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Longitudinal Analysis of the T-cell Receptor Repertoire in Graft-infiltrating Lymphocytes Following Hand Transplantation

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Background. T lymphocyte–mediated acute rejection is a significant complication following solid organ transplantation. Standard methods of monitoring for acute rejection rely on assessing histological tissue damage but do not define the immunopathogenesis. Additionally, current therapies for rejection broadly blunt cellular immunity, creating a high risk for opportunistic infections. There is, therefore, a need to better understand the process of acute cellular rejection to help develop improved prognostic tests and narrowly targeted therapies. **Methods.** Through next-generation sequencing, we characterized and compared the clonal T-cell receptor (TCR) repertoires of graft-infiltrating lymphocytes (GILs) and blood-derived lymphocytes from a hand transplant recipient over 420 days following transplantation. We also tracked the TCR clonal persistence and V beta (BV) gene usage, evaluating overlap between these 2 compartments. **Results.** TCR repertoires of blood and GIL populations remained distinct throughout the sampling period, and differential BV usage was consistently seen between these compartments. GIL TCR clones persisted over time and were seen in only limited frequency in the blood T-lymphocyte populations. **Conclusions.** We demonstrate that blood monitoring of TCR clones does not reveal the pathogenic process of acute cellular rejection in transplanted tissue. GILs show clonal persistence with biased BV usage, suggesting that tissue TCR clonal monitoring could be useful, although a deeper understanding is necessary to prognosticate rejection based on TCR clonal repertoires. Finally, the distinct TCR BV usage bias in GILs raises the possibility for prevention and therapy of acute cellular rejection based on targeting of specific TCR clones.

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INTRODUCTION

Allogeneic solid organ transplantation is an essential treatment option for individuals with end-organ diseases, allowing for significant decreases in mortality risk and improved life span.[1] Vascularized composite allotransplantation (VCA) has also expanded considerably in clinical practice,[2] with encouraging reporting for hand and upper limb transplantation outcomes.[3] However, a serious consequence of all allogeneic transplantation is the development of acute T lymphocyte (cellular)–mediated rejection, which can lead to graft dysfunction and eventual organ failure. This complication is well described following VCA[4] with multiple episodes of rejection being reported in some individuals[5] and a potential link to the development of donor-specific antibodies.[6]

The current gold standard for diagnosing acute rejection relies on detecting histopathological changes in

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transplanted tissues, which occurs generally only after significant organ dysfunction has been detected and may have variable sensitivity. Moreover, current immunomodulatory therapies to prevent and treat acute rejection are broadly acting and lead to generalized cellular immunosuppression, placing transplant recipients at high risk for opportunistic infections[7] including after VCA.[4,8] There is therefore a need to better understand the process of acute cellular rejection to develop better prognostic testing and more targeted therapies to prevent and treat this complication.

Acute rejection is mediated by oligoclonal populations of recipient T lymphocytes that predominately target donor major histocompatibility complex (MHC)-derived alloantigens through direct, indirect, and semidirect allorecognition.[9] During rejection, these alloreactive T-lymphocytes infiltrate and expand in graft tissue.[10-12] Graft-infiltrating lymphocytes (GILs) can be detected throughout the period of histopathologically defined rejection.[13-16] The population of T lymphocytes that comprise alloreactive GILs can be established through mixed lymphocyte reactions involving ex vivo stimulation of recipient peripheral blood mononuclear cells (PBMCs) by donor MHC-presenting cells.[15,17-19]

Innovations in the application of high-throughput next-generation sequencing (NGS) technologies have allowed for highly robust characterization of these alloreactive T-lymphocyte populations by T cell–receptor (TCR) complementarity-determining 3 region (CDR3) parallel sequencing. Millions of TCR sequences can be identified within T-lymphocyte populations, providing a deep representation of the clonal composition and diversity of their TCR repertoires.[20] Use of this technology has demonstrated that during acute rejection, graft tissues become highly enriched in alloreactive TCR clones compared with when rejection is absent,[18] and that these clones are no longer detected in graft tissue of recipients who develop graft tolerance.[17] Therefore, detection of alloreactive TCR clones among GILs has the potential to be used as a powerful tool in clinical care of transplant recipients[21,22] by enhancing the prognostication and detection of acute rejection.

