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T-cell receptor-α repertoire of CD8+ T cells following allogeneic stem cell transplantation using next-generation sequencing

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ABSTRACT

Alloreactivity or opportunistic infections following allogeneic stem cell transplantation are difficult to predict and contribute to post-transplantation mortality. How these immune reactions result in changes to the T-cell receptor repertoire remains largely unknown. Using next-generation sequencing, the T-cell receptor alpha (TRα) repertoire of naïve and memory CD8+ T cells from 25 patients who had received different forms of allogeneic transplantation was analyzed. In parallel, reconstitution of the CD8+/CD4+ T-cell subsets was mapped using flow cytometry. When comparing the influence of anti-T-cell therapy, a delay in the reconstitution of the naïve CD8+ T-cell repertoire was observed in patients who received *in vivo* T-cell depletion using antithymocyte globulin or post-transplantation cyclophosphamide in case of haploidentical transplantation. Sequencing of the TRα identified a repertoire consisting of more dominant clonotypes (>1% of reads) in these patients at 6 and 18 months post transplantation. When comparing donor and recipient, approximately 50% and approximately 80% of the donors' memory repertoire were later retrieved in the naïve and memory CD8+ T-cell receptor repertoire of the recipients, respectively. Although there was a remarkable expansion of single clones observed in the recipients' memory CD8+ TRα repertoire, no clear association between graft-*versus*-host disease or cytomegalovirus infection and T-cell receptor diversity was identified. A lower TRα diversity was observed in recipients of a cytomegalovirus-seropositive donor (*P*=0.014). These findings suggest that CD8+ T-cell reconstitution in transplanted patients is influenced by the use of T-cell depletion or immunosuppression and the donor repertoire.

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Introduction

Allogeneic stem cell transplantation (SCT) remains an essential component in the treatment of hematologic malignancies such as acute leukemia, lymphoma and myelodysplastic syndrome.[1,2] Despite advances in transplantation regimens and supportive care, alloreactivity following SCT remains difficult to predict, and a large proportion of post-transplantation mortality is due to severe graft-*versus*-host reactions.[3,4]

T cells play an important role in the initiation of graft-*versus*-host disease (GvHD),[5] in which the T-cell receptor (TCR) is a key element in the generation of an immune response against presented antigens. The heterodimeric TCR consists of an alpha (TRα) and beta (TRβ) chain. Quantitative analyses of the TCR repertoire by next-generation sequencing (NGS) allow a more sophisticated assessment of the adaptive immune system.[6,7]

The TCR repertoire has been proposed to modulate post-SCT outcomes. So far, TCR sequencing has largely been performed by sequencing the TRβ in patients fol-

lowing allogeneic SCT.[8] For example, assessing the recovery of the TRβ repertoire following allogeneic transplantation revealed a restricted TCR repertoire diversity in patients affected by viral infections.[9] We have previously shown that the TRα repertoire becomes oligoclonal and is dominated by a few expanded clonotypes following cytomegalovirus (CMV) infections in transplanted patients.[10] Furthermore, GvHD and the relapse of acute myeloid leukemia (AML) correlate with lower TRβ diversity,[11] and the expansion of individual TCR clonotypes was observed in GvHD patients in selected studies.[12] Formation of the TCR repertoire starts within months of transplantation, resulting in a more donor-like repertoire after the first year following transplantation.[13]

Little is known about how TCR changes are influenced by the choice of transplant regimen, especially by different forms of T-cell depletion. One study revealed lower TRβ diversity in patients receiving an *in vitro* T-cell-depleted stem cell graft than in patients who received a non-T-cell-depleted cord blood graft.[9] GvHD prophylaxis using post-transplantation cyclophosphamide (PTCy) on day +3 following SCT is an established therapeutic option in patients receiving haploidentical transplantation.[14] Furthermore, the application of antithymocyte globulin (ATG) prior to transplantation as part of the conditioning therapy has become a common procedure to prevent GvHD, especially in patients with a mismatched donor.[15] However, there have been no studies comparing these different regimens of *in vivo* T-cell depletion (ATG or PTCy) and their impact on the TCR repertoire.

Here we analyzed the TRα repertoire of naïve and memory CD8+ T cells in 25 patients following different forms of allogeneic transplantation. This study addressed the question of whether the recipient TRα repertoire is influenced by anti-T-cell therapies such as ATG or PTCy, and if there are differences in response to haploidentical transplantation between fully matched or mismatched donor transplants. Furthermore, we analyzed to what extent the donor TRα repertoire is transferred to the recipient. Finally, the correlation between TRα repertoire diversity and the clinical manifestation of GvHD or CMV reactivation were addressed.

Methods

Patients

Patients (n=25) and donors were recruited after obtaining written informed consent and the approval of the local ethical review board (EK-279072013). To qualify, patients needed to have received their first SCT for an underlying hematologic malignancy. Patients who suffered a relapse during the observation period were excluded from the study. All SCTs were performed at Dresden University Hospital. Patients' characteristics are shown in Table 1. Patients were stratified into different groups according to their SCT protocol. In the first group, 5 patients received matched unrelated donor transplants and ATG (UD-ATG) as an addition to conditioning chemotherapy. The second group contained 5 patients who received mismatched unrelated donor transplants (9/10 allele match) and ATG (mmUD-ATG). Group three contained 5 patients who received transplants from matched unrelated donors without the application of ATG (UD-noATG), whereas the fourth group (Haplo-PTCy) was made up of patients who underwent haploidentical transplantation and the use of PTCy. Lastly, 5 patients with matched related donors without any use of

T-cell depletion were recruited (SIB-noATG), and samples from the 5 patient donors were analyzed in parallel. Acute GvHD (aGvHD) was defined as GvHD diagnosed within the first 100 days following SCT. In contrast, chronic GvHD (cGvHD) was diagnosed in cases with GvHD after the first 100 days or the typical clinical presentation of cGvHD features.[16] CMV reactivation was determined by detection of CMV virus load in the peripheral blood.

Immunophenotyping by flow cytometry

Routine assessment of differential blood counts was used to define the engraftment of neutrophil leukocytes and reconstitution of whole lymphocytes. Samples for immunophenotyping were taken on day 60, day 120 and day 180 following transplantation. Twenty healthy stem cell donors were analyzed to define normal ranges (controls).

CD4+ and CD8+ T cells were characterized according to the expression of CCR7 and CD45RA as naïve (CCR7+CD45RA+), central memory (CM, CCR7+CD45RA−), effector memory (EM, CCR7−CD45RA−), and terminally differentiated effector memory (TEMRA, CCR7− CD45RA+) T cells.[17] Staining was performed using the following antibodies as previously described:[18] CD45-V500, CD3-PerCP-Cy5.5, CD8-APCH7, CCR7-FITC, CD45RA-PE (all BD Biosciences, San Jose, CA, USA) and CD4-eFluor450 (eBioscience, San Diego, CA, USA) (*Online Supplementary Figure S1*). Immunophenotyping was performed using a BD FACSCanto II (BD Bioscience, San Jose, CA, USA).

