not express aldehyde dehydrogenase and may be killed by cyclophosphamide.[27] Previous studies have already shown an immature NK phenotype in haploidentical transplantation in more intensive immunossupression with positive CD34 selection.[25,28]

In the present study, we identified a population of NK cells characterized as $CD45^{pos}$, $CD3^{neg}$, $CD11b^{neg}$, $CD14^{neg}$, $CD19^{neg}$, $CD56^{neg}$, $CD16^{pos}$, $NKG2D^{pos}$ that was higher in PTCy group on d+180. Human NK cells are generally defined as $CD3^{-}CD56^{+}$ lymphocytes. However, a subset of lymphocytes, $CD56^{-}CD16^{+}$ ($CD56^{-}$), displaying associations with NK markers, expands during chronic viral infections such as HIV-1 and HCV, and herpesvirus.[29] Many questions persist regarding the origin, development, phenotype, and function of the $CD56^{-}$ NK-cell population. Gaddy J *et al*. have shown a subset of NK cells $CD56^{-}$ $CD16^{-}$ in cord blood transplants that has low lytic activity and are possible precursors of mature NK cells.[30] A meta-analysis evaluating 757 individuals from a total of 28 studies found that $CD56^{-}$ cells constitute 5.67% of NK cells in healthy peripheral blood, while HIV-1 infection increases this population to 10.69% with no evidence of increased exhaustion or decreased proliferation within the $CD56^{-}$ subset.[31] In another study with healthy elderly donors, co-infection with CMV and EBV was associated with expansion of $CD56^{-}$ NK cells with reduced cytotoxic capacity, what correlated with end-stage-differentiated T cells and reduced CD4/CD8 ratio, reflecting immune risk profile.[32] The immune dysfunction after transplant and high prevalence of CMV and EBV serology in the population of the study may explain the identification of this NK cell in the present study.

The current study observed an increase in $CD8^{+}$ T lymphocytes, leading to inversion of the CD4/CD8 ratio. $CD4^{+}$ T lymphocytes at all evaluated time points and $CD8^{+}$ T lymphocytes at d+30 were phenotypically represented mostly by memory lymphocytes, as previously described in patients who received PTCy immunosuppression.[33] We observed an elevation in $CD8^{+}$ naive and transitional $CD4^{+}$ T cells in both groups from d+90, with a predominance of $CD8^{+}$ naive T cells at d+180 in both groups. This increase was accompanied by low counts of naive and transitional $CD4^{+}$ T lymphocytes compared to $CD4^{+}$ memory T cells, indicating slow reconstitution of $CD4^{+}$ T cells.[19,34] Kanakry *et al*. also described that the reconstitution of $CD4^{+}$ and $CD8^{+}$ T lymphocytes primarily involved effector memory cells ($CD45RA^{-}CCR7^{-}$) and terminally differentiated cells ($CD45RA^{+}CCR7^{-}$) in patients undergoing transplantation with PTCy.[33] It is known that the first “wave” of T cells post-transplant comprises donor T cells undergoing proliferation induced by lymphopenia and alloactivation, resulting in polyclonal T cells with a restricted T-cell receptor (TCR) repertoire, limited antigenic specificity or alloreactivity activity, causing GVHD. Complete T-cell reconstitution, with TCR diversity, requires thymic regeneration and endogenous T-cell development, making this process very slow.[35]

Besides that, in the PTCy group, we found a higher number of double-positive T lymphocytes ($CD4^{+}CD8^{+}$) at d+180. The recovery of *de novo* T lymphocytes is dependent on a functional thymus. The infused hematopoietic stem cells (CD34), are able to recover the bone marrow function and generate *de novo* lymphoid progenitors that migrate to the thymus. These progenitors, initially double-negative (without CD4 and CD8 expression), undergo a maturation process, becoming double-positive. At this stage, these cells undergo negative selection, where T cells that recognize self-antigens undergo apoptosis, an essential step for GVHD control.[36] If double-positive T cells do not pass through negative selection, escaping thymic selection, it may be related to the development of GVHD. Hussen *et al*.[37] observed the development of double-positive T cells during xenogeneic GVHD and Hess *et al*.[38] identified, in an observational study with 35 patients submitted to PTCy, that CD4/CD8 double-positive cells is a predictive biomarker of GVHD. In a xenogeneic model, the authors identified this population as transcriptionally, metabolically, and functionally distinct from single-positive CD4 and CD8 T cells, demonstrating that these are chronically activated CD8 T cells, highly inflammatory, secreting IFN-γ, TNF-α, GM-CSF, IL-17A, IL-22, granzyme, and perforin. These cells acquire effector functions of the CD4 lineage and are directly implicated in the pathogenesis of GVHD. As all patients in this study received PTCy, it is uncertain whether the predictive value of GVHD would also apply to the classic immunosuppression protocol. However, in our study, these lymphocytes were more frequent in the PTCy population.

