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Deletion of donor-reactive T cell clones following human liver transplantation

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Abstract

We recently developed a high throughput T cell receptor β chain (TCRβ) sequencing-based approach to identifying and tracking donor-reactive T cells. To address the role of clonal deletion in liver allograft tolerance, we applied this method in samples from a recent randomized study, ITN030ST, in which immunosuppression withdrawal was attempted within 2 years of liver transplantation. We identified donor-reactive T cell clones via TCRβ sequencing following a pre-transplant mixed lymphocyte reaction and tracked these clones in the circulation following transplantation in 3 tolerant and 5 non-tolerant subjects. All subjects showed a downward trend and significant reductions in donor-reactive TCRβ sequences were detected post-transplant in 6 of 8 subjects, including 2 tolerant and 4 non-tolerant recipients. Reductions in donor-reactive TCRβ sequences were greater than those of all other TCRβ sequences, including 3rd party-reactive sequences, in all 8 subjects, demonstrating an impact of the liver allograft after accounting for repertoire turnover. Although limited by patient number and heterogeneity, our results suggest that partial deletion of donor-reactive T cell clones may be a consequence of liver transplantation and does not correlate with success or failure of early immunosuppression withdrawal. These

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DISCLOSURE

The authors of this manuscript have conflicts of interest to disclose as described by the *American Journal of Transplantation*. H.R. owns stock in Adaptive Biotechnologies, Inc. The other authors have no conflicts of interest to disclose

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

observations underscore the organ- and/or protocol-specific nature of tolerance mechanisms in humans.

1. INTRODUCTION

Liver transplantation has been associated with relatively high rates of successful immunosuppression withdrawal in recipients who have been free of rejection for years[1,2]. Despite T cell infiltration associated with Hepatitis C Virus (HCV) and recurrent HCV post-transplant[3], a subset of HCV-infected liver transplant recipients has successfully achieved tolerance[4].

Spontaneous liver allograft tolerance occurs in rodent models across major histocompatibility complex barriers[5] and is associated with donor-specific tolerance to subsequent skin[6] or cardiac allografts[7]. Moreover, co-transplantation of liver allografts in humans appears to provide immunoprotection compared to single heart[8] or kidney[9–11] grafts, suggesting the liver allograft may promote systemic suppression of the anti-donor response. Using apoptosis analyses and TCR-transgenic approaches in rodent models, deletion of donor-reactive T cells has been associated with liver transplantation[12–14], but mechanisms of tolerance in humans are unclear. The Immune Tolerance Network-sponsored clinical trial ITN030ST ("A-WISH") randomized liver transplant recipients, stratified as HCV infected and not, to begin weaning immunosuppression within 1–2 years post-transplant, aiming for complete withdrawal[15]. Of 77 patients randomized to immunosuppression withdrawal, 10 achieved full withdrawal and were defined as tolerant[15].

Based on the above rodent studies and on an ITN-sponsored trial of combined kidney and bone marrow transplantation (CKBMT) in which we obtained evidence for long-term clonal deletion of donor-reactive T cells as a mechanism of renal allograft tolerance[16], we hypothesized that patients defined as tolerant in ITN030ST might demonstrate deletion of donor-reactive T cells in the circulation. We addressed this hypothesis by using a high throughput TCR sequencing-based method that we developed for identifying and tracking a significant fraction of the alloreactive T cell repertoire in any donor-recipient pair[16,17]. The method involves a pre-transplant recipient anti-donor mixed lymphocyte reaction (MLR) followed by high throughput TCRβ CDR3 sequencing of proliferating cells. Individual T cells have one or two TCR α chains and a single TCR β chain (TCRβ). Sequencing of the complementarity-determining region 3 (CDR3) of the TCRβ chain, therefore, approximates identification of T cell clones, permitting tracking of T cell clones with antigen specificity. This method has been validated by demonstrations of the biological relevance of clones identified in this manner in kidney and intestinal transplant recipients[16,18]. In this study, we tracked donor-reactive TCRβ sequences from a group of tolerant and non-tolerant patients from ITN030ST and obtained evidence consistent with deletion of donor-reactive T cells post-transplant in both groups.

