HHS Public Access

Author manuscript

Nat Med. Author manuscript; available in PMC 2013 September 01.

Published in final edited form as:

Nat Med. 2013 March ; 19(3): 372–377. doi:10.1038/nm.3100.

Quantitative assessment of T-cell repertoire recovery after hematopoietic stem cell transplantation

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Abstract

Delayed T-cell recovery and restricted T-cell receptor (TCR) diversity after allogeneic hematopoietic stem cell transplantation (allo-HSCT) are associated with increased risks of infection and cancer relapse. Technical challenges have limited faithful measurement of TCR diversity following allo-HSCT. Here we combined 5′-RACE PCR with deep sequencing, to quantify TCR diversity in 28 allo-HSCT recipients using a single oligonucleotide pair. Analysis of duplicate blood samples confirmed that the frequency of individual TCRs was accurately determined. After 6 months, cord blood graft recipients approximated the TCR diversity of healthy individuals, whereas recipients of T-cell-depleted peripheral blood stem cell grafts had a 28-fold and 14-fold lower $CD4^+$ and $CD8^+$ T-cell diversity, respectively. After 12 months, these deficiencies had improved for the $CD4^+$, but not the $CD8^+$ T-cell compartment. Overall, this method provides unprecedented views of T-cell repertoire recovery after allo-HSCT and may identify patients at high risk of infection or relapse.

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AUTHOR CONTRIBUTIONS

J.W.J.v.H. designed and performed the study, analyzed data and wrote the manuscript. I.C. collected clinical data. L.B.L. performed Roche/454 sequencing. D.W.S. compared naïve and memory $CD8^+$ T cells. G.D.W., A.M.R.G. and J.L.N. provided patient samples. M.R.M.v.d.B. designed and supervised the study. M.A.P. designed and supervised the study, and provided patient samples. E.G.P. designed and supervised the study, analyzed data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

INTRODUCTION

Allo-HSCT is a potentially curative treatment for a variety of hematologic diseases, including lymphoid and myeloid malignancies. Prior to transplantation, patients undergo conditioning with chemotherapy with or without irradiation, which results in severe immunodeficiency that particularly for the T-cell compartment can take months or years to restore[1,2]. This prolonged T-cell deficiency predisposes patients to infection and cancer relapse[3–6]. Strategies that improve T-cell reconstitution and recovery of high TCR diversity could therefore greatly reduce transplant-associated morbidity and mortality[7].

Restoration of TCR diversity after allo-HSCT heavily depends on the thymic generation of new naïve T cells[8–10]. Thymic function, however, diminishes markedly after the onset of puberty, and, in the allo-HSCT setting, is further impaired due to conditioning-associated damage and graft-versus-host disease (GVHD)[11,12]. Thus, it is unclear how well TCR diversity can be restored, particularly in older patients.

Over the past two decades, several strategies have been developed to probe human TCR diversity. One strategy aims to identify the presence of different TCR families, by using flow cytometry or PCR to determine the usage of different TCR variable (V) genes[13,14]. A second strategy, called CDR3 size spectratyping, aims to determine polyclonality of the repertoire, by using fluorescent primers to measure length variation of the CDR3 region within each TCR V family[15,16]. Spectratyping in particular has been useful to document substantial abnormalities in T-cell repertoire composition after allo-HSCT[17–19]. However, as neither of these strategies is able to measure the frequency of individual TCRs, they can only provide an estimate of repertoire complexity. With the advent of deep sequencing technology, it has now become possible to directly measure TCR diversity with high resolution[20–26]. Here, we have built on this approach to address two fundamental questions related to T-cell reconstitution after allo-HSCT: how TCR diversity recovers I) over time and II) as a function of different stem cell sources[27,28].

ONLINE METHODS

Patients

28 patients underwent allo-HSCT at Memorial Sloan-Kettering Cancer Center (MSKCC) from April 2010 through September 2011. Patient and treatment characteristics are summarized in Supplementary Table 1. Pre-transplant conditioning varied according to patient age, diagnosis, remission status, extent of prior therapies, and co-morbidities; and consisted of high-dose, reduced-intensity myeloablative and nonmyeloablative regimens[35]. GVHD prophylaxis for peripheral blood stem cell transplantation was either with T-cell depletion[34] or calcineurin inhibitor-based, and anti-thymocyte globulin was used according to protocol or physician preference. Cord blood recipients received mycophenolate mofetil and calcineurin inhibitors[36]; however, no patient received anti-thymocyte globulin[33]. Post-transplant granulocyte colony-stimulating factor was used in all patients. Acute and late acute or chronic GVHD were diagnosed clinically with histological confirmation when possible. Staging of GVHD was graded according to standard criteria[37,38]. All subjects

provided Institutional Review Board-approved informed consent for collection of blood samples. Graft samples were not available for analysis.

T-cell isolation and flow cytometry

From each ~8 ml heparinized blood sample, mononuclear cells were isolated by density centrifugation (Lymphocyte Separation Medium, MP Biomedicals). Recovered cells were lysed in RLT buffer (QIAGEN), homogenized using QIAshredder columns (QIAGEN) and stored at −80 °C. For $CD4^+$ and $CD8^+$ T-cell separation, two blood samples were pooled, followed by isolation of the mononuclear cell fraction. Recovered cells were split into two fractions and incubated with either CD4 or CD8 MicroBeads (Miltenyi Biotec). $CD4^+$ and $CD8^+$ T cells were separated using MS columns (Miltenyi Biotec). Eluted cells were lysed, homogenized and stored as above. To determine the efficiency of T-cell separation, eluted cells were stained with antibody to CD14 (clone M5E2, 1:5), antibody to CD4 (clone SK3, 1:20) and antibody to CD8 (clone RPA-T8, 1:5; all BD Pharmingen), and measured on an LSRII flow cytometer (BD Biosciences). Data was analyzed using FlowJo software (TreeStar). For separation of naïve and memory $CD8^+$ T cells, isolated mononuclear cells were stained with antibody to CD45RA (clone HI100, 1:5), antibody to CD45RO (clone UCHL1, 1:5; both BD Pharmingen) and antibody to CD8. Cells were sorted using a FACSAria cell sorter (BD Biosciences) into $CD8^+CD45RA^+CD45RO^-$ (naïve) and $CD8^+CD45RA–CD45RO^+$ (memory) fractions.