In our study of the correlations of clinical outcomes with immune reconstitution, at d+30, a higher absolute number of $CD4^{+}$ transitional cells were associated with worse survival. CD4 transitional cells represent an intermediate stage between naive and memory T cells. The transition from $CD45RA^{+}$ naive T cells to $CD45RO^{+}$ memory T cells, initiated by TCR-mediated signaling in the G1 phase of the cell cycle, suggests that these transitional cells are in an active state of differentiation and proliferation.[39] This hyperactivation can lead to an increased risk of GVHD, what may be related to increased mortality risk. Additionally, the immune reconstitution involving these highly reactive transitional T cells may not provide effective protection against infections due to their immature functional state, further contributing to increased mortality. Therefore, the presence of a higher number of transitional $CD4^{+}$ T cells may indicate an imbalance in immune homeostasis, resulting in adverse clinical outcomes post-transplantation. Besides that, higher quantity of $CD4^{+}$ naive, transitional $CD4^{+}$, and $CD8^{+}$ naive cells at d+60 were risk factors for aGVHD. Additionally, a higher quantity of $CD4^{+}$ memory cells at d+180 was a risk factor for cGVHD. In aGVHD, after activation, al-

loreactive memory T cells cause damage to various tissues, such as the gastrointestinal tract, liver, skin, and thymus, through direct cytotoxicity or cytokine-mediated mechanisms. However, uncertainties persist regarding whether these effector memory cells present in the patient's blood with aGVHD are derived from the graft, proliferating upon encountering the antigen, or if they differentiate from naive cells in the graft.[40] Some studies suggest that naive T cells are even more potent inducers of aGVHD and may be associated with more severe forms, possibly due to being the most potent inducers of alloreactive responses *in vitro*. A possible explanation could be that naive T cells have a broader TCR repertoire, and a higher proliferative capacity, resulting in a greater potential for clonal expansion and for recognizing antigens presented by antigen-presenting cells (APC). A study evaluating the association of donor-infused $CD4^+$ T cells with aGVHD found that a higher quantity of infused $CD4^+$ naive T cells was associated with a higher risk of grade 2-4 aGVHD and cGVHD.[41] The increased risk of GVHD associated with naive $CD4^+$ T cells may explain the worse overall survival found in the present study with more transitional $CD4^+$ T cells at d+60.

We found higher counts of naïve $CD4^+$ T cells on d+30 and higher counts of T cells, $CD4^+$ T cells, and $CD8^+$ T cells on d+60 in peripheral blood compared to the bone marrow graft source. Other authors have found higher $CD4^+$ T cell counts with peripheral blood, which may be explained by the higher number of lymphocytes infused, approximately one log higher with peripheral blood compared to bone marrow.[42,43] Reduced-intensity conditioning was associated with higher counts of $CD4^+$ naive T cells on d+30 and higher NK $CD56^{high}$ cells on d+30 and NK $CD56^{dim}$ on d+60. Petersen *et al.*[43] also compared non-myeloablative and myeloablative conditioning regimens in HLA-identical sibling donor transplants and found earlier normalization of NK cells, $CD8^+$ T cells, and $CD4^+$ T cells but delayed B-cell recovery with less intensive conditioning.

The present study has some limitations, primarily related to the number of patients included in the analyses. However, this prospective study comprehensively analyzed the immune reconstitution of patients using ATG or PTCy, providing valuable information for clinical practice regarding this innovative form of post-transplant immunosuppression. In conclusion, in the context of unrelated allogeneic transplantation, the use of PTCy compared to classical immunosuppression with ATG is associated with a lower number of total lymphocytes, T cells, NK cells, and B cells on d+30, and a lower number of $CD56^{dim}$ NK cells on d+60 and d+90, but with a higher proportion of double-positive T cells, B cells, and a higher proportion of $CD56^-CD16^+NKG2D^+$ NK cells on d+180. These results may contribute to a deeper understanding of the differences in immune reconstitution and their impact on clinical practice, which is crucial for refining transplant strategies, such as vaccinations, minimizing complications, and improving patient survival. Collaborative research efforts hold the potential to further advance our comprehension of reconstitution biology and translate these findings into customized therapeutic interventions for HSCT recipients.