Limited data thus far suggest that the oligoclonal population of alloreactive GILs mediating acute rejection demonstrates biased gene expression of recombined TCR gene segments, including the V beta (BV) gene segment families.[16,22-25] Therefore, characterization of the alloreactive T-lymphocyte population and detection in graft tissues could guide targeted therapies against GILs based on TCR BV usage bias, inhibiting acute rejection while preserving most of the recipient T-lymphocyte population to minimize generalized immunosuppression.

In this study, we tracked the TCR clonal composition of GILs in a hand transplant recipient over an extended period of 420 days and used high throughput TCR CD3 sequencing to provide in-depth analysis of the clonal composition of these cells over time. This work demonstrates the feasibility of tracking GILs for the purposes of monitoring alloreactive T lymphocytes in graft tissues of transplant recipients, particularly in VCA.

MATERIALS AND METHODS

Study Subject

A 51-year-old man (HLA*A11, A31, B38, B52, Cw15, Cw5, DRB1 4, DRB1 15, DRB4 53, DRB5 51, DQB1 8, DQB1 6) received a left-hand transplant from a male donor (HLA-A23, A24, B62, B41, Bw6, C9, C17, DR 7, DR11, DR52, DR53, DQB2, DQB7, DQA2, DQA5, DPB03:01, DPB23:01). The HLA profile was obtained from the United Network for Organ Sharing. Institutional review board approval was obtained for this work (institutional review board protocol and approval number 11-001249).

Pathological Analysis

Skin of the donor hand was sampled by punch biopsy and histopathologic analysis performed by the University of California, Los Angeles Pathology and Laboratory Medicine Department. Samples were examined for the presence of acute rejection and classified with standard Banff criteria.[26]

Isolation and Separation of T-lymphocyte Populations From GILs and PBMCs

GIL populations were isolated as previously described.[16] Briefly, 2-mm skin punch biopsies were taken from the transplant hand and placed in tissue culture with 2×10^6 irradiated (3000 rad) feeder PBMCs and 0.2 μg/mL 12F6 murine anti-CD3 monoclonal antibodies[27] in a 24-well plate with Roswell Park Memorial Institute 1640 (Sigma, USA) supplemented with 10% heat-inactivated fetal calf serum, 10 mmol/L N-2-hydroxyethylpiperazine-N9-2-ethanesulfonic acid, 2 mmol/L glutamine, 0.5 μg/mL of piperacillin/tazobactam, 0.125 μg/mL amphotericin B, and 50 U/mL recombinant human interleukin-2 (NIH AIDS Reference and Reagent Repository). Cells were refed every 4–5 days and cultured for 14 days total. PBMCs T-lymphocyte populations were generated from whole blood by initial isolation with Ficoll-Hypaque gradient and expansion in parallel identically as tissue but without the addition of irradiated feeder PBMCs. At the end of 14 days of culture, if PBMC T-lymphocyte populations contained $>1 \times 10^6$ cells, then $CD8^+$ and $CD8^-$ fractions were separated (EasySep $CD8^+$ T Cell–Enrichment Kit, STEMCELL Technologies, Inc., Canada). Due to lower cell numbers among tissue-derived T lymphocytes, $CD8^+$ and $CD8^-$ separation was done for 1 biopsy-derived GIL population only.

RNA Isolation and TCR cDNA Library Creation

Total RNA from T-cell populations was isolated (PicoPure spin columns, Applied Biosystems, USA) and reverse transcribed (SMARTScribe Reverse Transcriptase, Clontech, Japan) with the use of an oligo-dT primer along with a template-switch oligonucleotide (TSO) to create a common adaptor sequence at the 5′ end of cDNA generated from mRNAs. Full-length TCR cDNA was then amplified using primers specific for the constant region of the TCR beta chain and a primer specific for TSO adaptor for 21 cycles. These preamplified TCR-specific polymerase chain reaction (PCR) products were then purified (AMPure XP beads, Beckman Coulter, USA), and semi-nested PCR was performed using the same TSO adaptor-specific forward primer and a nested reverse primer for the TCR beta chain constant region. Samples were gel purified (NucleoSpin Gel and PCR Clean-up Kit, Macherey-Nagel, Germany) and processed for sequencing library creation.