2. MATERIALS AND METHODS

2.1 Study Design

Patients were enrolled ITN030ST as described in detail previously[15]. Briefly, post-transplant management included tacrolimus and steroids with or without mycophenolate mofetil. Patients randomized to immunosuppression withdrawal underwent gradual minimization of immunosuppression beginning 12–24 months post-transplant. Patients were considered tolerant if complete immunosuppression withdrawal was achieved for ≥1 year in the absence of allograft dysfunction, where allograft dysfunction was defined as elevation beyond twice the upper limit of normal of liver enzymes (aspartate aminotransferase, alanine aminotransferase, bilirubin). Patients were considered non-tolerant if allograft dysfunction occurred during immunosuppression minimization or shortly after immunosuppression withdrawal, at which time immunosuppression was returned to previous dosage. The institutional review board of each institution approved the study (Columbia University Protocol #AAAJ5056 and as described[15]) and informed consent was obtained from each subject.

2.2 Sample preparation

CFSE-MLRs were performed using pre-transplant recipient and either donor PBMCs, splenocytes or lymph node cells, or HLA-mismatched 3rd party PBMCs as described[16]. For unstimulated CD4 and CD8 samples, pre- and post-transplant PBMC samples were thawed and stained with anti-CD3 (OKT3; BD Biosciences), CD4 (OKT4; Tonbo Biosciences), and CD8 (SK1; BD Biosciences). Samples were then FACS-sorted for $CD3^+CD4^+$ and $CD3^+CD8^+$ populations, followed by DNA extraction. Genomic DNA was isolated using the Qiagen DNeasy Blood and Tissue Kit (Germantown, MD) as per manufacturer's instructions.

2.3 TCRβ sequencing

DNA was frozen at −20°C and shipped on dry ice to Adaptive Biotechnologies (Seattle, WA) for high-throughput TCRβ CDR3 sequencing. TCRβ sequencing data were retrieved from Adaptive's ImmunoSEQ software. PCR amplification, read sequencing, and mapping, with bias correction and internal controls, were performed by Adaptive Biotechnologies, returning tabulated productive template counts corresponding to unique productive CDR3 sequences across all samples. Sequencing data are available at https://clients.adaptivebiotech.com/pub/savage-2019-ajt. Unless otherwise specified, unique productive TCRβ sequences were defined by CDR3 nucleotide sequence and V and J gene. To identify unique productive TCRβ sequences, individual samples were downloaded from the ImmunoSEQ software, productive rearrangements were filtered, a new column was created combining the nucleotide sequence with V and J genes, and samples were merged according to the new column. When analyzed by amino acid sequence, unique productive TCRβ sequences were defined by CDR3 amino acid sequence. From these, sample template counts across unique productive TCRβ sequences were normalized to frequency of detection. Donor-reactive TCRβ sequences were defined by ≥5-fold expansion in the $CFSE^{lo}$ sample compared to unstimulated pre-transplant cells and frequency of ≥0.01% in the $CFSE^{lo}$ sample. Jensen-Shannon-Divergence was calculated as described previously[19].

TCRβ repertoire analysis was performed in R and Rstudio using standard commands. R codes are available in file https://github.com/Aleksobrad/AWISH.

2.4 Power calculations

Power calculations were performed as described previously[16], where minimum frequency thresholds for all unstimulated CD4 and CD8 populations for a given patient were set based on the minimum number of productive templates from the entire set of unstimulated CD4 or CD8 samples, respectively, at all timepoints for ≥90% detection power.