Sorting for T-cell receptor-α sequencing

For TRα sequencing, naïve and memory cells were sorted from CD45+CD3+CD8+ T cells as previously described[18] on day 60 and day 180 following transplantation. Sorted memory cells contained all memory subsets (CM, EM, TEMRA). Both T-cell subsets were sorted to a purity of more than 99% as checked by flow cytometry after sorting. For naïve CD8+ T cells and memory CD8+ T cells, a maximum of 1,000,000 cells were sorted, with a mean of 133,590 and 808,726 sorted cells for naïve and memory CD8+ T cells, respectively.

Library preparation for T-cell receptor-α sequencing

Library preparation was performed as previously described.[10,18,19] The final PCR product contained the nucleotide sequence for the variable region of the TRα (V and J segments), including the complementary determining region (CDR3).

Next-generation sequencing

Next-generation sequencing was performed using an Illumina HiSeq 2500 (Illumina, San Diego, CA, USA), generating 150-base-pair reads. Extraction of CDR3 sequences was performed using MiTCR as previously described.[19,20] MiTCR used error correction with the highest stringency. Sequencing was performed, calculating 20 reads per cell individually for each sample for normalization. Samples that failed quality control were excluded from the analyses and indexed as "not available". TRα chains with identical CDR3 region amino acid (AA) sequences were defined as clonotypes. Clonotypes with a TRα read frequency of more than 1% of reads were defined as "dominant" and more than 10% as "highly dominant" clonotypes. Sequence data are available at VDJServer under project UUID 35447650152632857336-242ac11d-0001-012 (*https://vdjserver.org*).

Statistical analysis

Data analysis was performed using GraphPad Prism (v.5.01, La Jolla, CA, USA), KNIME 2.5.2 and R (v.2.15.2 and Studio v.0.98.945, Boston, MA, USA). Immunophenotyping results were

Table 1. Patients' characteristics.

Group ID#	Patient ID#	Disease	Age	Sex	CMV-Status	Treatment status at SCT	Transplant regimen allele-matching	Stem cell source	T-cell depletion	Conditioning regimen	Donor Sex	Donor CMV-Status	Donor Age	Post grafting immunosuppression
UD-ATG	TCR_001	ALL	47	m	neg	1st CR	UD-SCT 10/10	PBSC	ATG	TBI 12 Gy/Eto	m	neg	23	Mtx, CsA
UD-ATG	TCR_003	T-ALL	34	m	pos	1st CR	UD-SCT 10/10	PBSC	ATG	TBI 12 Gy/Eto	m	pos	34	Mtx, CsA
UD-ATG	TCR_023	AML	54	m	neg	1st CR	UD-SCT 10/10	PBSC	ATG	Flu/Bu 8	m	neg	23	Mtx, CsA
UD-ATG	TCR_041	CLL	71	m	pos	PR	UD-SCT 10/10	PBSC	ATG	Flu/Bu 8	f	pos	55	CsA, MMF
UD-ATG	TCR_056	AML	68	m	pos	1st CR	UD-SCT 10/10	PBSC	ATG	Flu/Treo	m	pos	51	Mtx, CsA
mmUD-ATG	TCR_013	B-ALL	63	f	pos	2nd CR	UD-SCT 9/10	PBSC	ATG	Flu/TBI 8 Gy	f	pos	32	Mtx, CsA
mmUD-ATG	TCR_038	AML	49	f	neg	1st CR	UD-SCT 9/10	PBSC	ATG	Bu/Cy	m	pos	19	Mtx, CsA
mmUD-ATG	TCR_048	AML	63	m	pos	1st CR	UD-SCT 9/10	PBSC	ATG	Flu/TBI 2Gy	m	pos	50	Mtx, CsA
mmUD-ATG	TCR_054	AML	68	m	neg	1st CR	UD-SCT 9/10	PBSC	ATG	Flu/Bu 8	m	pos	48	Mtx, CsA
mmUD-ATG	TCR_055	AML	63	f	neg	Induction failure	UD-SCT 9/10	PBSC	ATG	Flu/Mel	f	neg	46	Mtx, CsA
UD-noATG	TCR_005	B-NHL	61	f	pos	2nd relapse	UD-SCT 10/10	PBSC	no	Flu/Bu 8	f	neg	27	Mtx, CsA
UD-noATG	TCR_008	CLL	73	m	pos	stable	UD-SCT 10/10	PBSC	no	Flu/Bu 8	m	neg	23	Mtx, CsA
UD-noATG	TCR_017	AML	46	m	neg	1st CR	UD-SCT 10/10	PBSC	no	Flu/Bu 8	m	neg	42	Mtx, CsA
UD-noATG	TCR_019	MDS	64	m	neg	no response	UD-SCT 10/10	PBSC	no	Flu/Bu 8	m	neg	20	Mtx, CsA
UD-noATG	TCR_027	AML	72	f	pos	1st CR	UD-SCT 10/10	PBSC	no	Flu/Bu 8	m	pos	41	Mtx, CsA
Haplo-PTCy	TCR_012	AML	62	m	neg	> 2nd relapse	Haplo-SCT	BM	PTCy	Flu/Cy/TBI 2Gy	m	pos	33	Tac, MMF
Haplo-PTCy	TCR_014	AML	21	m	pos	Induction failure	Haplo-SCT	BM	PTCy	Flu/Cy/TBI 2Gy	f	neg	44	Tac, MMF
Haplo-PTCy	TCR_026	MDS	65	f	pos	2nd relapse	Haplo-SCT	BM	PTCy	Flu/Cy/TBI 2Gy	m	pos	29	Tac, MMF
Haplo-PTCy	TCR_036	AML	51	f	neg	2nd CR	Haplo-SCT	BM	PTCy	Flu/Cy/TBI 2Gy	m	neg	25	Tac, MMF
Haplo-PTCy	TCR_049	AML	65	m	pos	Induction failure	Haplo-SCT	BM	PTCy	Flu/Cy/TBI 2Gy	m	pos	38	Tac, MMF
SIB-noATG	TCR_002	AML	53	f	pos	1st CR	SIB-SCT 10/10	PBSC	no	Flu/TBI 8Gy	f	pos	60	CsA
SIB-noATG	TCR_011	AML	50	f	pos	1st CR	SIB-SCT 10/10	PBSC	no	Flu/Bu 8	f	neg	49	Mtx, CsA
SIB-noATG	TCR_024	MDS	63	m	pos	no response	SIB-SCT 10/10	PBSC	no	Flu/Bu 8	f	neg	67	Mtx, CsA
SIB-noATG	TCR_040	T-NHL	49	m	pos	3rd relapse	SIB-SCT 10/10	BM	no	Bu/Cy	m	neg	45	Mtx, CsA
SIB-noATG	TCR_063	AML	59	m	neg	1st relapse	SIB-SCT 10/10	PBSC	no	Flu/Bu 8	f	pos	64	Mtx, CsA

ALL: acute lymphoblastic leukemia; T-ALL: T-cell acute lymphoblastic leukemia; B-ALL: B-cell acute lymphoblastic leukemia; AML: acute myeloid leukemia; CLL: chronic lymphocytic leukemia; MDS: myelodysplastic syndrome; NHL: non-Hodgkin lymphoma; T-NHL: T-cell non-Hodgkin's lymphoma; B-NHL: B-cell non-Hodgkin lymphoma; m: male; f: female; CR: complete remission; PR: partial remission; UD-SCT: unrelated donor stem cell transplant; Haplo-SCT: haploidentical donor stem cell transplant; SIB-SCT: identical sibling donor stem cell transplant; PBSC: peripheral blood stem cells; BM: bone marrow; ATG: antithymocyte globulin; PTCy: post-transplantation cyclophosphamide; TBI: total body eradication; Eto: etoposide; Flu: fludarabine; Bu: busulfan; Mtx: methotrexate; CsA: cyclosporine; Tac: tacrolimus; MMF: mycophenolate mofetil; pos: positive; neg: negative.

analyzed using ANOVA and Dunnett's multiple comparison test. Simpson's diversity index (D_s) was used as a general measure of diversity, as previously described; this index is known to be insensitive to differences in sample sizes.[18,21] Differences in D_s between groups were tested with Wilcoxon tests or Kruskal-Wallis rank sum tests. $P=0.05$ was considered statistically significant for all tests.