TCR Sequencing Library Preparation and Sequence Analysis

Amplified TCR beta chain PCR products were then tagged with Illumina sequencing-platform–specific adaptor sequences at both 5′ and 3′ ends using Nextera XT DNA library preparation kit and subsequently sequenced by Illumina MiSeq system (Illumina, San Diego, CA). The resulted indexed TCR sequence reads obtained from MiSeq systems were aligned to annotate V, J, and C genes, and further, these aligned reads were assembled into clonotypes based on similar clonotypes (ie, based on CDR3 region) by the use of MiXCR software.[28]

Statistical Analysis

TCR clone counts were generated for individual samples using MIXCR. Two clone count matrices were generated from individual samples: best BV, based on the V annotation, and CDR3 sequence. V, J, and C family annotations were included with CDR3 sequences such that clones with identical CDR3 sequences but different V, J, or C families were considered different features. Principle Component Analysis (PCA) plots were generated to check for any potential outliers. One sample derived from PBMCs taken on day 28 after transplantation was removed as an extreme outlier in the PCA plot, and the outlier was also confirmed based on the very low number of CDR3 sequences present.

Differential analyses of BV families or TCR clones between skin tissue and blood were performed using the R package edgeR on raw clone counts.[29] Before analysis, the data were filtered to remove any clones that have at least 10% samples containing nonzero counts. Data were normalized as counts per million, including the TMM normalization factor in edgeR. *P* values were adjusted for multiple testing using the false discovery rate (FDR) correction of Benjamini and Hochberg.[30] Significantly differentially abundant features were based on FDR <0.05.

For beta diversity analysis, Jensen-Shannon Divergence (JSD) was calculated with a linear empirical method using philentropy R library on percent abundances based on the counts per million normalized values for both BV families and TCR clones. Boxplots of within-group sample to sample JSD indices were computed for skin tissue and blood, respectively, and the significance of difference between these groups was computed using the Wilcoxon test.

RESULTS

Clinical Course and Blood and Tissue Sampling

Skin biopsies of the donor hand were taken 16 times from the time of transplantation to day 475 into the subject's clinical course, and histopathologic analysis performed using Banff criteria (Figure 1). Blood samples were taken concomitantly during this period. T lymphocytes from 12 skin and 14 blood samples were expanded in culture and T-lymphocyte populations isolated for TCR CDR3 cDNA library sequencing and analysis. Of note, on day 371, dorsal and volar biopsies were obtained