3. RESULTS

3.1 Reduced detection of donor-reactive T cell TCRβ sequences following liver transplantation

We investigated the fate of donor-reactive TCRβ sequences in 8 patients from ITN030ST, 3 of whom were considered to be tolerant (Subjects 206, 186, 159) and 5 of whom were non-tolerant (Subjects 129, 166, 222, 036, 176). Subjects 129, 166, and 186 were HCV-positive, while the remainder were transplanted for non-autoimmune and non-viral indications. Subjects did not undergo induction therapy and received standard immunosuppression until 12–24 months post-transplant, when they were randomized to continue receiving standard immunosuppression or to immunosuppression withdrawal (Figure 1A)[15]. Subjects who underwent complete immunosuppression withdrawal without elevation in liver enzymes beyond twice the upper level of normal for >1 year were considered tolerant; those who were randomized to immunosuppression withdrawal but had elevation in liver enzymes more than twice the upper limit of normal during immunosuppression weaning or shortly after withdrawal were not fully withdrawn from immunosuppression or were restarted on immunosuppression, respectively, and were considered non-tolerant[15]. Clinical information on the 8 subjects studied is summarized in Tables S1 and S2; all 8 subjects studied were randomized to immunosuppression withdrawal. We identified donor-reactive clones via the pre-transplant CFSE-MLR as unique TCRβ sequences with frequency ≥0.01% in the $CFSE^{lo}$ sample that were at least 5-fold expanded in the $CFSE^{lo}$ sample relative to the pre-transplant unstimulated sample as described and validated previously (Figure 1B)[16,18]. We applied minimum frequency and fold change criteria in identifying the donor-reactive TCRβ sequences to avoid including FACS contaminants and high frequency but not donor-specific T cells undergoing bystander proliferation in this dataset.

We first counted the number of donor-reactive T cell TCRβ sequences in blood samples pre-transplant, pre-randomization before immunosuppression withdrawal, and post-randomization when immunosuppression was fully withdrawn or immunosuppression withdrawal had failed. Sequencing data are summarized in Table S3. Based on the unstimulated sample with the lowest TCRβ productive template counts, we applied a minimum frequency threshold across all CD4 or CD8 samples within a subject to ensure ≥90% detection power, corresponding to ≥90% probability of detecting a unique TCR β sequence that is present in circulation with the minimum frequency threshold. Applying a minimum frequency threshold allowed us to appropriately test for clonal deletion. We observed significant reductions in the number of circulating donor-reactive TCRβ CD4

sequences post-transplant, both pre- and post-randomization, compared to pre-transplant in 5 of 8 subjects studied, irrespective of tolerance or HCV status (Figure 2A–2D; Table S4). Despite a downward trend following the transplant, we did not observe statistically significant reductions in tolerant subject 206 or non-tolerant subject 176, in whom the measurable donor-reactive repertoire was limited due to low numbers of available donor cells, or in tolerant subject 159. The reductions in donor-reactive sequences appeared stable over time. Although the detected CD8 donor-reactive repertoires were limited in size, we also observed statistically significant reductions in the number of donor-reactive CD8 sequences in 5 of 8 subjects, including 2 of 3 tolerant and 3 of 5 non-tolerant subjects, and a downward trend in all 8 subjects. The fold change in numbers of donor-reactive TCRβ sequences in the circulation for CD4 and CD8 cells in all 8 subjects is shown in Figure 2E–2F. We also observed a reduction in sum frequencies of donor-reactive CD4 and CD8 TCRβ sequences in circulation post-transplant in 7 of 8 patients (Figure S1). Similar results were obtained when, instead of nucleotide sequences, we defined donor-reactive TCRβ by amino acid sequences. We observed reduced post-transplant detection of donor-reactive amino acid TCRβ sequences both by clone counts and sum frequency (Figure S2A–S2C). These results were consistent with the possibility that partial deletion of donor-reactive T cells occurred following human liver transplantation, regardless of whether or not tolerance was achieved.

3.2 Reduced detection of donor-reactive TCRβ sequences after accounting for repertoire turnover

To determine whether the observed reductions in circulating donor-specific T cell clones were related to the presence of donor antigens or reflected a more non-specific effect of treatments given to the patients, we examined overall T cell repertoire turnover. There was considerable patient-to-patient variability in the level of repertoire turnover after the transplant, as measured by Jensen-Shannon-Divergence (JSD), which ranges from 0 (no change in TCR sequences) to 1 (complete turnover of TCR sequences). CD4 repertoire turnover was low in most patients (JSD<0.2) and did not surpass 0.5 in any patient (Figure 3). CD8 repertoire turnover appeared greater in non-immune, non-viral patients, achieving levels close to 0.8. Turnover did not correlate with tolerance status, but was uniformly low for both CD4 and CD8 cells in the 3 HCV patients (Figure 3B).