Results

Engraftment

On day 60 following transplantation, 19 out of 25 patients had recovered leukocyte counts. The median day of neutrophil granulocyte engraftment (defined as 0.5×10^9 neutrophils/L) was day 20 post transplantation. In contrast, lymphocytes remained significantly below control levels on day 60 ($P<0.001$) and day 120 ($P<0.01$), and recovered to control levels only by day 180 (*Online Supplementary Figure S2A*).

Suppression of naïve T cells in patients with *in vivo* T-cell depletion

Assessment of CD4+ T cells revealed suppressed numbers on days 60, 120 and 180 compared to controls (all $P<0.001$). Notably, patients who received ATG had significantly lower CD4+ counts on day 180 than those who did not receive ATG as part of their conditioning regimen ($P<0.001$). Analyses of CD4+ T-cell subsets revealed the sustained suppression of naïve T cells compared to controls ($P<0.001$). The lowest mean naïve CD4+ numbers were found in both groups receiving ATG and PTCy at all time points ($P<0.001$). In all groups, the CD4+ T-cell compartment was dominated by CCR7−CD45RA− EM cells with significantly higher values ($P<0.001$) for patients than for controls (Figure 1A).

In contrast to the low CD4+ counts in transplanted patients, the CD8+ T-cell numbers from all samples were increased on days 120 and 180 compared to those of controls ($P<0.05$ and $P<0.01$, respectively) (*Online Supplementary Figure S2B*). The proportion of
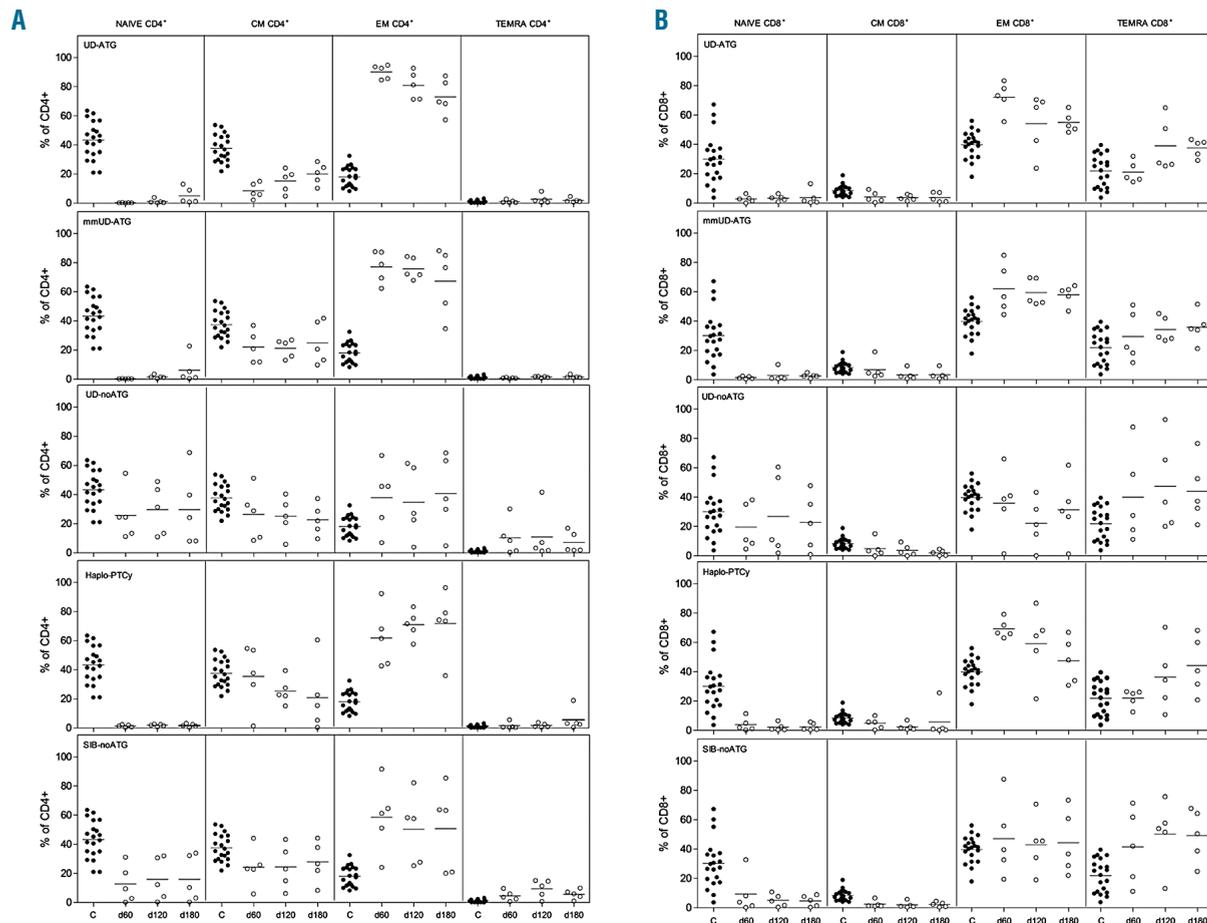
Figure 1. CD4+ and CD8+ T-cell subsets. Immune reconstitution of T-cell subsets of CD4+ T cells (A) and CD8+ T cells (B) on day 60 (d60), day 120 (d120) and day 180 (d180) post transplantation. Patient groups with 5 patients were analyzed separately and compared to the control population (C, n=20).

CCR7+CD45RA+ naïve CD8+ T cells was also suppressed within all groups compared to those of controls (*P*<0.001). Again, the lowest counts were seen in patients receiving ATG and PTCy, but these counts were not statistically significant. CD8+ T cells were primarily composed of EM cells and TEMRA cells for all groups. Both the EM and TEMRA fractions were comparable to or higher than those in the reference group, with differentiated cells becoming the dominant population over time (EM, *P*<0.01 on day 60; TEMRA, *P*<0.01 on day 120 and *P*<0.001 on day 180); no significant differences were seen between the transplantation groups (Figure 1B).

T-cell receptor-α repertoire composition and diversity are shaped by the memory T-cell receptor-α repertoire of the donor

We had access to blood samples from 5 donors at the time of transplant donation (*Online Supplementary Table S1*) allowing us to directly compare their repertoires with those of the recipients. While the correlation of memory diversity between donor and recipient was not significant (likely due to the low number of samples), the correlation was stronger on day 180 (r²=0.6602) than on day 60 (r²=0.5484).