5′-RACE PCR and Roche/454 sequencing

Total RNA from frozen homogenates was extracted using an RNeasy mini kit (QIAGEN). RACE-Ready cDNA was generated using a SMARTer RACE cDNA Amplification kit (Clontech) and oligo(dT) or random (N–15) primers. 5′-RACE PCR was performed using Advantage 2 Polymerase (Clontech) with Clontech's universal forward primer and a self-designed universal TCRβ-constant reverse primer compatible with both *TRBC* genes (5′-GCACACCAGTGTGGCCTTTTGGG-3′). Amplification was performed on a Mastercycler pro (Eppendorf) and was 1 min at 95 °C; 5 cycles of 20 sec at 95 °C and 30 sec at 72 °C; 5 cycles of 20 sec at 95 °C, 30 sec at 70 °C and 30 sec at 72 °C; 25 cycles of 20 sec at 95 °C, 30 sec at 60 °C and 30 sec at 72 °C; 7 min at 72 °C. PCR products were loaded on 1.2% agarose gels (Bio-Rad) and bands centered at ~600 bp were excised and purified using a MinElute Gel Extraction kit (QIAGEN). Purified products were subjected to a second round of amplification to introduce adaptor sequences compatible with uni-directional Roche/454 sequencing. $1/50^{th}$ of first-round PCR product was amplified using Advantage 2 Polymerase with a hybrid forward primer consisting of Roche's Lib-L primer B and Clontech's nested universal primer (5′-CCTATCCCCTGTGTGCCTTGGCAGTCTCAGAAGCAGTGGTATCAACGCAGAGT-3′) and a hybrid reverse primer consisting of Roche's Lib-L primer A and a self-designed nested universal TCRβ-constant primer (5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG-MID-AACACAGCGACCTCGGGTGGGAA-3′, wherein MID represents the multiplex identifier used to separate pooled samples during sequence analysis). Multiplex identifiers were 6–7 bp long. Amplification was 1 min at 95 °C; 25 cycles of 20 sec at 95 °C, 30 sec at 68 °C and

30 sec at 72 °C; 7 min at 72 °C. PCR products were purified from agarose gels as above. Purified products were sequenced using the GS Junior 454 platform (Roche).

Sequence analysis

Raw sequence data was converted to FASTA format using MOTHUR software[39]. Sequences shorted than 125 bp, with uncalled bases, with a Phred quality score average below 30 (base call accuracy < 99.9%)[30], or with no exact match to the TCRβ-constant primer or a multiplex identifier were discarded. Resulting FASTA files were uploaded to the IMGT/HighV-QUEST database (http://www.imgt.org/HighV-QUEST/index.action)[40]. Using IMGT summary files, sequences with out-of-frame rearrangements, with a V- and J-region identity < 80%, with V-region pseudogenes or a CDR3β amino acid junction lacking a 5′ cysteine and 3′ phenylalanine were discarded. Resulting sequences were sorted using Excel (Microsoft) and graphed using Prism 5 software (GraphPad). The inverse Simpson's diversity index (1/*D*s) was calculated using MOTHUR.

Statistical analysis

TCR diversity was compared using an unpaired Student's *t*-test (two groups) or one-way ANOVA with Bonferroni's multiple comparison test (three groups or more). A *P* value of < 0.05 was considered statistically significant.

RESULTS

Strategy and reproducibility of T-cell repertoire analysis

T cells typically express only one TCRβ chain, making sequence analysis of TCRβ cDNA a useful measure of TCR diversity. To faithfully evaluate the TCRβ repertoire we made use of 5′-RACE PCR, which allows amplification of all 48 Vβ genes using a single oligonucleotide pair. To test the reproducibility of this approach[29], we separately amplified two ~8 ml blood samples from a single patient, 138 days following T-cell-depleted (TCD) peripheral blood stem cell transplantation (TCD no.1; Supplementary Table 1). For comparison, we also amplified two blood samples from four healthy donors (Healthy nos.1–4). All samples were analyzed by Roche/454 sequencing, using a Phred quality score average of 30 to minimize sequence errors[30]. Among total TCR sequences, all 48 Vβ genes were found (Supplementary Fig. 1), confirming that our approach covers the entire TCRβ repertoire. Comparison of the two patient blood samples showed a highly reproducible pattern of Vβ usage, which differed markedly from healthy donors (Fig. 1a). This suggested substantial clonal expansions in the patient's repertoire, which were confirmed by digital CDR3 size spectratype profiles (Fig. 1b).

Next, we determined the frequency at which each distinct TCRβ chain, or TCRβ clonotype was present. In 15,902 reads obtained from both patient blood samples, we detected 1,097 clonotypes, with the most frequent clonotype comprising 11.7% of reads (Fig. 1c). In fact, 19 clonotypes were present above 1% and together constituted 70.8% of reads. In contrast, the most abundant clonotype in healthy donors comprised just 2.8 ± 2.6% of reads, and on average only 3 clonotypes were found above 1% (Supplementary Fig. 1). To establish the accuracy of clonotype frequencies, we compared the clonotype distribution of both patient

blood samples. Importantly, abundant clonotypes found in one blood sample were found with almost identical frequency in the other blood sample, resulting in a near-perfect correlation of both clonotype distributions (*r*: 0.98; Fig. 1d). In healthy donors, expanded clonotypes were also detected with high reproducibility, however the average correlation between two blood samples was lower (*r*: 0.44; Supplementary Fig. 1), primarily because fewer clonotypes passed the threshold for physical presence in a second blood sample (8 ml of ~5 liter total = ~0.16%).