To account for repertoire turnover and its potential effect on the post-transplant detection of donor-reactive T cell clones identified prior to transplant, we compared the ratio of post-transplant circulating donor-reactive and total TCRβ sequences to the corresponding pre-transplant sequences sets in the unstimulated repertoires. We observed significant reductions in detection of donor-reactive CD4 and CD8 TCRβ sequences compared to total pre-transplant unstimulated sequences in 7 of 8 subjects and 8 of 8 subjects, respectively (Figure 4; Table S5). We did not observe a significant reduction in CD4 donor-reactive sequences when accounting for repertoire turnover in subject 129 (Figure 4B), who did show a reduction in numbers of circulating CD4 and CD8 donor-reactive sequences following the transplant (Figure 2D). While we did not observe significant reductions in numbers of circulating CD4 and CD8 donor-reactive sequences in subjects 176 and 159 (Figure 2), we did observe significant reductions in relative numbers of donor-reactive sequences when accounting for repertoire turnover in these subjects. A similar overall pattern was observed

when sequences were compared at the amino acid rather than the nucleotide level (Figure S2D). These results, taken together, identified significant reductions in detection of donor-reactive clones post-transplant that in most cases could not be explained by overall repertoire turnover, consistent with antigen-specific deletion of donor-reactive T cells.

3.3 Tracking of 3rd party-reactive TCRβ sequences

To further understand the reduction in donor-reactive TCRβ sequences, we identified 3rd party-reactive TCRβ sequences via pre-transplant CFSE-MLRs using irradiated HLA-mismatched 3rd-party PBMCs as stimulators (HLA information in Table S2). We hypothesized that 3rd party-reactive T cells would not encounter the same antigenic pressures as donor-reactive T cells following transplantation and could therefore serve as an additional comparator. Donor-reactive and 3rd party-reactive repertoires had minimal overlap (Table S6). We performed the same analyses on the 3rd party-reactive TCRβ sequences as for the donor-reactive TCRβ sequences described above. Pre-randomization samples demonstrated significant reductions in 3rd party-reactive sequences only in Subject 036 (Figure S3A–S3F, Table S7). Sum frequencies of 3rd party-reactive sequences in circulation were not consistently reduced post-transplant (Figure S3G). In contrast to consistent reductions in relative detection of donor-reactive sequences, we observed significantly increased detection of 3rd party-reactive CD4 sequences relative to the unstimulated repertoire in subjects 222, 206 and 186, along with significantly reduced relative detection of 3rd party-reactive CD4 and CD8 sequences in subject 159 and subjects 176, 159, 206, and 129, respectively (Figure S3H–S3I, Table S8). When compared in a paired fashion, we observed significantly greater fold reductions in detection of donor-reactive than 3rd party-reactive CD4 and CD8 TCRβ sequences and significantly greater reductions in detection relative to the pre-transplant repertoire of donor-reactive CD4 sequences than 3rd party-reactive sequences (Figure 5), altogether suggesting that donor-reactive TCRβ sequences were reduced in blood in an antigen-specific manner. Consistently, we observed significantly greater reductions in detection of TCRβ amino acid sequences that were donor-reactive than 3rd party-reactive (Figure S4).

4. DISCUSSION

We investigated the fate of donor-reactive TCRβ sequences in a study in which human liver allograft recipients were randomized to attempted early immunosuppression withdrawal. Although histological assessment may better distinguish tolerant and non-tolerant recipients than biochemical-only definitions of tolerance and non-tolerance due to reduced sensitivity of liver chemistry tests[20,21], tracking donor-reactive sequences in blood did not identify tolerant patients prospectively in this study, where tolerance was defined biochemically without histological confirmation. Despite the lack of induction therapy, our results were consistent with deletion of donor-reactive TCRβ sequences among liver transplant recipients. This interpretation cannot be conclusive in the absence of corresponding tissue specimens, as it is possible that donor-reactive T cells entered the allograft in non-tolerant recipients. In addition, larger, more homogeneous cohorts of patients studied at standardized post-transplant time points, are needed to confirm our preliminary conclusions. These reductions in circulating donor-reactive T cells in liver allograft recipients contrast strikingly