The recipients' repertoires were heavily shaped by the memory repertoires of the donors. We found that 77.2% and 80.0% of the TRα reads from the donors' memory repertoires were populated by clonotypes shared by donors' and patients' memory repertoires on days 60 and 180, respectively. Notably, 41.5% (on day 60) and 61.0% (on day 180) of the donors' memory repertoires were also found in the naïve repertoires of the recipients. In contrast, only 8.9% and 10.1% of the donors' naïve repertoires were recovered in the recipients' memory repertoires, and 6.0% and 18.6% were recovered in the recipients' naïve repertoires on days 60 and 180, respectively (Figure 2A).

Clinical context of T-cell receptor-α repertoire composition in SIB-noATG patients

Among SIB-noATG patients, 3 out of 5 patients (TCR_011, TCR_024, TCR_040) revealed highly dominant memory clonotypes (defined as >10% of repertoire) that either were not dominant or were not even found in their donor's repertoire (Figure 2C).

The medical history of 2 patients (TCR_024 and TCR_040) who developed highly dominant clonotypes accompanied by a decrease in TCR memory diversity

A

Read frequency (%) of shared clonotypes

d60	found in sample:			
	PN	PM	DN	DM
PN (%)		43.0	8.6	18.7
PM (%)	63.3		34.6	57.9
DN (%)	6.0	8.9		14.6
DM (%)	41.5	77.2	60.5	

Read frequency (%) of shared clonotypes

d180	found in sample:			
	PN	PM	DN	DM
PN (%)		13.3	14.9	11.5
PM (%)	87.9		30.9	60.6
DN (%)	18.6	10.1		14.6
DM (%)	61.0	80.0	60.5	

B

C
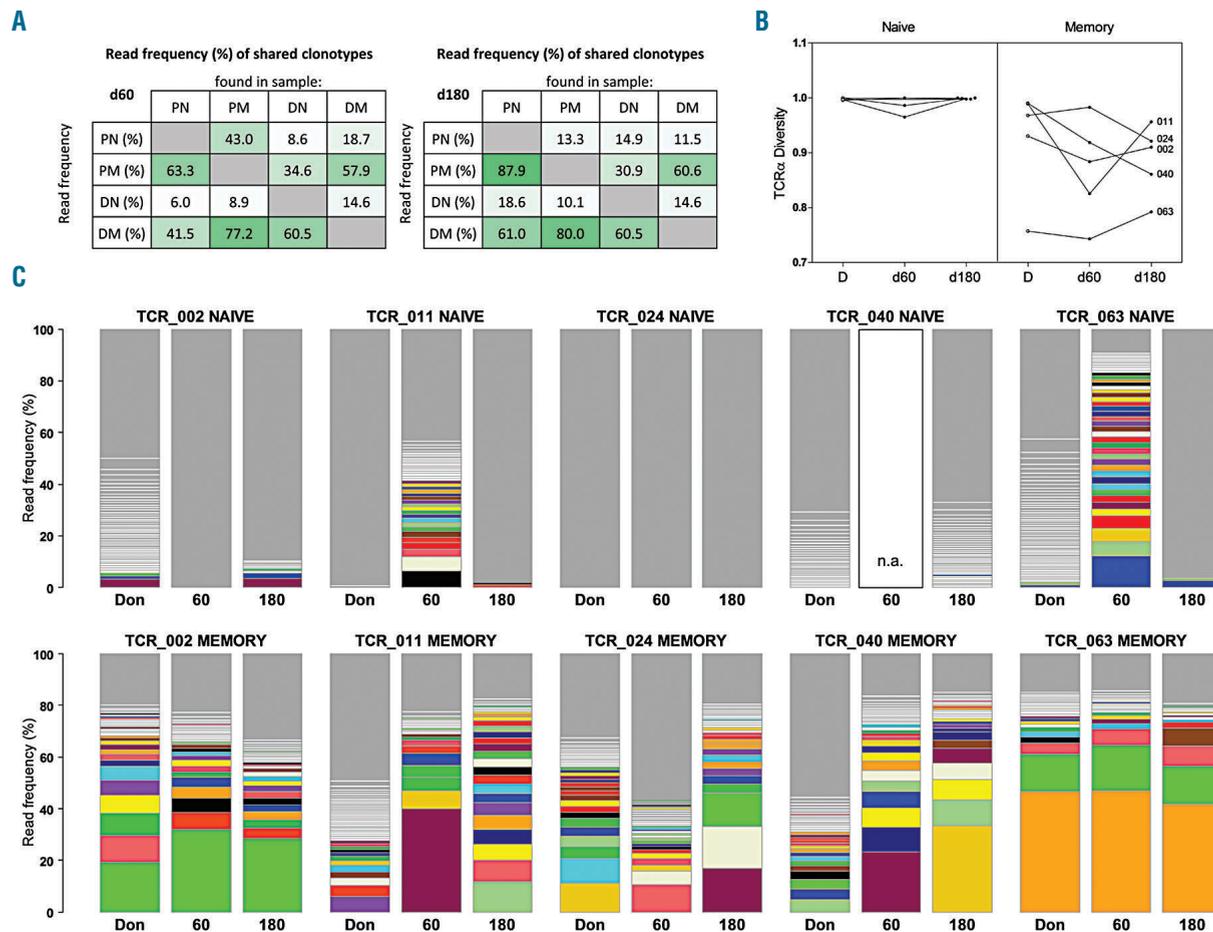
Figure 2. T-cell receptor alpha (TRα) repertoire of SIB-noATG patients and their donors. (A) Read frequencies (%) representing shared clonotypes between patient and donor repertoires on day 60 (left) and day 180 (right) are shown. Darker shading indicates higher read frequencies. (B) Simpson's diversity index (Ds) of the naïve and memory TRα repertoires for patients and their donors (D). TRα diversity on day 60 post transplantation (d60) and day 180 (d180) post transplantation for the patients TCR_002, TCR_011, TCR_024, TCR_040 and TCR_063 are plotted. (C) Distribution of TRα clonotypes in the CD8^{+} naïve (upper panels) and memory (lower panels) repertoires of each patient and donor. Starting on the left, the first bar represents the donor's repertoire (Don), followed by the patient's repertoire on day 60 post transplantation (60) and day 180 post transplantation (180). Frequent clonotypes (frequency of >1% of TRα reads) are shown in color. Each pair of donor and recipient identical clonotypes is shown in the same color (separately for the naïve and memory repertoires). All clonotypes with a TRα read frequency between 0.1 and 1.0% are in white; frequencies less than 0.1% are in gray. PN: patient's naïve repertoire; PM: patient's memory repertoire; DN: donor's naïve repertoire; DM: donor's memory repertoire; n.a.: not available.

between days 60 and 180 showed that both of these patients suffered from CMV reactivation on days 93-100 in TCR_024 and days 10-59 and day 152 in TCR_040 (Figure 2B and C). In addition, TCR_024 was diagnosed with aGvHD Grade II on day 88. TCR_040 suffered from Grade I aGvHD on day 74 and extensive cGvHD on day 136 following transplantation. Of note, the highly dominant clonotypes in the repertoire of patient TCR_040 were not found in the donor, in contrast to patient TCR_024.