To quantify TCR diversity, we used the inverse Simpson's diversity index (1/*D*s), which sums the frequency of each clonotype[31]. This index ranges from 1 to ∞ (no to infinite diversity) and is highest when all clonotypes are equally distributed. To test the usefulness of this index, we sorted naïve and memory $CD8^+$ T cells from Healthy no.1 and found a 20-fold higher TCR diversity in the naïve T-cell compartment (Supplementary Fig. 2). Analysis of the patient's repertoire revealed a very low TCR diversity (1/*D*s: 23), which was more than 100-fold lower than the average diversity of four healthy donors (1/*D*s: 2,525; Fig. 1e). Therefore, at 138 days after transplant this patient's T-cell repertoire was highly restricted.

To monitor repertoire recovery over time, we measured three additional timepoints of TCD no.1 at days 147, 194 and 377 after transplant. To our surprise, TCR Vβ usage was very different at each timepoint examined (Fig. 2a), indicating high variability of the T-cell repertoire. To evaluate individual clonotypes, we first determined whether clonotype frequencies were reliably measured. On day 147, we again found a near-perfect correlation between two blood samples (*r*: 0.99), and the same held true for days 194 and 377 (*r*: 1 and 0.98, respectively; Fig. 2b). Comparison of the repertoire on days 138 and 147, however, revealed dramatic shifts in clonotype frequencies (Fig. 2c). Although some clonotypes were present at roughly similar frequency on both days, several others differed by more than 100-fold; resulting in a low similarity between both T-cell repertoires measured just 9 days apart (*r*: 0.24). Importantly, these repertoire shifts coincided with Epstein-Barr virus (EBV) reactivation in the patient, which was first detected on day 145 and peaked on day 147 (Supplementary Fig. 3). We identified the 9th most abundant clonotype on day 147 (*TRBV29–1*, CDR3β CSVGTGGTNEKLFF) as being specific for the HLA-A2-restricted $BMLF1_{280}$ epitope from EBV[32]. While undetectable on days 138 and 194, this clonotype comprised 2.9% of reads on day 147 and 0.1% on day 377, highlighting the potential of our method to track antigen-specific clonotypes. Despite apparent resolution of EBV reactivation by day 158, subsequent blood samples continued to reveal clonotype frequencies fluctuating over orders of magnitude. Thus, the repertoire of two samples on the same day was highly similar (*r*: 0.99), whereas on different days it was markedly distinct (*r*: 0.25; Fig. 2d). A notable exception was the repertoire of days 138 and 377, which revealed a surprisingly large degree of similarity (*r*: 0.87; Supplementary Fig. 3). Despite profound changes in repertoire composition, TCR diversity did not increase over time (1/*D*s: 23 and 19 for days 138 and 377, respectively; Fig. 2e). Therefore, between 4.5 and 12.5 months after transplant, the complexity of this patient's T-cell repertoire did not improve.

T-cell repertoire recovery by different stem cell sources

To evaluate whether our method might enable stratification of allo-HSCT recipients according to their T-cell repertoire, we measured TCR diversity in 27 patients at either 6 or 12 months after conventional (Conv) or TCD peripheral blood stem cell transplantation, or double-unit umbilical cord blood (DUCB) transplantation (Fig. 3a–c). Since analysis of TCD no.1 had suggested substantially greater TCR diversity in $CD4^+$ compared to $CD8^+$ T cells (Supplementary Fig. 4), we separately analyzed both T-cell compartments for all ensuing patients, as well as for five healthy donors (Fig. 3d). After determining the TCR diversity of each individual, we established the following: I) for all stem cell sources as well as for healthy donors, $CD4^+$ T-cell diversity was ~50-times higher than $CD8^+$ T-cell diversity (1/*D*s: 4,665 and 81, respectively; Fig. 3e,f). II) Regarding $CD4^+$ T cells, healthy donors had the highest TCR diversity (1/*D*s: 15,470), followed by DUCB after 12 months (1/*D*s: 5,069), DUCB after 6 months (1/*D*s: 3,745), Conv after 12 months (1/*D*s: 3,298), TCD after 12 months (1/*D*s: 1,871), Conv after 6 months (1/*D*s: 674) and TCD after 6 months (1/*D*s: 132). Therefore, DUCB recipients had the highest TCR diversity of all patients and had a significantly (28-fold; $P = 0.033$) more diverse $CD4^+$ T-cell repertoire compared to TCD recipients after 6 months. Importantly, this increased TCR diversity also correlated with a substantially greater fraction of naïve $CD4^+$ T cells in DUCB compared to TCD recipients (Supplementary Fig. 5). Although TCD recipients had limited $CD4^+$ T-cell diversity after 6 months, this diversity was 14-fold higher after 12 months, reducing the difference with DUCB recipients to 3-fold. III) Regarding $CD8^+$ T cells, DUCB recipients again had the highest TCR diversity of all patients, which was 14-fold higher than TCD recipients after 6 months and 17-fold higher after 12 months, thereby reaching statistical significance ($P = 0.012$).

Using above data, we investigated several clinical parameters that could influence T-cell repertoire recovery. We found no significant impact of age or donor on TCR diversity (Fig. 4a,b). Interestingly, acute GVHD (grade 2 or 3) and systemic steroid treatment were associated with higher TCR diversity, suggesting that these variables do not restrict repertoire recovery (Fig. 4c,d). In contrast, cytomegalovirus (CMV) or EBV infection were associated with lower TCR diversity (Fig. 4e).