to those in kidney allograft recipients, in whom we have previously observed an increase of circulating donor-reactive TCRβ sequences post-transplant (ref[16] and unpublished data). In that study, reductions in circulating donor-reactive TCRβ sequences were specifically associated with achievement of tolerance following CKBMT[16]. We have also tracked donor-specific TCRs in the circulation of intestinal allograft recipients and have detected increases in their number following the transplant, though to a lesser extent than is detected in the allograft itself during rejection (ref[18] and J.Fu and M.Sykes, unpublished data). Therefore, the reduction in circulating donor-reactive TCRs in liver allograft recipients is anomalous compared to other types of transplant in the absence of demonstrable tolerance and more closely resembles our observations in tolerant CKBMT recipients. We postulate, therefore, that the liver inherently induces some deletion of T cells that recognize it, as observed in rodent models[12–14], but that deletion is incomplete (as observed) and other factors are decisive in determining whether or not the recipient is fully tolerant of the donor. We utilized pre-transplant CFSE-MLRs to identify donor-reactive TCR sequences in our study. This approach allowed evaluation of the effect of transplantation on this repertoire. The effects of immunosuppression on MLRs and our previously observed lack of correlation between post-transplant MLRs and tolerance in CKBMT recipients[16], combined with the above-mentioned evidence for the biological significance of the donor-reactive repertoire identified pre-transplant, support the relevance of pre-transplant as opposed to post-transplant MLRs for analysis. Our previous studies have shown that a given alloreactive repertoire is stable over at least one year in healthy donors[16] and the relatively low repertoire turnover over time in the cohort studied here suggests that any contribution from *de novo* development of donor-specific T cells post-transplant to the donor-reactive repertoire would likely be small.

Our study could have implications for attempts to promote tolerance. Time from transplant is predictive of tolerance in liver transplant recipients[2,22]. Combined with our data showing that deletion of donor-reactive clones did not evolve further after 1–2 years, we speculate that other mechanisms such as regulatory phenomena may develop over longer periods in most patients who achieve tolerance. Tregs are enriched in tolerant liver transplant recipients[23–25], and donor-specific Treg infusion promoted tolerance in 7 of 10 liver transplant recipients[26]. In contrast to the sequence proposed here for liver allograft tolerance, we have proposed a sequential Treg-dependent/deletion-dependent model to explain the tolerance observed in CKBMT recipients with transient chimerism[16,27–29]. In those patients, high throughput TCRβ sequencing demonstrated the expansion of donor-specific Tregs by 6 months post-transplant only in patients who successfully achieved tolerance[30] followed by later deletion of donor-reactive TCRs, again only in tolerant patients[16]. While we may postulate that patients in ITN030ST who achieved tolerance were those with the greatest late expansion of donor-specific Tregs, exploration of this hypothesis would require larger numbers of pre-transplant cells than are currently available in order to optimally identify the donor-specific Treg repertoire as described[30].

In conclusion, we have obtained preliminary evidence that donor-reactive T cells undergo partial deletion in patients receiving liver allografts. While not in itself predictive of a tolerant state, liver-induced clonal deletion may contribute to the successes that have been achieved in liver allograft tolerance induction in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ITN	Immune Tolerance Network
TCRβ	T cell receptor β chain
CDR3	complementarity-determining region 3
HCV	Hepatitis C virus
CKBMT	combined kidney and bone marrow transplantation
MLR	mixed lymphocyte reaction
JSD	Jensen-Shannon-Divergence