Patient TCR_011 developed a highly dominant clonotype on day 60 (AA: CATDAPPSNDYKLSF; TRAV17, TRAJ20), representing 40.1% of the memory TRα repertoire that was not present in the donor's memory repertoire. In contrast to the other 2 patients who developed highly dominant clonotypes, this patient did not have any documented viral infections during the observation period but did experience extensive cGvHD on day 142. Patients TCR_063 and TCR_002 did not show any new "highly dominant" clonotypes in their repertoires at either time point because the documented highly dominant clono-

types were already frequently present in the donor's repertoire. Patient TCR_063 did not have any viral complications and had limited cGvHD only on day 93 following transplantation. Patient TCR_002 first developed aGvHD only on day 93; CMV viral load was detectable in this patient but remained below the quantifiable level of 300 IU/mL and was not considered clinically relevant.

Diversity of T-cell receptor-α in relation to the application of ATG or PTCy

The sequencing results obtained for the UD-ATG, mmUD-ATG, UD-noATG and Haplo-PTCy groups, including the obtained TRα reads and clonotypes, are shown in *Online Supplementary Table S2*. Interestingly, patients who did not receive ATG or PTCy (UD-noATG) had the highest mean diversity in the naïve repertoire of all groups, with a D_s of 0.998761 on day 60 and 0.999677 on day 180 following transplantation. Indeed, these patients did not show any highly frequent clonotypes at all, and clonotypes with frequencies of more than 1.0%
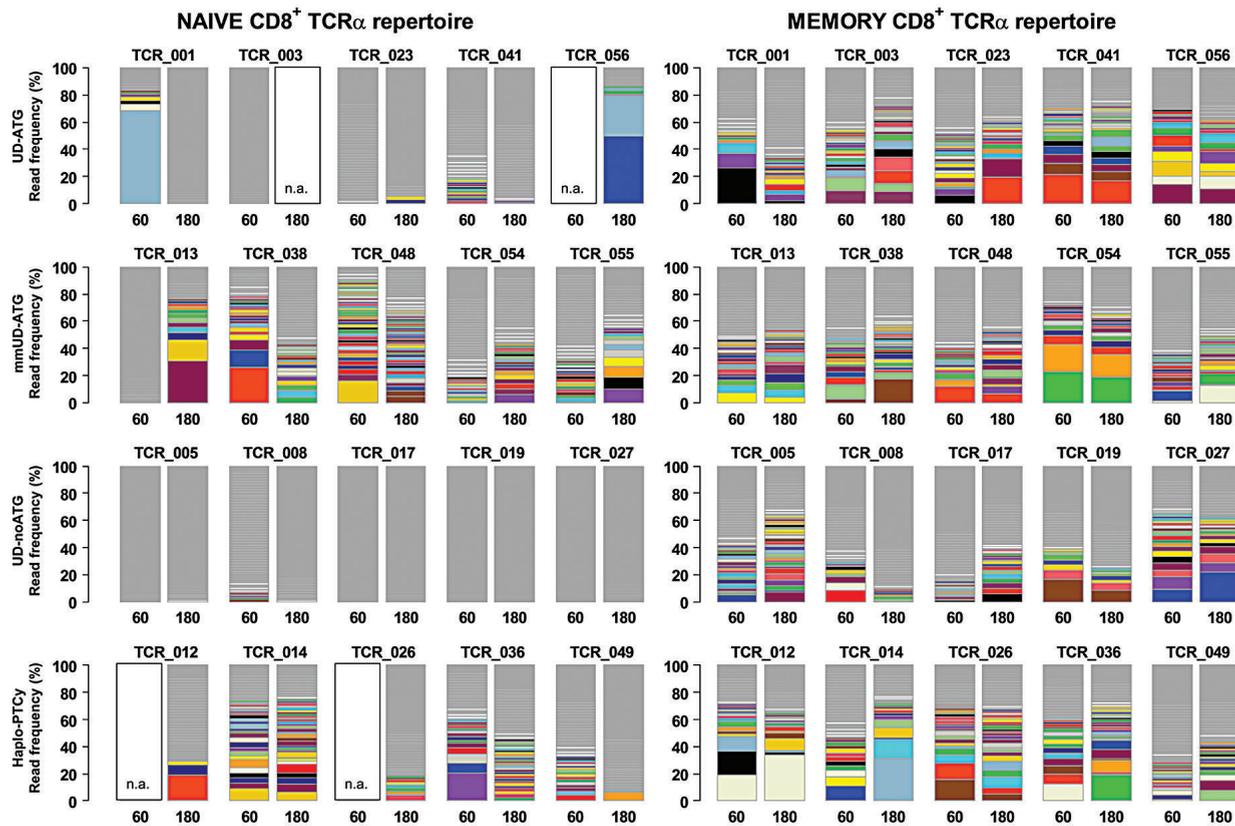
Figure 3. T-cell receptor alpha (TRα) repertoire composition in the context of T-cell depletion. Distribution of TRα clonotypes in the CD8+ naïve (left) and memory (right) repertoires of patients from four groups: UD-ATG, mmUD-ATG, UD-noATG and Haplo-PTCy. The patients' repertoires on day 60 post transplantation (60) and day 180 post transplantation (180) are mapped for each patient. Frequent clonotypes (frequency >1% of TRα reads) are shown in color. Each pair of identical clonotypes on day 60 and day 180 are shown in the same color (separately for the naïve and memory repertoire). All clonotypes with a TRα read frequency between 0.1% and 1.0% are colored in white; frequencies less than 0.1% are in gray. n.a.: not available.

were present in only one patient on day 60, representing 7.4% of TRα reads (TCR_008) (Figure 3).

The lowest mean naïve TRα repertoire diversity was seen in the UD-ATG group (d60 D_s=0.880519 and d180 D_s=0.915102). Lower diversity was generally caused by a few highly dominant clonotypes in individual patients. For example, in TCR_001 (D_s=0.530540), one dominant clonotype represented 68.2% of the naïve TRα repertoire (AA: CAYSPYDKVIF; TRAV38-2/DV8, TRAJ50). Similarly, on day 180 following transplantation, two highly dominant clonotypes with frequencies of 49.7% and 29.4% in the repertoire of patient TCR_056 disproportionately contributed to the lower diversity (D_s=0.665819) than the diversity in other groups.

In the TRα memory repertoire on day 60, most dominant clonotypes were seen in the UD-ATG group, in which a mean of 59.0% of clonotypes had frequencies of more than 1% of TRα reads, followed by the Haplo-PTCy group, in which a mean of 54.6% of the TRα was represented by frequent clonotypes. The diversities of the UD-ATG and Haplo-PTCy groups were 0.949665 and 0.958078, respectively, which were the two lowest diversities among the four groups. The high amount of frequent clonotypes within these two groups was sustained over time: on day 180 more than 50% of the repertoire was again represented by frequent clonotypes [58.0% (D_s=0.957527) and 61.8% (D_s=0.928325) for the UD-ATG

and Haplo-PTCy groups, respectively] (Figure 3). The spatial distributions of the clonotypes, which were visualized by normalizing within the group, supported the distributions described for each patient individually. These distributions showed that no frequent clonotypes were seen in the naïve repertoire of UD-noATG-patients, and the space occupied by rare clonotypes in their memory repertoire was larger than that in the groups receiving T-cell-depleting transplant regimens (Figure 4). There were too few observations within one group to perform statistical testing of TRα diversity within the treatment cohorts.