Within each stem cell group, we identified patients who had normal T-cell counts, but very low TCR diversity after one year (Supplementary Fig. 6). To investigate repertoire recovery during the second year of transplant, we reanalyzed TCR diversity in three of these patients after 19–21 months. For comparison, we also reanalyzed three healthy donors. We found stability in the $CD4^+$ T-cell repertoires of Conv no.6 and TCD no.8, and the $CD8^+$ T-cell repertoire of DUCB no.7 (Fig. 5a). Similar stability was observed in the $CD8^+$ T-cell repertoires of healthy donors when measured over 109 days, whereas over 299 days there was somewhat greater divergence (Fig. 5b). Despite occasional changes in clonotype frequencies, TCR diversity in healthy donors was stable (Supplementary Fig. 7). Interestingly, in Conv no.6 the frequency of abundant clonotypes had substantially decreased over time, resulting in a 10-fold higher $CD4^+$ T-cell diversity (Fig. 5c). In contrast, there was no diversification in the other two patients. Together, these data illustrate

the potential of our method to identify patients as well as transplant protocols that are associated with either greater or lesser T-cell repertoire recovery.

DISCUSSION

In this study, we established a method to reproducibly and accurately measure human TCR diversity. By combining 5′-RACE PCR with deep sequencing, this method assesses the entire TCRβ repertoire using a single oligonucleotide pair, thereby eliminating amplification bias. This contrasts with TCR sequencing methods based on gDNA[20–23], which have to use many different oligonucleotides for amplification, making some degree of bias unavoidable. While 5′-RACE PCR provides a clear advantage, a limitation is that it requires RNA, and thus changes in TCR transcription could skew the frequency of particular clonotypes. Although we used Roche/454 sequencing here, our approach should be readily adaptable to other platforms with greater sequence coverage, which could help to identify infrequent TCRs.

We validated our method by measuring T-cell repertoire recovery in allo-HSCT recipients, in whom limited TCR diversity is linked to susceptibility to infection and cancer relapse. Although we found significant improvement in TCR diversity over time, there was substantial variability in the rate of recovery between different stem cell sources. Most notably, DUCB recipients had a 28-fold higher $CD4^+$ T-cell diversity compared to TCD recipients after 6 months. Importantly, this is highly consistent with clinical findings, which have shown that after 6 months, DUCB recipients have a low incidence of infection[33], have higher $CD4^+$ T-cell numbers than TCD recipients[33,34] and have a lower rate of leukemia relapse[27]. Although many variables could contribute to this differential repertoire recovery, at least partially it can be explained by the fact that DUCB recipients receive ~7,000-fold more T cells in their graft and transplantation is performed without T-cell-depleting regimens[33].

Besides allo-HSCT, this method should be useful to characterize T-cell immunity in other clinical settings of immune deficiency, autoimmunity and tumor immunity. Ultimately, the ability to measure T-cell repertoire complexity with great precision should guide the way for novel therapeutic approaches aimed at immune regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We are grateful to all patients and healthy donors who kindly volunteered to participate in this study. We thank G. Redelman-Sidi for collection of blood samples, I. Leiner for processing of blood samples, C. Ubeda-Morant for assistance with data analysis, J. Li and A. Viale of the MSKCC Genomics Core Laboratory for performing Roche/454 sequencing and R. Jenq for critical reading of the manuscript. This work was supported by the Netherlands Organization for Scientific Research (NWO) (Rubicon fellowship to J.W.J. van Heijst), the US National Institutes of Health (NIH) (grants HL069929, CA107096 and AI080455 to M.R.M. van den Brink, and AI042135, AI039031 and CA023766 to E.G. Pamer), and The Tow Foundation (to E.G. Pamer). Additional support was received from the US Department of Defense (USAMRAA Award W81XWH-09-1-0294), the Radiation Effects Research Foundation (RERF-NIAID), The Lymphoma Foundation, Alex's Lemonade Stand, The Geoffrey Beene Cancer Research Center at Memorial Sloan-Kettering Cancer Center, and The Peter Solomon Fund (to

M.R.M. van den brink), The Translational and Integrative Medicine Research Fund of Memorial Sloan-Kettering Cancer Center, Cycle for Survival, The New York Community Trust, and When Everyone Survives (to M.A. Perales), and The Experimental Therapeutics Center of Memorial Sloan-Kettering Cancer Center funded by Mr. W.H. Goodwin and Mrs. A. Goodwin (to M.R.M. van den brink and M.A. Perales).

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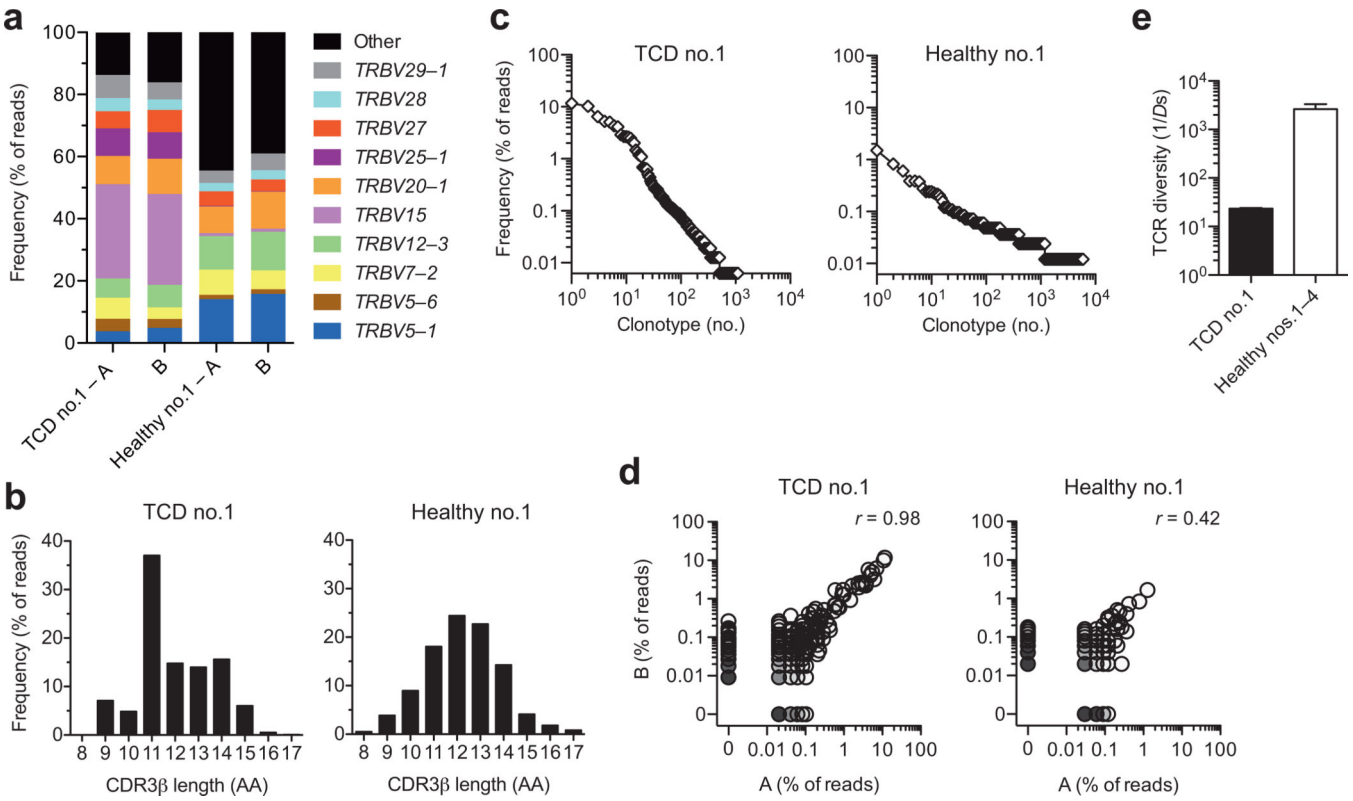