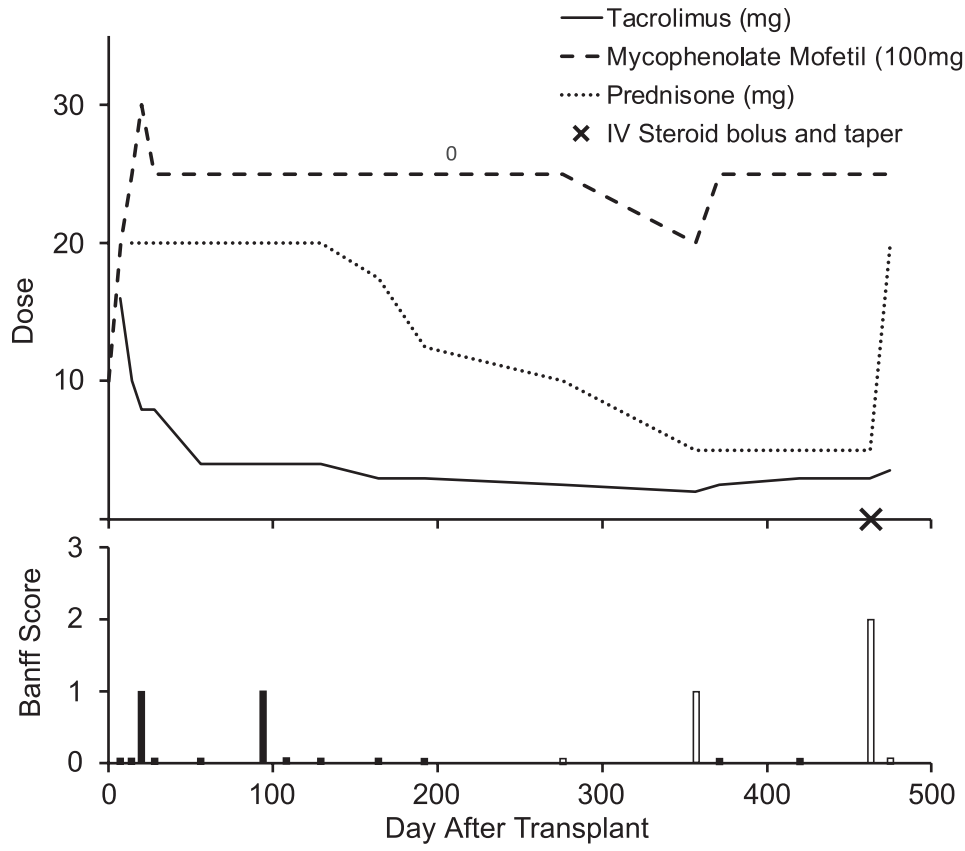

FIGURE 1. Immunosuppressive drug regimen and rejection status. (Top) immunosuppressive drug doses shown over time; (bottom) histopathologic Banff scores of collected skin biopsies (filled in bars represent samples used for ex vivo expansion and TCR library analysis). TCR, T-cell receptor.

simultaneously and expanded separately. One tissue-derived and 10 blood-derived T-lymphocyte populations were separated into $CD8^+$ and $CD8^-$ populations before analysis. The patient experienced relative clinical stability during this period with 3 episodes of mild rejection. There was 1 episode of moderate rejection on day 463 requiring a steroid bolus and taper. A follow-up biopsy on day 475 showed no evidence of rejection. A summary of Banff scores, biopsy dates, as well as immunomodulatory treatment regimens are shown in Figure 1.

Characterization of TCR Sequence Repertoires

TCR cDNA libraries of tissue-derived and blood-derived T-lymphocyte populations were sequenced to establish TCR repertoires. After alignment and clustering, an average of 761 and 1007 TCR clones were seen with >10 counts among tissue- and blood-derived TCR repertoires, respectively. The number of distinct clones (>10 counts) for each T-lymphocyte population repertoire is shown in Table 1. To compare the clonal compositions of TCR repertoires between sample populations, non-metric multidimensional scaling plots were generated after combining $CD8^+$ and $CD8^-$ TCR repertoires of separated samples. For these combined analyses, the dorsal and volar TCR repertoires from day 371 were also combined. As shown in Figure 2A, there is tight clustering among blood-derived TCR repertoires, and more widely distributed but distinct in area clustering for tissue-derived TCR repertoires. Sample to sample distances in JSD are shown in Figure 2B, also showing significantly less variability among blood repertoires. To assess the statistical robustness of TCR sequencing, rarefraction analysis was performed to 5% of the minimum depth, with similar separation and clustering of blood and tissue-derived populations, suggesting sufficient TCR sequence richness (Figure S1, SDC, http://links.lww.com/TP/C56). These findings demonstrate that there is more uniformity among blood T-lymphocyte populations and that clonal composition is distinct between skin and blood populations.

TABLE 1.

Number of TCR clones identified with counts >10 in each T-lymphocyte population isolated from tissue (left) or blood (right), arranged by day after transplantation each sample was taken

	Skin-derived TCR repertoire		Peripheral blood-derived TCR repertoire	
D after transplant	Number of clones identified		Number of clones identified	
7	665		94	
14	1017		$CD8^+$	999
			$CD8^-$	775
20	273		$CD8^+$	807
			$CD8^-$	862
28	1340			
56	793		$CD8^+$	907
			$CD8^-$	919
94	978		$CD8^+$	1589
			$CD8^-$	1069
108	1615		212	
129	1675		543	
164	$CD8^+$	728	$CD8^+$	1014
	$CD8^-$	201	$CD8^-$	1111
192	43		$CD8^+$	1419
			$CD8^-$	1318
276			$CD8^+$	950
			$CD8^-$	1305
371	Dorsal	315	$CD8^+$	640
	Volar	863	$CD8^-$	1481
420	152		$CD8^+$	1259
			$CD8^-$	1207
475			$CD8^+$	1210
			$CD8^-$	1468

Some populations were separated by $CD8^+$ and $CD8^-$ fractions, and on d 371, 2 tissue samples were taken (1 from a dorsal and 1 from a volar site).
TCR, T-cell receptor.