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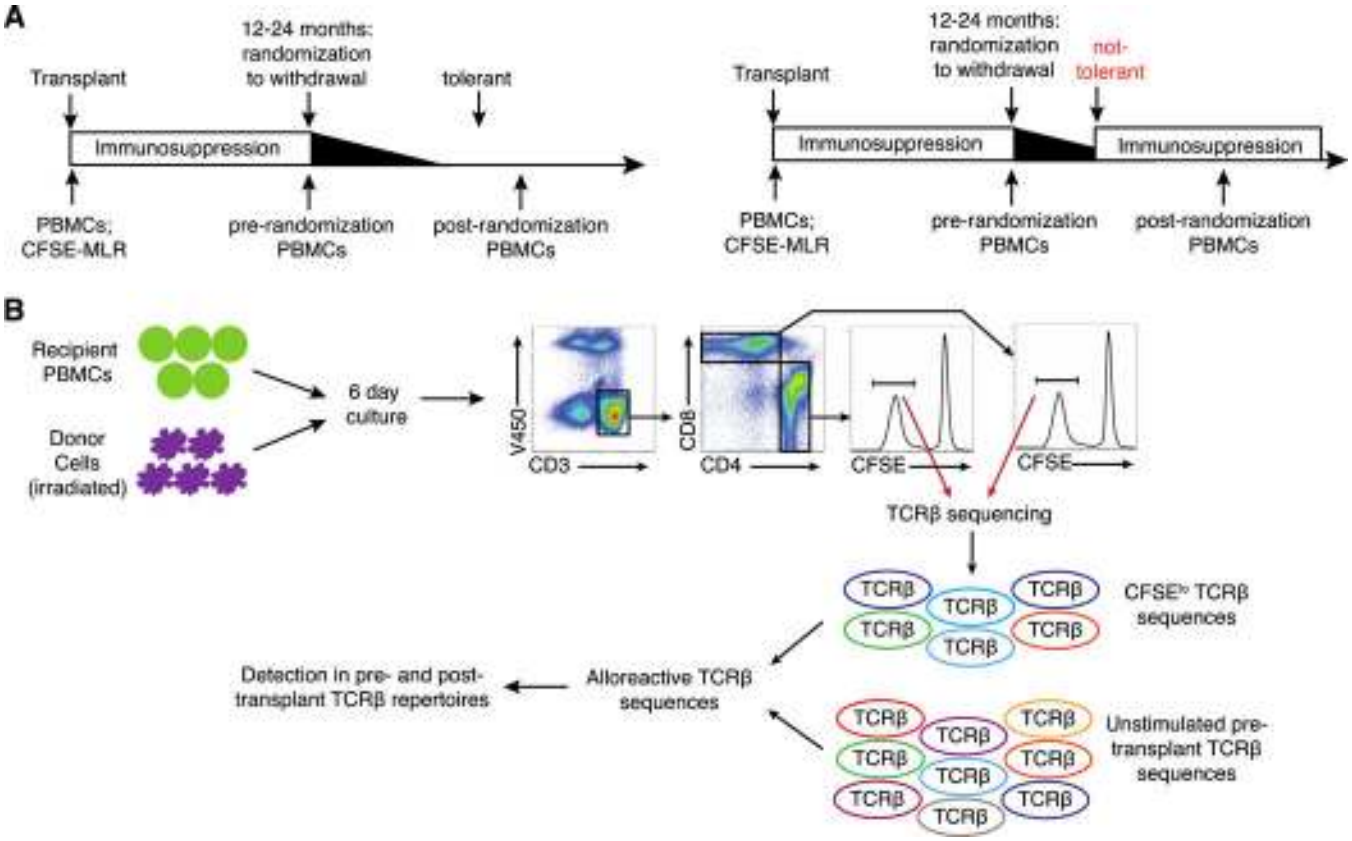

Fig. 1. Study design.

(A) Schematic of subjects in ITN030ST study randomized to immunosuppression withdrawal. Tolerant subjects diagrammed on left and non-tolerant subjects on right. Key time points shown at top and sample collection at bottom. (B) Schematic of identification and tracking of the donor-reactive TCRβ repertoire via the pre-transplant CFSE-MLR.