Figure 1.

Quantifying T-cell repertoire recovery after allo-HSCT. (**a**) Vβ gene usage of TCRs recovered from two separately processed blood samples of TCD no.1 at 138 days after transplant (TCD no.1 – A and B) as well as a representative healthy donor (Healthy no.1 – A and B). The 10 most frequent Vβ genes of TCD no.1 are indicated in color, the remaining 38 Vβ genes are grouped in black. Nomenclature is according to the ImMunoGeneTics information system (IMGT). Number of reads: TCD no.1, A (4,858) and B (11,044); Healthy no.1, A (3,318) and B (5,009). (**b**) Digital CDR3 size spectratype plots of total TCRβ sequences from TCD no.1 (15,902 reads) and Healthy no.1 (8,327 reads). CDR3β length is defined as all amino acids (AA) in between the conserved 5′ cysteine and 3′ phenylalanine of the CDR3β region. (**c**) Clonotype distribution plots of total TCRβ sequences from TCD no.1 and Healthy no.1. Each diamond represents a distinct CDR3β AA sequence. (**d**) Dot plots comparing the clonotype distribution of two blood samples (A and B) from TCD no.1 and Healthy no.1. Each dot represents a distinct TCRβ clonotype. Dot opacity reflects multiple clonotypes of the same frequency. Values in the upper right corner depict the Pearson correlation. (**e**) TCR diversity of TCD no.1, as well as the average TCR diversity of four individually-measured healthy donors (Healthy nos.1–4). Error bars depict 95% confidence intervals.

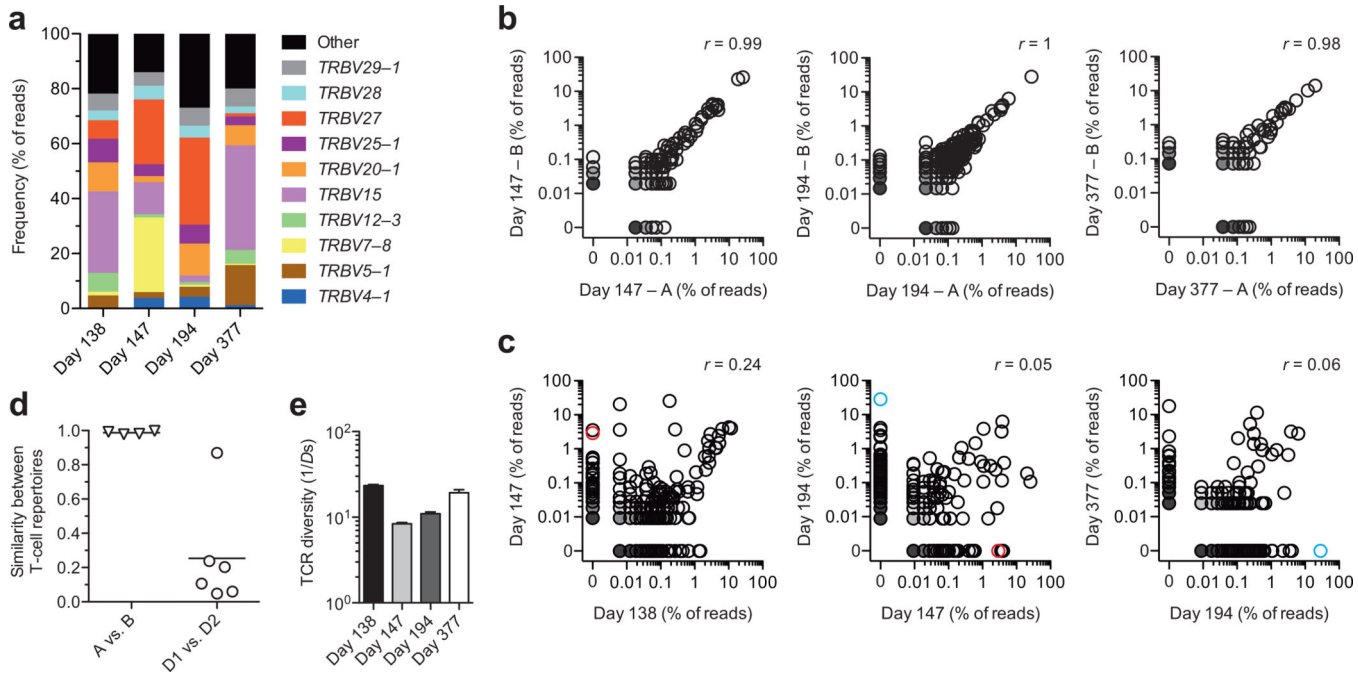

Figure 2.