Differences Among Skin and Blood-derived TCR Repertories

Alloreactive GILs are significantly enriched in graft tissues compared with peripheral blood,[15] even in the absence of histopathologically defined rejection.[18] Therefore, these T lymphocytes would be highly represented among the TCR clones in the tissue-derived repertoires and minimally among blood repertoires. Conversely, nonalloreactive T lymphocytes in blood would be noted in greater frequency in blood versus tissue TCR repertoires. Because these differentially abundant clones are likely driving the distinctions between tissue- and blood-derived TCR repertoires in our subject, we sought to characterize these TCR clones and survey them for the persistence of these over time. We, therefore, performed a differential analysis of TCR clones between the combined $CD8^+$ and $CD8^-$ TCR repertoires. As shown in Figure 3A, a significant number of differentially abundant clones are present in both the tissue and blood-derived TCR repertoires and had varying degrees of persistence over time. Differential analysis among $CD8^+$, $CD8^-$, and nonseparated samples separately was performed but did not produce statistical significance likely due to the limited number of samples in each category. This analysis shows that there are distinct clonal compartments within blood- and tissue-derived T-lymphocyte populations, and these can be traced through repeated sampling over time.

To determine if along with these distinctions in clonal composition, usage of BV gene segments was distinct between tissue and blood populations, we performed a differential analysis of for BV family usage among combined TCR repertoires. As shown in Figure 3B, there is noted differential usage of BV families between tissue and blood T-lymphocyte populations, most markedly in BV3, BV20, BV4, and BV18 families.

Comparison of Clonal Persistence in Tissue and Blood TCR Repertoires

To characterize the persistence of TCR clones within tissue and blood-derived TCR repertoires over time, we calculated the fraction of clones present in each tissue-derived and blood sample-derived TCR repertoire. Clone presence in a sample was established if there were ≥10 counts, and persistence was computed as the fraction of total blood or skin repertoires ($CD8^+$ and $CD8^-$ combined) that had the clone present. As displayed in Figure 4A, there is a

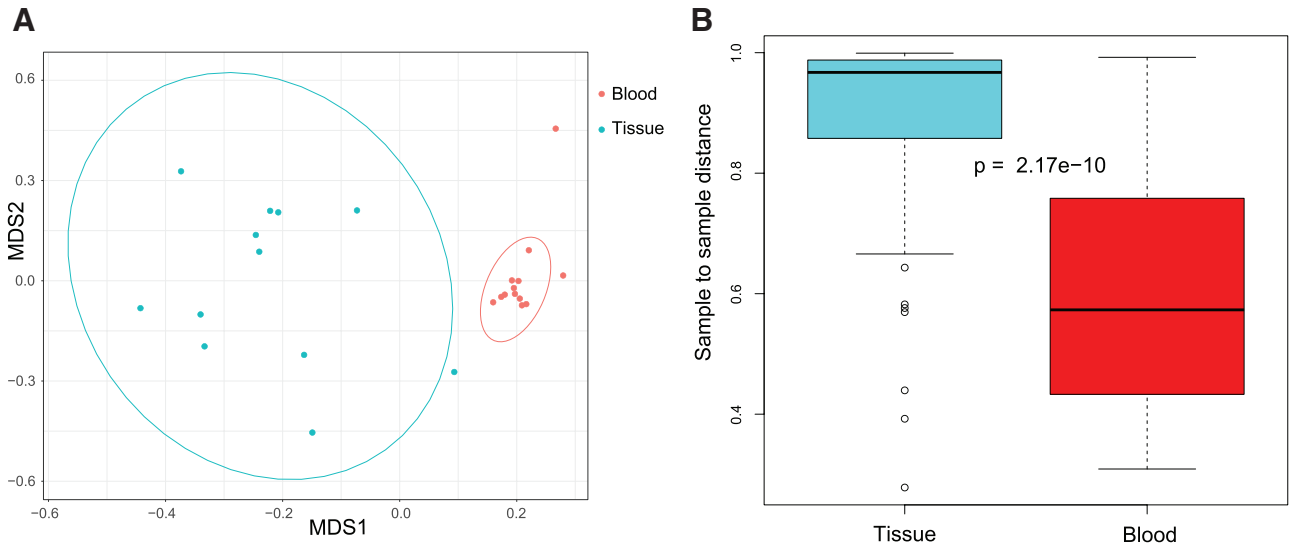

FIGURE 2. A, Non metric MDS plot for TCR repertoires in tissue and blood. B, Sample to sample Jensen-Shannon distances plotted within tissue- and blood-derived samples. MDS, multi dimensional scaling; TCR, T-cell receptor.

greater fraction of clones persisting in T-lymphocyte populations among blood-derived samples. However, a significant proportion of TCR clones among tissue samples is also persisting. To determine if these persisting clones had a significant overlap between skin and blood populations, we compared the persistence per clone as a 2-dimensional histogram (Figure 4B). TCR clones seen in multiple tissue-derived T-lymphocyte populations were present only in limited frequency among blood-derived populations, and vice versa when assessing for clones seen in multiple blood populations among tissue populations. This suggests that clones persisting among tissue and blood TCR repertoires are generally distinct.