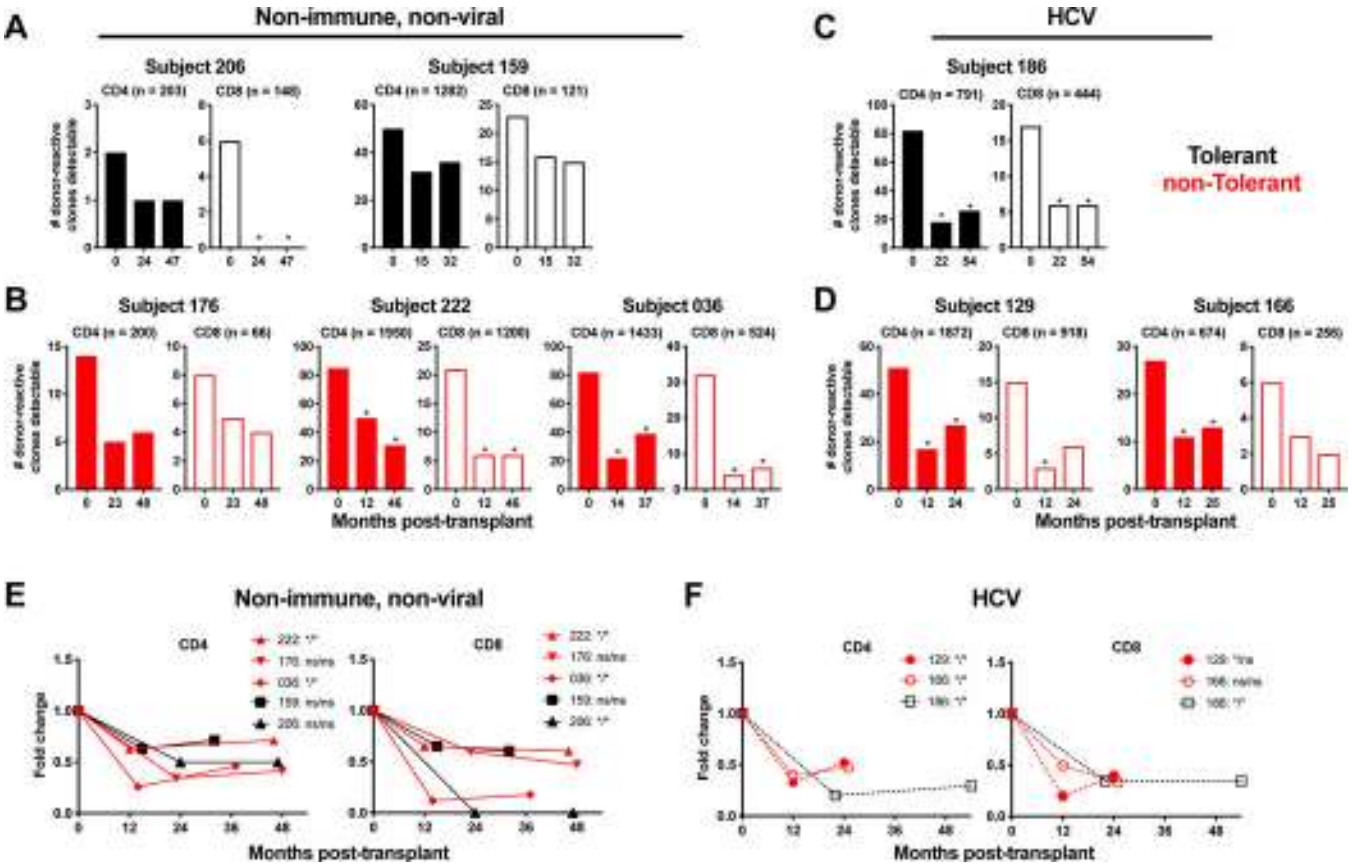

Fig. 2. Reduction in detection of donor-reactive TCRβ sequences after liver transplantation.

Counts of donor-reactive TCRβ sequences in pre- and post-transplant CD4 and CD8 samples with ≥90% detection power for (A) tolerant and (B) non-tolerant non-immune, non-viral subjects, and for (C) tolerant and (D) non-tolerant HCV subjects. n corresponds to the number of total donor-reactive CD4 or CD8 sequences. Fold change in detection of donor-reactive sequences among (E) non-immune, non-viral subjects and (F) HCV subjects. Fold change is defined as the ratio of the odds of detecting donor-reactive sequences post-transplant relative to pre-transplant. Notation following each subject in the key refers to p-value at each time point pre-randomization/post-randomization. *p<0.05 reduction or increase in odds of detection of donor-reactive sequences compared to pre-transplant (two-sided Fisher's exact test); tabulated data in Table S4.

