Longitudinal Analysis of the T Cell Receptor Repertoire in Graft Infiltrating Lymphocytes Following Hand Transplantation

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Authorship Page

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Abbreviations Page

BV, Beta chain variable
CDR3, Complementarity-determining 3 region
cDNA, Complementary DNA
CPM, Counts per million
FDR, False discovery rate
gDNA, Genomic DNA
GIL, Graft infiltrating lymphocyte
HLA, Human leukocyte antigen
JSD, Jensen-Shannon Divergence
MHC, Major histocompatibility complex
MLR, Mixed lymphocyte reaction
NGS, Next-generation sequencing
PBMC, Peripheral blood mononuclear cell
PCA, Principle Component Analysis
TCR T cell receptor
VCA, Vascularized composite allotransplantation
Abstract

Background: T lymphocyte mediated acute rejection is a significant complication following solid organ transplantation. Standard methods of monitoring for acute rejection rely on assessing histologic 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 (NGS), 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 repertories 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.
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. Vascularized composite allotransplantation (VCA) has also expanded considerably in clinical practice, with encouraging reporting for hand and upper limb transplantation outcomes. 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 with multiple episodes of rejection being reported in some individuals, and a potential link to the development of donor specific antibodies.

The current gold standard for diagnosing acute rejection relies on detecting histopathological changes in 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 including after VCA. 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 semi-direct allore cognition. During rejection, these alloreactive T lymphocytes infiltrate and expand in graft tissue. Graft infiltrating lymphocytes (GILs) can be detected throughout the period of histopathologically defined rejection. The population of T
lymphocytes that comprise alloreactive GILs can be established through mixed lymphocyte reactions (MLRs) 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 to 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 recipients21,22 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 human leukocyte antigen (HLA) profile was obtained from the United Network for Organ Sharing (UNOS). Institutional review board (IRB) approval was obtained for this work (IRB protocol and approval number 11-001249).

Pathological Analysis

Skin of the donor hand was sampled by punch biopsy and histopathological analysis performed by the UCLA 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, 2mm skin punch biopsies were taken from the transplant hand and placed in tissue culture with 2 x 10^6 irradiated (3000 rad) feeder PBMCs and 0.2ug/ml 12F6 murine anti-CD3 monoclonal antibodies in a 24-well plate with RPMI 1640 (Sigma, USA) supplemented with 10% heat inactivated fetal calf serum, 10mM N-2-hydroxyethylpiperazine-N9-2-ethanesulfonic acid (HEPES), 2mM glutamine, 0.5ug/ml of pipercllin/tazobactam, 0.125ug/ml amphotericin B, and 50U/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 more than $1 \times 10^6$ 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. This preamplified TCR specific PCR products were then purified (AMPure XP beads, Beckman Coulter, USA), and semi-nested PCR performed using 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, USA). 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 (i.e., based on CDR3 region) by the use of MiXCR software.\textsuperscript{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. PCA (Principle Component Analysis) 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\textsuperscript{29}. Prior to 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 (CPM), 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\textsuperscript{30} (Benjamini and Hochberg, 1995). Significantly differentially abundant features were based on FDR < 0.05.

For beta diversity analysis, Jensen-Shannon Divergence (JSD) were calculated with linear empirical method using philentropy R library on percent abundances based on the CPM 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 histopathological 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 simultaneously and expanded separately. One tissue derived and 10 blood derived T lymphocyte populations were separated into CD8+ and CD8- populations prior to 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 is 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 1,007 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 are shown in Table 1. In order to compare the clonal compositions of TCR repertoires between sample populations, NMDS 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. In order to assess the statistical robustness of TCR sequencing, rarefaction 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 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.

**Differences among skin and blood derived TCR repertories**

Alloreactive GILs are significantly enriched in graft tissues compared to 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 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 and blood sample derived TCR repertoire. Clone presence in a sample was established if there were greater than or equal to 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 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. In order to determine if these persisting clones had 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.\textsuperscript{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 [http://links.lww.com/TP/C56](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 to 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. In our prior work, we identified individual CD8+ TCR clones within the tissue derived T lymphocyte population that remained remarkably stable prior to 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, utilizing 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. In a prior report, this approach was explored in kidney transplant recipients over a 3-month period. 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.\textsuperscript{13,15,16,23-25,32-35} Our NGS based TCR repertoire analysis confirms this phenomenon, in agreement with an earlier study.\textsuperscript{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 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 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 utilizes a skewed repertoire of TCR BV gene segments.\textsuperscript{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.\textsuperscript{17,18} If the process of BV over-representation 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 (e.g. 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 (e.g. 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 to 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 and colleagues similarly expanded T lymphocytes in culture and noted that this led to changes in frequencies of TCR clones19. 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 expansion of T lymphocytes ex vivo was performed by CD3 stimulation, there should have been 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 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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**Figure Legends**

**Figure 1:** Immunosuppressive drug regimen and rejection status. (Top) immunosuppressive drug doses shown over time, (bottom) histopathological Banff scores of collected skin biopsies (filled in bars represent samples used for ex vivo expansion and TCR library analysis)

**Figure 2:** (A) NMDS plot for TCR repertoires in tissue and blood; (B) Sample to sample Jensen-Shannon distances plotted within tissue and blood derived samples.

**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.

**Figure 4** (A) Fraction of TCR clones (y-axis) persisting in blood and skin TCR repertoires 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 more than 10 counts.

**Figure S1:** (A) Rarefraction analysis to 5% of the minimum depth with NMDS calculations for combined TCR repertoires performed and plotted; (B) Sample to sample similarity distances plotted among tissue infiltrating and blood derived samples.

**Figure S2:** Distribution of pairwise distances between tissue infiltrating TCR repertoires; location of distance between dorsal and volar samples collected on day 371 indicated by dotted blue line.
Tables

Table 1: Number of TCR clones identified with counts greater than 10 in each T lymphocyte population isolated from tissue (left) or blood (right), arranged by day after transplantation each sample was taken. Some populations separated by CD8+ and CD8-fractions, and on day 371 2 tissue samples were taken (one from a dorsal and one from a volar site).

| Day after transplant | Skin derived TCR repertoire | Peripheral blood derived TCR repertoire |
|----------------------|-----------------------------|----------------------------------------|
|                      | Number of clones identified | Number of clones identified             |
|                      | CD8+                        | CD8+                                   |
| 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                              |
Figure 2

A

B

p = 2.17e-10
Figure 3

A

Day After Transplant

B

Day After Transplant