Assessing TCR Repertoire Heterogeneity Among Tissue-derived T-lymphocyte Populations

Our prior work has shown that punch biopsy sampling was likely inadequate for representing the entire GIL population of a transplanted hand.[16] To determine if the TCR repertoire was likely underestimated in this subject, we plotted the distribution of pairwise distances between tissue-derived TCR repertoires. As shown in Figure S2 (SDC, http://links.lww.com/TP/C56), the distance between dorsal and volar samples obtained on day 371 was shorter than only about two-thirds of all pairwise distances. This suggests that these 2 T-lymphocyte populations do not share significantly more identity than when compared with populations derived from tissues at different time points, again confirming that biopsy sampling likely underestimates the whole GIL population.

DISCUSSION

Alloreactive GILs can be detected in graft tissues throughout the period of acute rejection.[13,14] In our prior work,[16] we identified individual CD8$^+$ TCR clones within the tissue-derived T-lymphocyte population that remained remarkably stable before and throughout acute rejection, although we used a relatively insensitive technique to define only highly enriched TCR sequences. These persistent clones were detectable for periods ranging from 41 to 171 days and likely reflected alloreactive GILs. In this work, we extend these findings by detecting a persistent fraction of tissue infiltrating TCR clones likely representing alloreactive T lymphocytes in a VCA recipient over an extended period of 475 days following transplantation, using NGS TCR sequencing to provide granular characterization of the TCR repertoire.

Understanding the determinants of the TCR repertoire mediating acute cellular rejection would be useful for developing novel diagnostic approaches. Alloreactive T lymphocytes specifically target donor-derived alloantigens, and so detection and quantification of these clones among GILs would specifically indicate when acute rejection is present, even in the presence of other pathologies, such as BK nephropathy.[19,22] In a prior report, this approach was explored in kidney transplant recipients over a 3-month period.[31] Although alloreactive clones were not specifically defined, changes in TCR clonal frequencies were monitored, and TCR repertoire diversity was compared between blood- and graft-derived samples, providing proof-of-concept that such monitoring is possible. Further development of this approach could, therefore, establish how to characterize the alloreactive clonal population and the key quantitative or qualitative parameters that predict acute cellular rejection.

Prior characterization of TCR repertoires by cruder spectratyping-based approaches demonstrated that the expansion of alloreactive GIL clones was not mirrored in the peripheral blood, resulting in a clear distinction in TCR repertoires of oligoclonal tissue-derived and more broadly distributed blood-derived T-lymphocyte populations.[13,15,16,23-25,32-35] Our NGS-based TCR repertoire analysis confirms this phenomenon, in agreement with an earlier study.[18] In greater detail, we now show further that tissue- and blood-derived TCR repertoires have distinct clonal compositions that remain consistent over time. The blood repertoires demonstrated a longer duration of uniformity in clonal composition, which could either reflect the more dynamic shifting of TCR clones in graft tissues or potentially reflect sampling limitations of tissue T-lymphocyte populations through skin punch biopsies. Although sampling limitations may have restricted