To understand how the naïve repertoire helps repopulate the memory repertoire, we looked for clonotype overlap between the naïve and memory repertoires in all patients. In the naïve repertoire, a mean of 31.9% of TRα reads on day 60 and 29.7% on day 180 were represented by clonotypes that were also found in the memory repertoire. In the memory repertoire, a higher proportion of TRα reads consisted of shared clonotypes. On days 60 and 180, a mean of 51.0% and 63.4% of memory TRα reads, respectively, were composed of clonotypes found in the naïve and memory repertoire.

Donor age and graft cell counts do not affect T-cell receptor-α diversity

Total number of T cells contained in the graft varied between the cohorts. Patients receiving a haploidentical

bone marrow transplant showed the lowest T-cell counts ($P=0.007$). Nevertheless, analyses of graft cell counts among the total number of CD34^{+} cells (x10^{6}/kg body weight) and total number of T cells showed no association with TRα diversity (*Online Supplementary Figure S3*). Furthermore, there was no relation between donor age and the diversity in the patient's repertoire.

Cytomegalovirus serostatus impairs T-cell receptor-α diversity

Cytomegalovirus infections impose a serious post-transplantation risk. The occurrence of CMV infections and CMV serostatus of patients and donors were analyzed for associations with TRα diversity. CMV infections were mainly observed within the first 60 days following transplantation. Ten of 25 patients suffered from CMV infections during the first 60 days (Figure 5A). The correlation between CMV infection and TRα diversity is shown in Figure 5B. Statistical analysis revealed no significant differences in TRα diversity for patients suffering from CMV reactivation and patients without detected CMV reactivation. Significantly lower diversity in the naïve TRα repertoire ($P=0.014$) was observed in patients transplanted from a CMV-seropositive donor. In the memory repertoire, a tendency towards lower diversity was also observed at both time points (mean D_s of 0.933183 and 0.936029 *vs.* 0.956337 and 0.950007, respectively).

Acute and chronic graft-*versus*-host disease in association with TCR diversity

The occurrence of aGvHD and TRα diversity for each patient is shown in Figure 6A. Sixteen (64.0%) out of 25 patients developed aGvHD, with 9 (36.0%) patients suf-

fering from aGvHD ≥Grade II. A tendency towards higher TRα diversity in the memory compartment was observed in patients suffering from aGvHD compared with patients without aGvHD on day 60 (0.958911 *vs.* 0.918315, respectively), but this difference was not statistically significant. On day 180 post transplantation, comparable memory D_s values were seen for patients with and without aGvHD (0.943686 *vs.* 0.941054, respectively). Nevertheless, diversity in aGvHD patients decreased over time, while non-GvHD patients showed increasing diversity. Similarly, the mean naïve D_s was higher in aGvHD patients on day 60 than in non-GvHD patients (0.990826 *vs.* 0.916534, respectively), with a decrease to a mean of 0.959410 on day 180 compared to 0.95290 in non-GvHD patients. No differences were observed between patients who developed aGvHD before day 60 (early) and those who developed aGvHD after day 60 (late).

Chronic graft-*versus*-host disease occurred in 12 out of 25 patients (48.0%), with 10 patients suffering from extensive cGvHD. The mean D_s of the naïve compartment was higher in cGvHD patients than in non-GvHD patients at both time points (0.983022 and 0.992050 *vs.* 0.947150 and 0.953680, respectively). In contrast, the TCR diversity of the memory compartment tended to be lower in cGvHD patients than in patients without cGvHD (0.932145 and 0.929679 *vs.* 0.955514 and 0.954794 on day 60 and day 180, respectively) (Figure 6B).

Discussion

Immune reconstitution following transplantation is essential to achieve optimal outcomes with allogeneic SCT. However, detailed knowledge of TCR repertoire
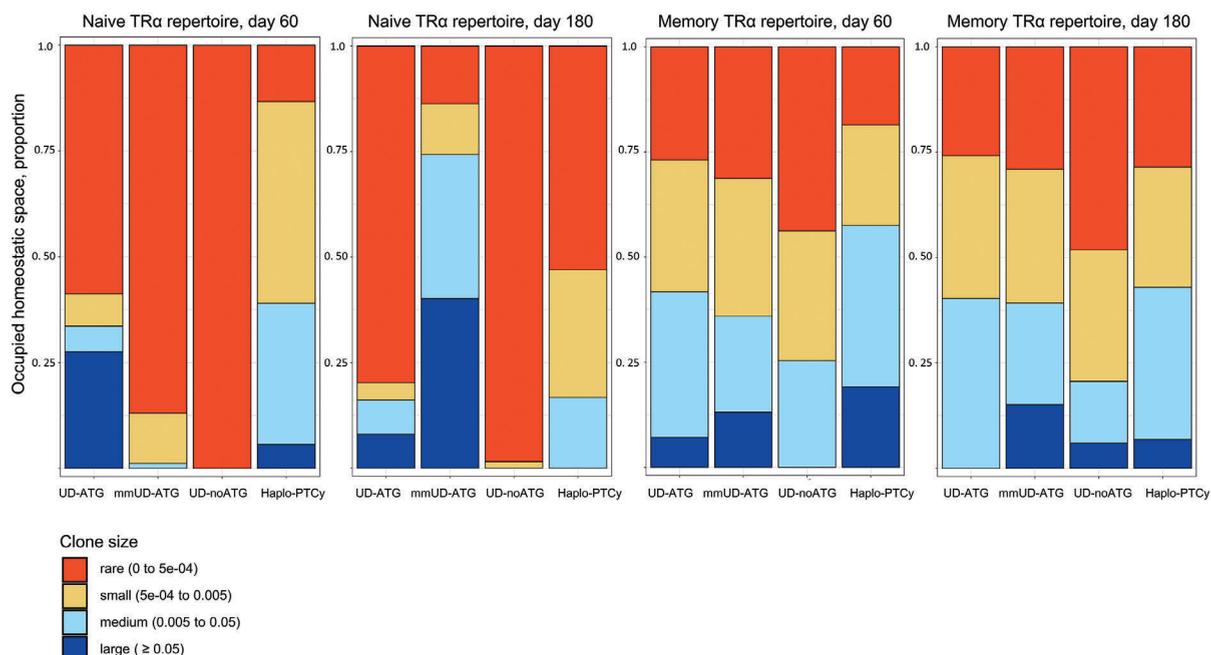
Figure 4. Normalized spatial clonotype distribution. Distribution of the clonotypes is visualized by grouping them according to their proportions (rare, small, medium and large) and normalization within the UD-ATG, mmUD-ATG, UD-noATG and Haplo-PTCy groups. Clonal space distribution was calculated using a R package provided for TCR repertoire data analysis.[28] Starting on the left, the first two panels represent the naïve repertoire (day 60 followed by day 180 post transplantation) and successive panels show the distribution of the memory repertoire on day 60 and day 180 post transplantation.

reconstitution following transplantation remains scarce. The recovery of neutrophil counts is achieved between day 14 and day 30 post transplantation. In contrast, reduced lymphocyte counts within the first 100 days following transplantation and an associated delay in T-cell reconstitution have been reported.[22] Consistent with this, only patients from two groups out of five in the current analysis achieved normal range lymphocytes by day 180.