T-cell repertoire dynamics during the first year of allo-HSCT. (**a**) Vβ gene usage of TCRs recovered from TCD no.1 at indicated timepoints after transplant. Number of reads: day 138 (15,902); day 147 (10,732); day 194 (11,220) and day 377 (3,980). (**b**) Dot plots comparing the clonotype distribution of two blood samples obtained on the same day from TCD no.1 at indicated timepoints. Number of reads: day 147, A (5,644) and B (5,088); day 194, A (4,445) and B (6,775); day 377, A (2,607) and B (1,373). (**c**) Dot plots comparing the clonotype distribution of blood samples obtained on different days from TCD no.1 at indicated timepoints. The red clonotype (*TRBV29–1* and CDR3β CSVGTGGTNEKLFF) is specific for the HLA-A2-restricted $BMLF1_{280}$ epitope from EBV. The cyan clonotype was below the limit of detection on days 138 and 147, comprised 28% of the T-cell repertoire on day 194 and was again below the limit of detection on day 377. (**d**) Similarity of T-cell repertoires recovered from blood samples of TCD no.1 obtained either on the same day (A vs. B) or on different days (D1 vs. D2). Values represent the Pearson correlation. Horizontal bars depict group mean. (**e**) Diversity of T-cell repertoires recovered from TCD no.1 at indicated timepoints. Error bars depict 95% confidence intervals.

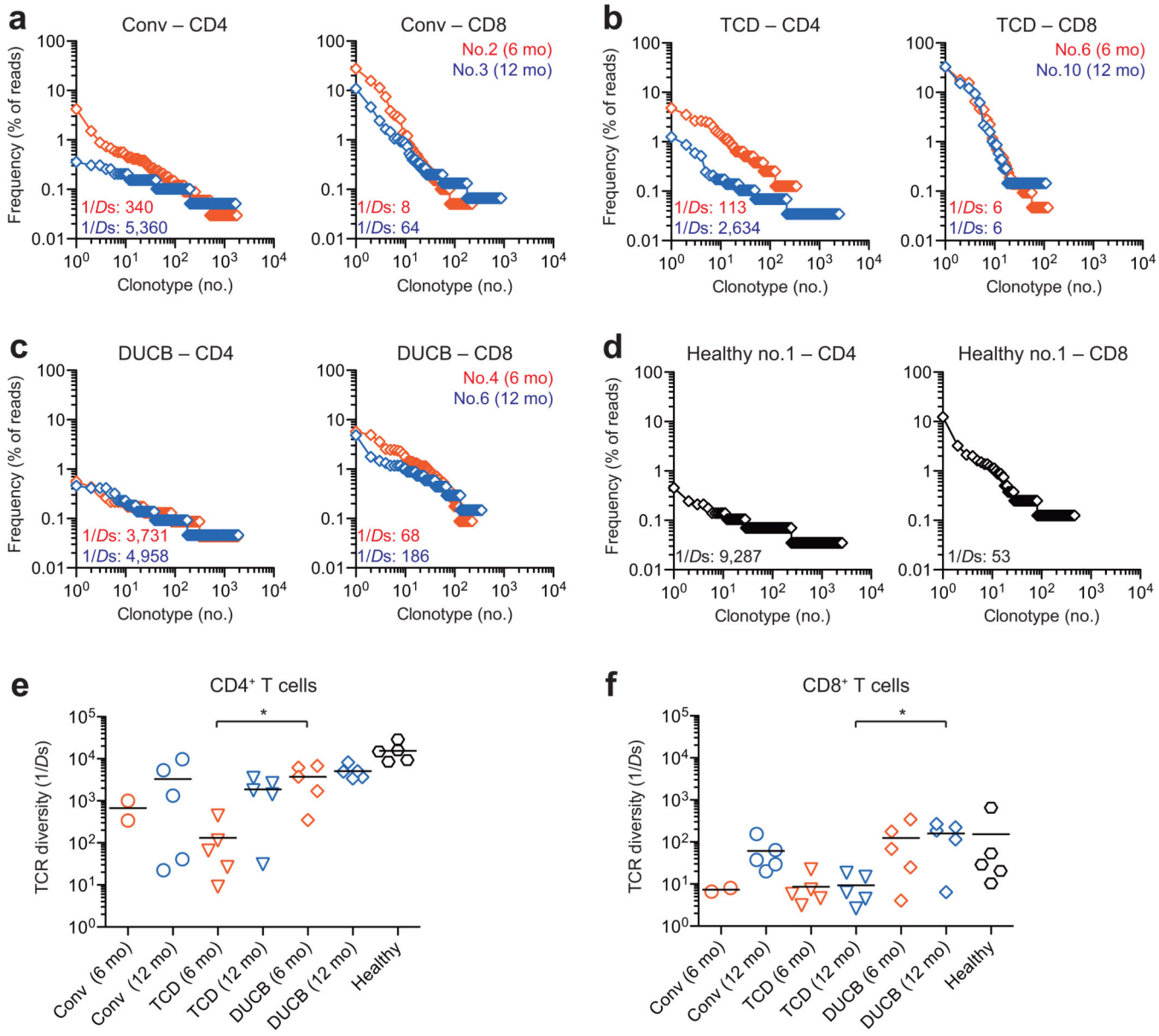

Figure 3.