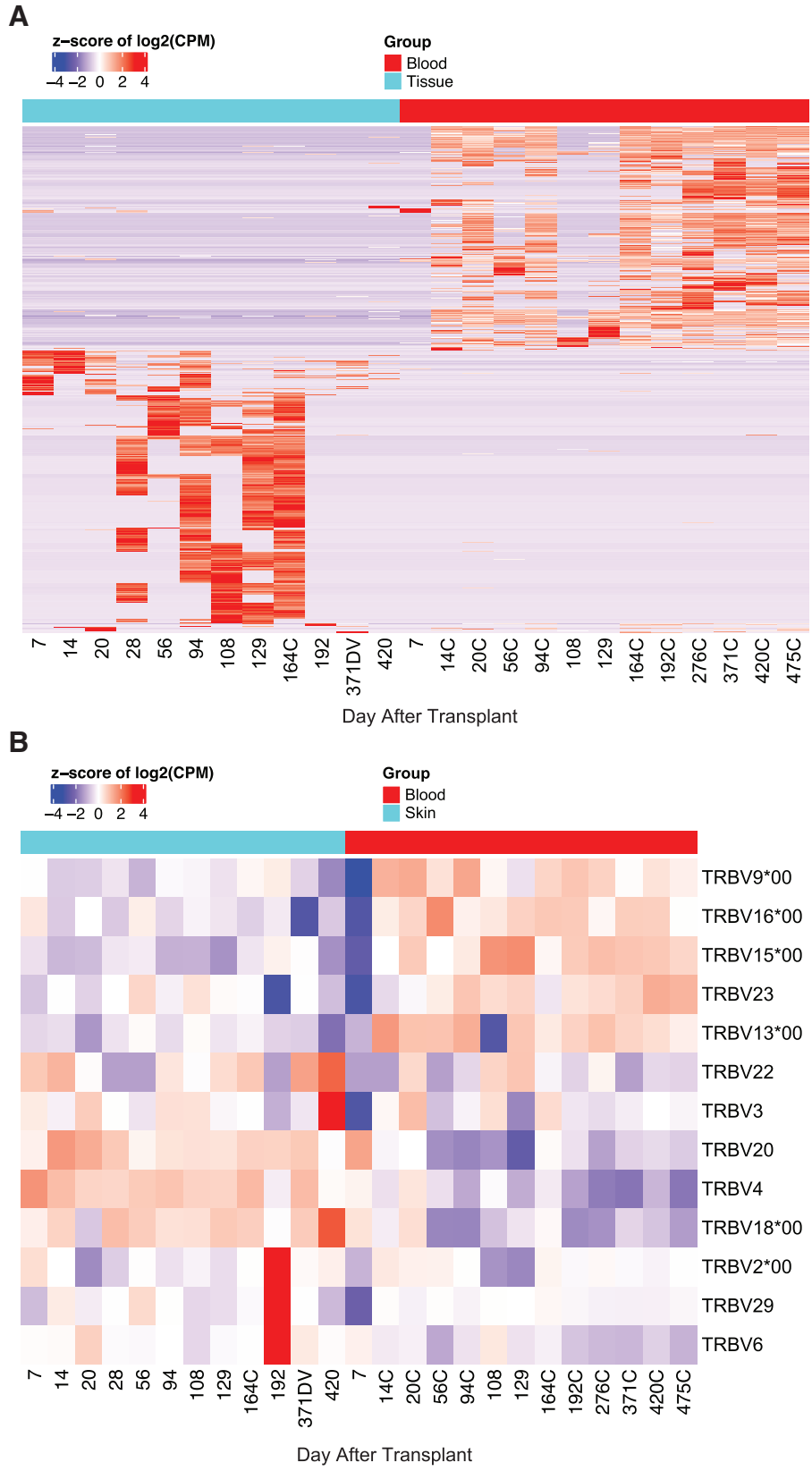

FIGURE 3. Differentially abundant TCR clones (A) and BV families (B) between combined $CD8^+$ and $CD8^-$ TCR repertoires, each column represents samples collected from a different day (C = combined $CD8^+$ and $CD8^-$ fractions; DV = combined dorsal and volar repertoires), and each row corresponds to a differentially expressed clone or BV family, respectively; representation of each clone quantified by z-score (red is highly represented, blue is sparsely represented); only differentially represented BV families and TCR clones are displayed. BVCPM, counts per million; CPM, counts per million; TCR, T-cell receptor.