T cells in transplanted patients primarily consisted of CD8^{+} T cells, contrasting the preferential CD4^{+}-dominated composition of the healthy reference population. This inverse ratio of CD4^{+} and CD8^{+} T cells after transplantation has been previously described. Our detailed analyses of CD8^{+} T-cell subsets revealed the dominance of memory cells (EM and TEMRA) and reduced naïve CD8^{+} T-cell frequencies. This finding is in line with previous reports demonstrating that following SCT, the T-cell compartment is mainly composed of memory cells with an associated low recovery of naïve cells. The dominance of memory cells is assumed to be based on the proliferation of T cells that were already present in the donor graft.[13]

In this context, the impact of *in vivo* T-cell depletion is of special interest, as we demonstrated the lowest naïve cell counts within these groups. The application of ATG prior to transplantation has been reported to impair the reconstitution of naïve CD4^{+} and naïve CD8^{+} T cells for up to one year while not affecting the reconstitution of effector memory cells.[23] Effects in patients receiving PTCy were described differently in prior studies. Approximately 70%

of memory and effector T cells were depleted by the application of cyclophosphamide, whereas naïve T cells were not affected by cyclophosphamide. The authors proposed that T-cell reconstitution is generated from naïve precursors with T cells acquiring an effector phenotype after antigen stimulation from naïve-derived T cells.[24,25]

Assessment of the dominant clonotypes revealed striking differences between groups, with dominant clonotypes in the naïve repertoire being highest in the groups receiving ATG and PTCy. The underlying mechanism for the enhanced clonal proliferation of naïve clonotypes under these conditions remains unknown. The participation of antigen-specific naïve T cells in immune reconstitution following SCT has been described.[25] These cells may stimulate the proliferation of certain T-cell clonotypes in the naïve compartment. Furthermore, the presence of stem cell-like memory T cells preceding the reconstitution of effector cells following transplantation originating from naïve T cells has been reported.[25,26]

The assumption that early immune reconstitution originates from naïve precursors gives reason to expect that the memory repertoire is mirrored by clonotypes that are also present in the naïve compartment. In our samples, we determined that 51% and 63% (day 60 and day 180, respectively) of the memory repertoire was composed of clonotypes that were also found in the naïve compartment. Approximately 40-50% of the TRα memory repertoire was not identified in the naïve compartment. Similar observations were previously described for TRβ.[13] The
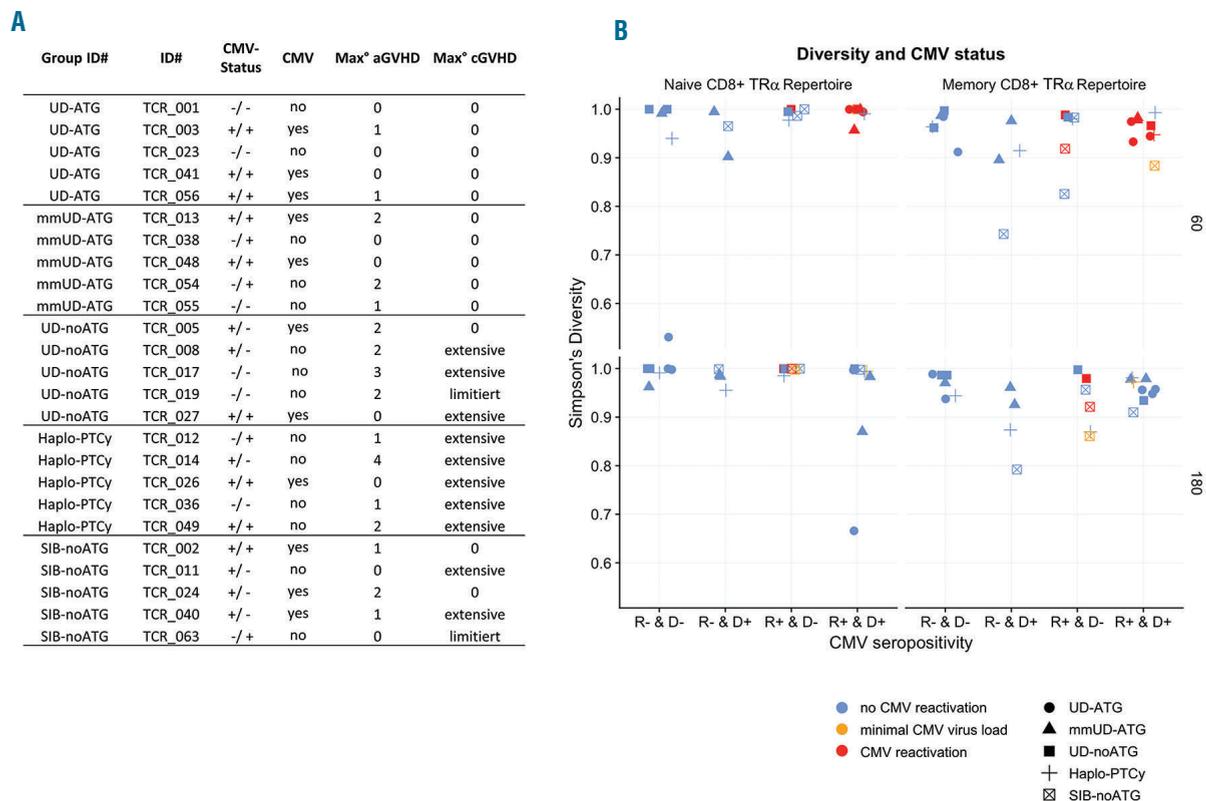
Figure 5. Cytomegalovirus (CMV) and T-cell receptor alpha (TRα) diversity. (A) Clinical observations of the development of CMV reactivation (CMV, yes - no), acute graft-*versus*-host disease (aGvHD) and chronic GvHD (cGvHD). (B) Simpson's diversity index (Ds) of the naïve and memory TRα repertoire of each patient on day 60 (60) and day 180 (180) post transplantation. Patients suffering from CMV reactivation close to the sampling point were mapped. Repertoire diversity is mapped in relation to CMV serostatus of the recipient (R) and donor (D). CMV seropositivity (+) and CMV seronegativity (-).
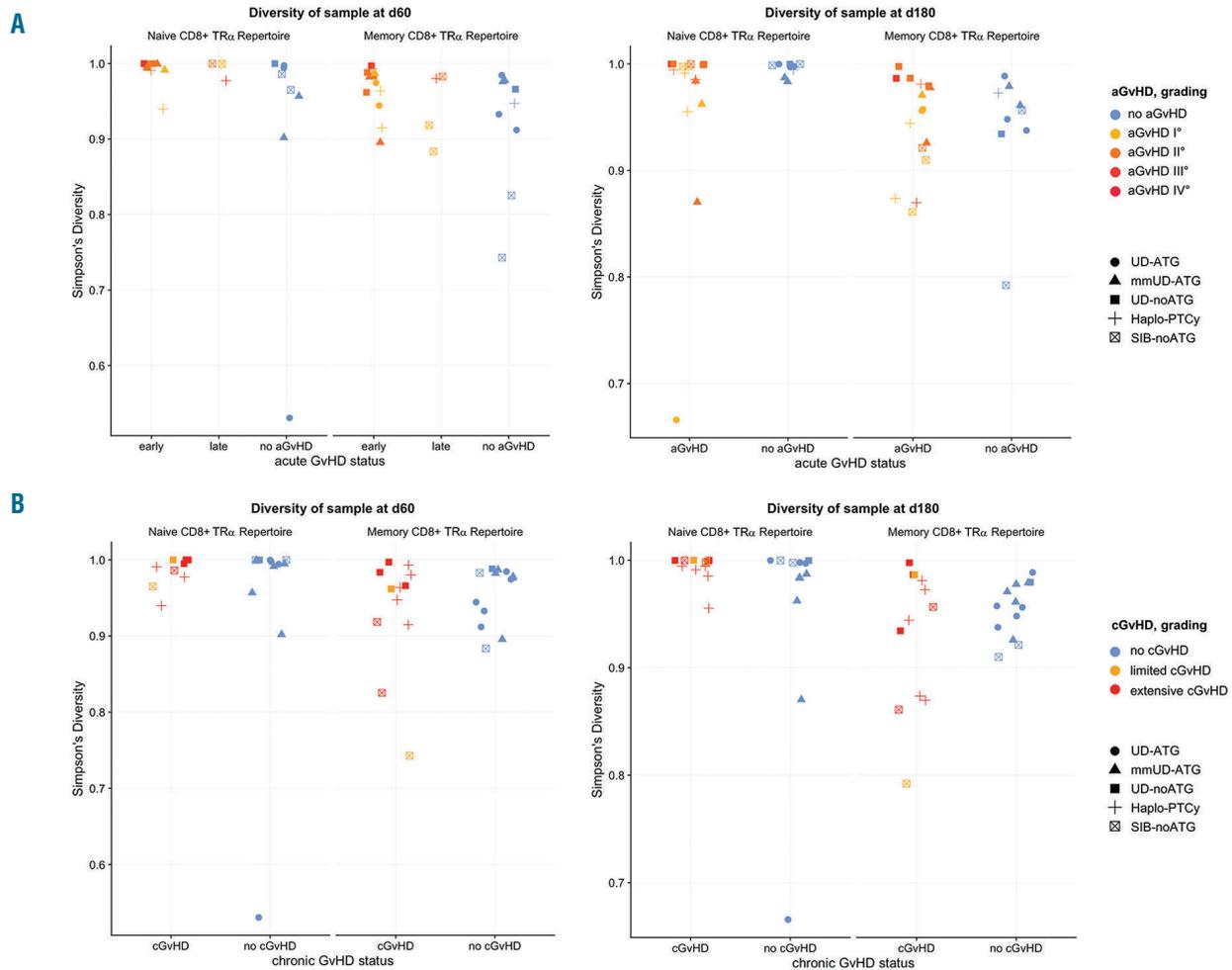
Figure 6. Graft-*versus*-host disease (GvHD) and T-cell receptor alpha (TRα) diversity. (A) Simpson's diversity index (D_S) on day 60 (left) and day 180 (right) of the naïve and memory TRA repertoire of each patient following transplantation. Repertoire diversity is mapped in relation to the occurrence of acute GvHD (aGvHD). Left: patients suffering from aGvHD prior to or at the first time of sampling (day 60 following transplantation) were designated "early". Patients with aGvHD past the first next-generation sequencing (NGS) sampling were designated "late". (B) Simpson's diversity index (D_S) on day 60 (left) and day 180 (right) following transplantation of the naïve and memory TRA repertoire of each patient. Repertoire diversity is mapped in relation to the occurrence of chronic GvHD (cGvHD).