Disclosures

PB, LNK and The University of Texas MD Anderson Cancer Center have institutional financial conflict of interest with Takeda Pharmaceutical for the licensing of the technology related to CAR-NK-cell research. MD Anderson has implemented an Institutional Conflict of Interest Management and Monitoring Plan to manage and monitor the conflict of interest with respect to MDACC's conduct of any other ongoing or future research related to this relationship. PB participates on the scientific advisory board for Dialectica.

Contributions

MNK contributed with study design and methodology, data collection and analysis, results interpretation, writing of the manuscript draft and subsequent revisions. FAR and PSC contributed with data collection and contribution to the execution of laboratory tests in flow cytometry. LJA contributed with statistical analyses of data and results interpretation. LNK, CMM and AAFR contributed with study conceptions, results interpretation and critical review of the manuscript drafts. PB contributed with Cytobank analysis. LCM and NH contributed with study conception and methodology, general supervision, results interpretation and critical review of the manuscript drafts. All authors reviewed and approved the final version submitted.

Acknowledgments

The authors thank Patricia Logullo, PhD, CMPP (Palavra Impressa) for manuscript editing services.

Funding

This study was funded by Amigo H.

Data-sharing statement

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

References

1. Ratanatharathorn V, Nash RA, Przepiorka D, et al. Phase III study comparing methotrexate and tacrolimus (prograf, FK506) with methotrexate and cyclosporine for graft-versus-host disease prophylaxis after HLA-identical sibling bone marrow