T-cell repertoire recovery by three different stem cell sources 6 and 12 months after allo-HSCT. Shown are representative clonotype distribution plots of $CD4^+$ and $CD8^+$ T cells obtained at either 6 or 12 months after conventional (Conv) or T-cell-depleted (TCD) peripheral blood stem cell transplantation, or double-unit umbilical cord blood (DUCB) transplantation. Healthy represents age-matched healthy donors. (**a**) Clonotype distribution plots of Conv no.2 (6 months; in red) and Conv no.3 (12 months; in blue). Values in the lower-left corner depict the TCR diversity. Number of reads: Conv no.2, CD4 (3,379) and CD8 (1,985); Conv no.3, CD4 (1,954) and CD8 (1,515). (**b**) Clonotype distribution plots of TCD no.6 (6 months; in red) and TCD no.10 (12 months; in blue). Number of reads: TCD no.6, CD4 (793) and CD8 (2,141); TCD no.10, CD4 (2,889) and CD8 (694). (**c**) Clonotype distribution plots of DUCB no.4 (6 months; in red) and DUCB no.6 (12 months; in blue).

Number of reads: DUCB no.4, CD4 (2,312) and CD8 (1,138); DUCB no.6, CD4 (2,173) and CD8 (680). (**d**) Clonotype distribution plots of Healthy no.1. Number of reads: CD4 (2,856) and CD8 (800). (**e**) $CD4^+$ T-cell diversity of indicated groups. Symbols depict individual subjects, bars depict group mean. *P = 0.033. (**f**) $CD8^+$ T-cell diversity of indicated groups. *P = 0.012.

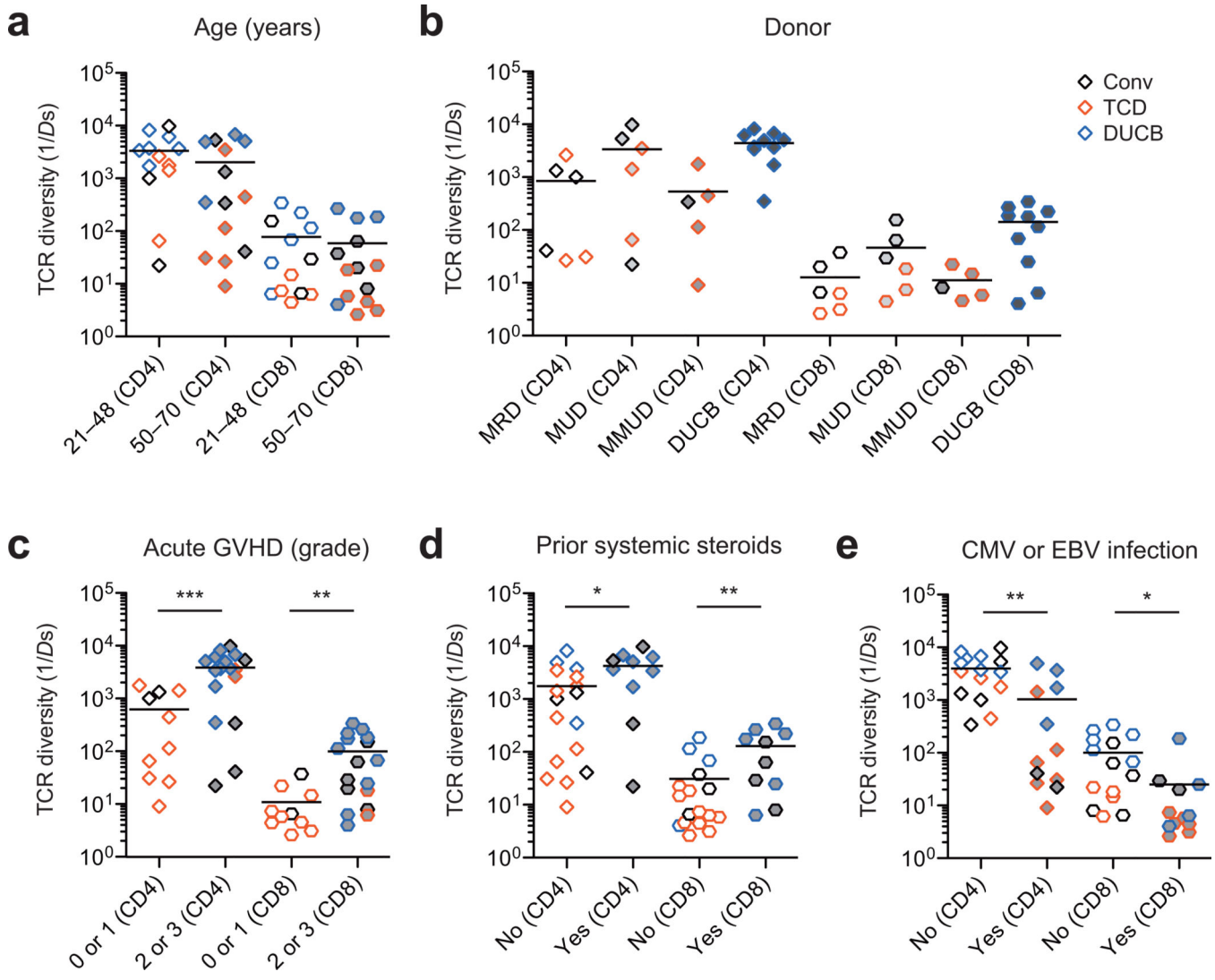

Figure 4.

T-cell repertoire recovery after allo-HSCT as a function of clinical variables. $CD4^+$ and $CD8^+$ T-cell diversity of 27 allo-HSCT recipients was divided according to clinical parameters that could influence T-cell repertoire recovery. (**a**) TCR diversity in patients that were either 21–48 years old (n = 13) or 50–70 years old (n = 14). (**b**) TCR diversity in patients that received either a matched related donor (MRD; n = 6), a matched unrelated donor (MUD; n = 6), a mismatched unrelated donor (MMUD; n = 5) or a DUCB (n = 10) transplantation. (**c**) TCR diversity in patients that either had no or grade 1 acute GVHD (n = 12) or grade 2 or 3 acute GVHD (n = 15). ***P < 0.001 and **P = 0.003. (**d**) TCR diversity in patients that either have not received (n = 17) or have received (n = 10) prior systemic steroid treatment. *P = 0.023 and **P = 0.006. (**e**) TCR diversity in patients that either have not been infected (n = 15) or have been infected (n = 12) with CMV or EBV. **P = 0.004 and *P = 0.033.