authors assumed that the naïve-derived TCR repertoire established in the first month following SCT does not persist longer, and memory-derived clonotypes dominate the long-lasting TCR repertoire.[13]

In our study, when comparing the TRα repertoires of donors and recipients, we observed that a high percentage of shared reads in both the naïve (approx. 50% of reads) and memory (approx. 80%) repertoires was derived from the memory compartment of the donor, but only approximately 10% of reads were derived from the naïve compartment. These results indicate that, in the absence of T-cell depletion, a large proportion of the donor's memory repertoire contributes to the initial TRα reconstitution in transplanted patients. Of note, the 2 patients (TCR_011 and TCR_040) with new highly dominant clonotypes in their memory repertoires that were not detected in the donor repertoires were diagnosed with extensive cGvHD during the observation period. It remains to be clarified in further studies whether there is an association between extensive GvHD and the appearance of newly dominant clonotypes.

T-cell receptor-α sequencing in patients receiving PTCy

has previously shown that these patients have a unique repertoire in the first month following transplantation but become donor-like during the first year post transplantation.[13] Given the small number of patients in our study, and our focus on the first six months following transplantation, no significant changes towards a more donor-like repertoire were observed in any of the patients in the observed time. However, a donor-like memory repertoire was evident in 2 patients, with more than 90% of the memory repertoire represented by clonotypes that were also identified in the donor's repertoire.

It is still not known whether certain changes in the TCR repertoire render patients more prone to immunological complications post transplantation, such as acute and chronic GvHD as well as viral infections. Published studies addressing the relation between TCR diversity and GvHD are controversial. One of the first studies to analyze the TRβ repertoire in transplanted patients found that patients with aGvHD (≥Grade II) had a higher TRβ diversity.[9] In contrast, other studies have shown the association of aGvHD and/or cGvHD with a more clonal TRβ reper-

toire.[11,13] In our study, we were unable to show statistical significance for any correlation between aGvHD or cGvHD and TRα diversity. However, despite the limited number of patients and the large deviation between individual samples, there was a tendency towards higher memory diversity in patients with aGvHD on day 60 and lower TRα memory diversity in patients with cGvHD at both time points. Evidence is emerging that the association between diversity and GvHD may be individualized in each patient: single clones that expand massively can induce aGvHD, while in other cases, hundreds of clonotypes may be required to achieve a similar effect.[12] Another potential explanation for the lack of correlation may be that, in this study, NGS sampling was performed on day 60 and day 180 but not specifically at the time of GvHD diagnosis. Previous publications have shown that diversity is significantly lower when the sample is drawn at the time of GvHD diagnosis.[11]

Similarly, no difference in TRα diversity was observed in association with CMV reactivation. CMV reactivation induces a skewed and less diverse repertoire, as shown in one of our previous publications[10] and by other groups.[9,13,27] Again, since this study did not primarily focus on CMV reactivation/infection, samples were not taken close to the day of detected CMV reactivation. Nevertheless, we observed a tendency towards lower diversity in patients transplanted from a CMV-seropositive donor. Lower diversity has previously been shown in patients with CMV-seropositive donors.[13]

In conclusion, this study is the first to analyze TRα repertoire reconstitution in transplanted patients focusing on different transplant regimens. Repertoire sequencing by NGS represents a new method for in-depth immune monitoring. Further studies are needed to clarify the effects and prognostic value of repertoire changes in various clinical settings.

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