transplantation. Blood. 1998;92(7):2303-2314.
2. Bacigalupo A, Lamparelli T, Barisione G, et al. Thymoglobulin prevents chronic graft-versus-host disease, chronic lung dysfunction, and late transplant-related mortality: long-term follow-up of a randomized trial in patients undergoing unrelated donor transplantation. Biol Blood Marrow Transplant. 2006;12(5):560-565.
3. Soiffer RJ, Kim HT, McGuirk J, et al. Prospective, randomized, double-blind, phase III clinical trial of anti-T-lymphocyte globulin to assess impact on chronic graft-versus-host disease-free survival in patients undergoing HLA-matched unrelated myeloablative hematopoietic cell transplantation. J Clin Oncol. 2017;35(36):4003-4011.
4. Arai Y, Jo T, Matsui H, Kondo T, Takaori-Kondo A. Efficacy of antithymocyte globulin for allogeneic hematopoietic cell transplantation: a systematic review and meta-analysis. Leuk Lymphoma. 2017;58(8):1840-1848.
5. Arcuri LJ, Kerbauy MN, Kerbauy LN, Santos FPS, Ribeiro AAF, Hamerschlak N. ATG in HLA-matched, peripheral blood, hematopoietic cell transplantation in acute myeloid leukemia and myelodysplastic syndrome: a secondary analysis of a CIBMTR database. Transplant Cell Ther. 2023;29(1):40.
6. Kanakry JA, Kasamon YL, Gocke CD, et al. Outcomes of related donor HLA-identical or HLA-haploidentical allogeneic blood or marrow transplantation for peripheral T cell lymphoma. Biol Blood Marrow Transplant. 2013;19(4):602-606.
7. Ciurea SO, Mulanovich V, Saliba RM, et al. Improved early outcomes using a T cell replete graft compared with T cell depleted haploidentical hematopoietic stem cell transplantation. Biol Blood Marrow Transplant. 2012;18(12):1835-1844.
8. Tischer J, Engel N, Fritsch S, et al. Virus infection in HLA-haploidentical hematopoietic stem cell transplantation: incidence in the context of immune recovery in two different transplantation settings. Ann Hematol. 2015;94(10):1677-1688.
9. Moiseev IS, Pirogova OV, Alyanski AL, et al. Graft-versus-host disease prophylaxis in unrelated peripheral blood stem cell transplantation with post-transplantation cyclophosphamide, tacrolimus, and mycophenolate mofetil. Biol Blood Marrow Transplant. 2016;22(6):1037-1042.
10. Mehta RS, Saliba RM, Chen J, et al. Post-transplantation cyclophosphamide versus conventional graft-versus-host disease prophylaxis in mismatched unrelated donor haematopoietic cell transplantation. Br J Haematol. 2016;173(3):444-455.
11. Rashidi A, Slade M, DiPersio JF, Westervelt P, Vij R, Romee R. Post-transplant high-dose cyclophosphamide after HLA-matched vs haploidentical hematopoietic cell transplantation for AML. Bone Marrow Transplant. 2016;51(12):1561-1564.
12. Gooptu M, Romee R, St Martin A, et al. HLA-haploidentical vs matched unrelated donor transplants with posttransplant cyclophosphamide-based prophylaxis. Blood. 2021;138(3):273-282.
13. Battipaglia G, Galimard JE, Labopin M, et al. Post-transplant cyclophosphamide in one-antigen mismatched unrelated donor transplantation versus haploidentical transplantation in acute myeloid leukemia: a study from the Acute Leukemia Working Party of the EBMT. Bone Marrow Transplant. 2022;57(4):562-571.
14. Bolaños-Meade J, Reshef R, Fraser R, et al. Three prophylaxis regimens (tacrolimus, mycophenolate mofetil, and cyclophosphamide; tacrolimus, methotrexate, and bortezomib; or tacrolimus, methotrexate, and maraviroc) versus tacrolimus and methotrexate for prevention of graft-versus-host disease with haemopoietic cell transplantation with reduced-intensity conditioning: a randomised phase 2 trial with a non-randomised contemporaneous control group (BMT CTN 1203). Lancet Haematol. 2019;6(3):e132-e143.
15. Luznik L, Pasquini MC, Logan B, et al. Randomized phase III BMT CTN trial of calcineurin inhibitor-free chronic graft-versus-host disease interventions in myeloablative hematopoietic cell transplantation for hematologic malignancies. J Clin Oncol. 2022;40(4):356-368.
16. Jurdi NE, Hoover A, O'Leary D, et al. Phase II study of myeloablative 7-8/8-matched allotransplantation with post-transplantation cyclophosphamide, tacrolimus, and mycophenolate mofetil. Transplant Cell Ther. 2023;29(9):576.
17. Bolaños-Meade J, Hamadani M, Wu J, et al. Post-transplantation cyclophosphamide-based graft-versus-host disease prophylaxis. N Engl J Med. 2023;388(25):2338-2348.
18. van den Brink MR, Velardi E, Perales MA. Immune reconstitution following stem cell transplantation. Hematology Am Soc Hematol Educ Program. 2015;2015:215-219.
19. Elfeky R, Lazareva A, Qasim W, Veys P. Immune reconstitution following hematopoietic stem cell transplantation using different stem cell sources. Expert Rev Clin Immunol. 2019;15(7):735-751.
20. Minculescu L, Marquart HV, Friis LS, et al. Early natural killer cell reconstitution predicts overall survival in T cell-replete allogeneic hematopoietic stem cell transplantation. Biol Blood Marrow Transplant. 2016;22(12):2187-2193.
21. Mackall CL, Fleisher TA, Brown MR, et al. Age, thymopoiesis, and CD4+ T-lymphocyte regeneration after intensive chemotherapy. N Engl J Med. 1995;332(3):143-149.
22. Kotecha N, Krutzik PO, Irish JM. Web-based analysis and publication of flow cytometry experiments. Curr Protoc Cytom. Jul 2010;Chapter 10:Unit10.17.
23. Rambaldi B, Kim HT, Reynolds C, et al. Impaired T- and NK-cell reconstitution after haploidentical HCT with posttransplant cyclophosphamide. Blood Adv. 2021;5(2):352-364.
24. Goldsmith SR, Abid MB, Auletta JJ, et al. Post-transplant cyclophosphamide (PTCy) is associated with increased cytomegalovirus infection: a CIBMTR analysis. Blood. 2021;137(23):3291-3305.
25. Nguyen S, Kuentz M, Vernant JP, et al. Involvement of mature donor T cells in the NK cell reconstitution after haploidentical hematopoietic stem-cell transplantation. Leukemia. 2008;22(2):344-352.
26. Abrahamsen IW, Sømme S, Heldal D, Egeland T, Kvale D, Tjønnfjord GE. Immune reconstitution after allogeneic stem cell transplantation: the impact of stem cell source and graft-versus-host disease. Haematologica. 2005;90(1):86-93.
27. Russo A, Oliveira G, Berglund S, et al. NK cell recovery after haploidentical HSCT with posttransplant cyclophosphamide: dynamics and clinical implications. Blood. 2018;131(2):247-262.
28. Nguyen S, Dhedin N, Vernant JP, et al. NK-cell reconstitution after haploidentical hematopoietic stem-cell transplantations: immaturity of NK cells and inhibitory effect of NKG2A override GvL effect. Blood. 2005;105(10):4135-4142.
29. Hu PF, Hultin LE, Hultin P, et al. Natural killer cell immunodeficiency in HIV disease is manifest by profoundly decreased numbers of CD16+CD56+ cells and expansion of a population of CD16dimCD56- cells with low lytic activity. J Acquir Immune Defic Syndr Hum Retrovirol. 1995;10(3):331-340.
30. Gaddy J, Broxmeyer HE. Cord blood CD16+56- cells with low lytic activity are possible precursors of mature natural killer cells. Cell Immunol. 1997;180(2):132-142.
31. Cocker ATH, Liu F, Djaoud Z, Guethlein LA, Parham P. CD56-negative NK cells: Frequency in peripheral blood, expansion