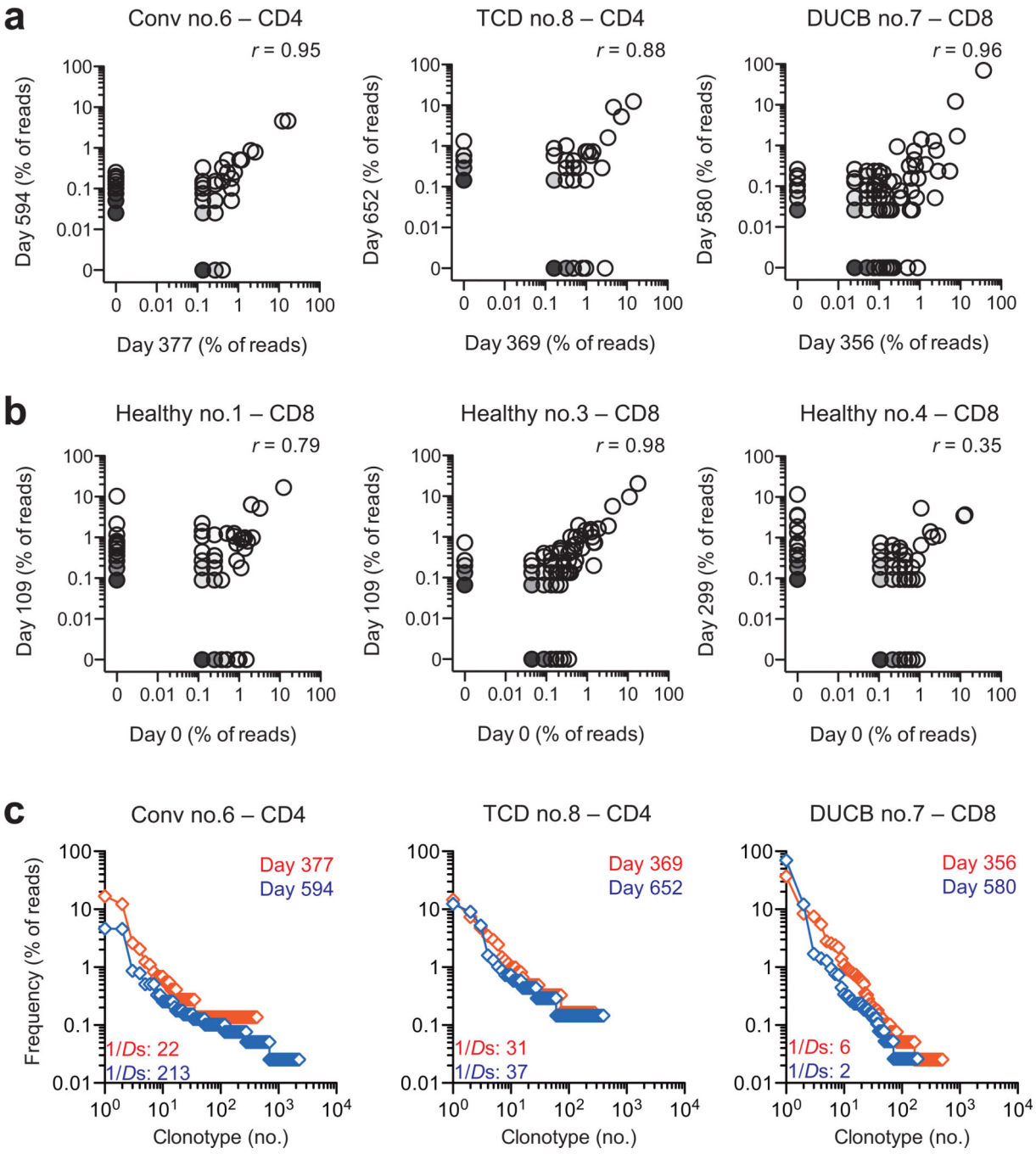

Figure 5.

Monitoring patients with poor T-cell repertoire recovery after allo-HSCT. Three patients identified with low TCR diversity after 12 months were reanalyzed after 19–21 months. (**a**) Dot plots comparing the clonotype distribution of T cells isolated on different days from Conv no.6 ($CD4^+$ T cells; 218 days apart), TCD no.8 ($CD4^+$ T cells; 284 days apart) and DUCB no.7 ($CD8^+$ T cells; 225 days apart). Number of reads: Conv no.6, day 377 (731) and day 594 (3,940); TCD no.8, day 369 (610) and day 652 (688); DUCB no.7, day 356 (3,932) and day 580 (3,825). (**b**) Dot plots comparing the clonotype distribution of $CD8^+$ T cells

isolated on different days from Healthy nos.1 and 3 (both 109 days apart) and Healthy no.4 (299 days apart). Number of reads: Healthy no.1, day 0 (800) and day 109 (1,120); Healthy no.3, day 0 (2,267) and day 109 (1,508); Healthy no.4, day 0 (917) and day 299 (1,068). (**c**) Clonotype distribution plots of $CD4^+$ T cells isolated from Conv no.6 and TCD no.8 as well as $CD8^+$ T cells isolated from DUCB no.7. Values in the lower-left corner depict the TCR diversity. Note that over 218 days, Conv no.6 had a 1.6-fold increase in $CD4^+$ T-cell count (from 452 to 722 cells μl^{-1}) and a 9.7-fold increase in $CD4^+$ T-cell diversity. In contrast, over 284 days, TCD no.8 had no increase in $CD4^+$ T-cell count (from 212 to 207 cells μl^{-1}) and no increase in $CD4^+$ T-cell diversity.