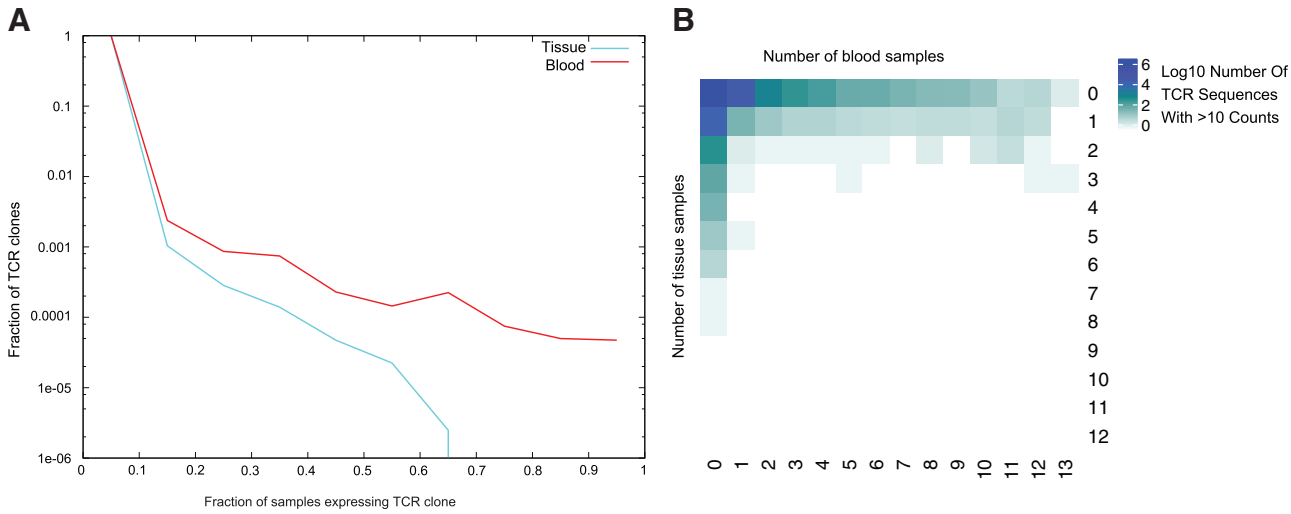

FIGURE 4. A, Fraction of TCR clones (y-axis) persisting in blood and skin TCR repertories displayed as fraction of clones seen in total blood or skin samples (x-axis). B, Two-dimensional histogram with each box representing the number of TCR clones seen in the corresponding number of TCR repertoires of blood (horizontal) and skin (vertical), represented as $\log_{10}$ of the number of TCR clones with >10 counts. TCR, T-cell receptor.

complete description of the TCR repertoires of GILs, the presence of persistent TCR clones in tissue infiltrating repertories suggests that distinct populations of alloreactive T-lymphocyte clones with particularly biased BV usage can be detected and characterized.

Our findings demonstrate in greater detail the previous reports that the oligoclonal population of alloreactive T lymphocytes mediating acute rejection uses a skewed repertoire of TCR BV gene segments.[16,22-25] While this skewing is not reflected in peripheral blood, some studies have suggested that it could be predicted through ex vivo culture of recipient PBMCs with donor target cells in a mixed lymphocyte reaction.[17,18] If the process of BV overrepresentation among alloreactive T lymphocytes can be defined by tissue sampling or such ex vivo predictions, targeted therapies against alloreactive GILs directed by their BV usage (eg, BV-specific depleting monoclonal antibodies) could potentially specifically deplete alloreactive cells and better preserve the overall recipient T-lymphocyte population. This would reduce the generalized immunosuppression transplant recipients currently receive (eg, OKT3) and minimize risks of posttransplant opportunistic infections and malignancies. Proof-of-concept of this strategy has been demonstrated in a mouse model of skin graft rejection, where administration of certain BV-specific lymphocyte-depleting antibodies improved graft survival.[36]

There are limitations to our study. The TCR repertoire is likely underrepresented by punch biopsy sampling of skin tissue. However, rarefaction analysis suggests that our TCR sampling is not highly limiting, and unbiased sampling limitation would not cause distinct compartmentalization compared with PBMC sampling, which was adequate. Another potential limitation was that GILs were expanded in ex vivo tissue culture to amplify the population for adequate TCR mRNA yield. Dziubianu et al[19] similarly expanded T lymphocytes in culture and noted that this led to changes in frequencies of TCR clones. They did note, however, that the majority of dominant and subdominant alloreactive clones were still amplified, and other work has shown that bulk expansion in culture produces minimal bias in antigen-specific T-cell populations.[37,38] Because the expansion of T lymphocytes ex vivo was performed by CD3 stimulation, there should have been the minimal bias of different TCR populations. Future work, however, may take advantage of technological advances such as single-cell sequencing without ex vivo expansion. A further improvement could be examination of genomic DNA to avoid the potential quantitative bias of differential expression of mRNAs.[19]

In conclusion, we compare TCR populations of GILs and peripheral T lymphocytes over time after VCA using deep sequencing. We confirm and extend findings using blunt techniques such as TCR spectratyping demonstrating the distinct profile of T-lymphocyte populations in graft tissues versus blood compartments. Our observation of biased and distinct BV usage in GILs offers a potential avenue for tailored prevention and therapy of acute cellular rejection after transplantation that avoids global T-lymphocyte suppression.

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