during HIV-1 infection, functional capacity, and KIR expression. Front Immunol. 2022;13:992723.
32. Müller-Durovic B, Grählert J, Devine OP, Akbar AN, Hess C. CD56-negative NK cells with impaired effector function expand in CMV and EBV co-infected healthy donors with age. Aging (Albany NY). 2019;11(2):724-740.
33. Kanakry CG, Coffey DG, Towlerton AM, et al. Origin and evolution of the T cell repertoire after post transplantation cyclophosphamide. JCI Insight. 2016;1(5):e86252.
34. Vivier E, Raulet DH, Moretta A, et al. Innate or adaptive immunity? The example of natural killer cells. Science. 2011;331(6013):44-49.
35. Velardi E, Tsai JJ, van den Brink MRM. T cell regeneration after immunological injury. Nat Rev Immunol. 2021;21(5):277-291.
36. Dulude G, Brochu S, Fontaine P, et al. Thymic and extrathymic differentiation and expansion of T lymphocytes following bone marrow transplantation in irradiated recipients. Exp Hematol. 1997;25(9):992-1004.
37. Alhaj Hussen K, Michonneau D, Biajoux V, et al. CD4+CD8+ T-lymphocytes in xenogeneic and human graft-versus-host disease. Front Immunol. 2020;11:579776.
38. Hess NJ, Turicek DP, Riendeau J, et al. Inflammatory CD4/CD8 double-positive human T cells arise from reactive CD8 T cells and are sufficient to mediate GVHD pathology. Sci Adv. 2023;9(12):eadf0567.
39. Johannisson A, Festin R. Phenotype transition of CD4+ T cells from CD45RA to CD45R0 is accompanied by cell activation and proliferation. Cytometry. 1995;19(4):343-352.
40. Dekker L, Sanders E, Lindemans CA, de Koning C, Nierkens S. Naive T cells in graft versus host disease and graft versus leukemia: innocent or guilty? Front Immunol. 2022;13:893545.
41. Chang YJ, Zhao XY, Huo MR, Huang XJ. Expression of CD62L on donor CD4(+) T cells in allografts: correlation with graft-versus-host disease after unmanipulated allogeneic blood and marrow transplantation. J Clin Immunol. 2009;29(5):696-704.
42. Fedele R, Martino M, Garreffa C, et al. The impact of early CD4+ lymphocyte recovery on the outcome of patients who undergo allogeneic bone marrow or peripheral blood stem cell transplantation. Blood Transfus. 2012;10(2):174-180.
43. Petersen SL, Ryder LP, Björk P, et al. A comparison of T-, B- and NK-cell reconstitution following conventional or nonmyeloablative conditioning and transplantation with bone marrow or peripheral blood stem cells from human leucocyte antigen identical sibling donors. Bone Marrow Transplant. 2003;32(1):65-72.