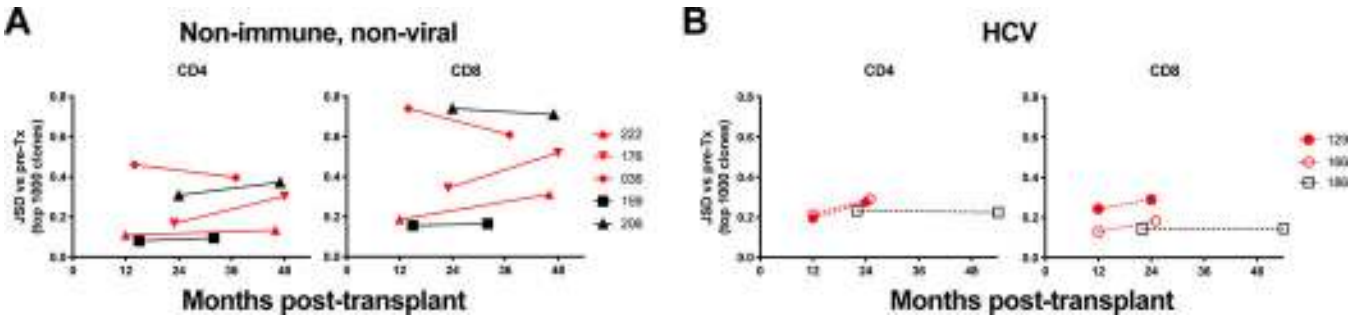

Fig. 3. Repertoire turnover post-transplant.

Jensen-Shannon-Divergence (JSD) comparing the top 1000 TCRβ sequences at each post-transplant time point to the top 1000 TCRβ sequences pre-transplant for (A) non-immune, non-viral subjects and (B) HCV subjects. JSD ranges from 0 to 1, with 1 representing complete divergence and 0 representing identical samples.

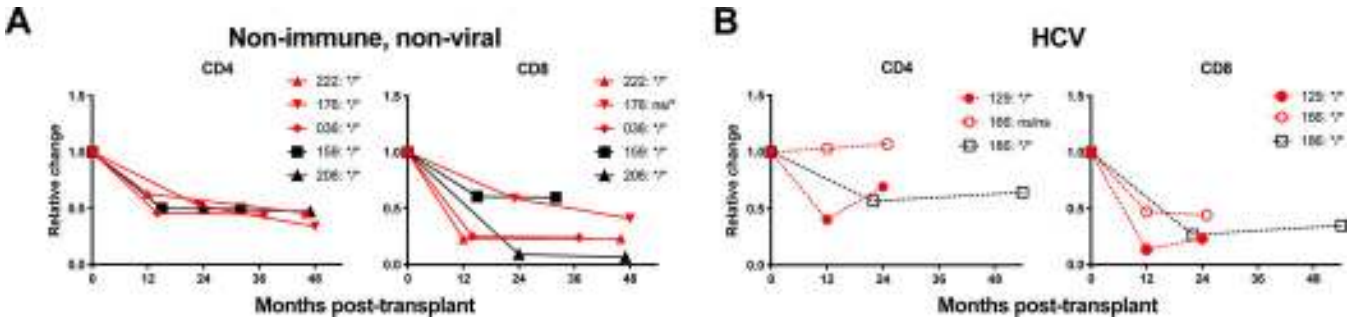

Fig. 4. Reduced detection of donor-reactive sequences after accounting for repertoire turnover.

Relative change in detection of donor-reactive TCRβ sequences for (A) non-immune, non-viral subjects and (B) HCV subjects, where black are tolerant and red are non-tolerant subjects. Relative change at a post-transplant time-point is the ratio of the odds of detecting pre-transplant-identified donor-reactive sequences to the odds of detecting any pre-transplant-identified (unstimulated) sequences at the same post-transplant timepoint. A value of 1 indicates that the proportion of pre-transplant-identified donor-reactive sequences detected at a given time point was equal to that for any pre-transplant-identified sequences. Values <1 and >1 indicate a lower rate and a greater rate, respectively, of detection of donor-reactive versus any pre-transplant sequences. Notation following each subject in the key refers to p-value for each time point pre-randomization/post-randomization. *p<0.05 reduction or increase in odds of post-transplant detection of pre-transplant-identified donor-reactive sequences compared to unstimulated pre-transplant sequences (two-sided Fisher's exact test); tabulated data in Table S5.

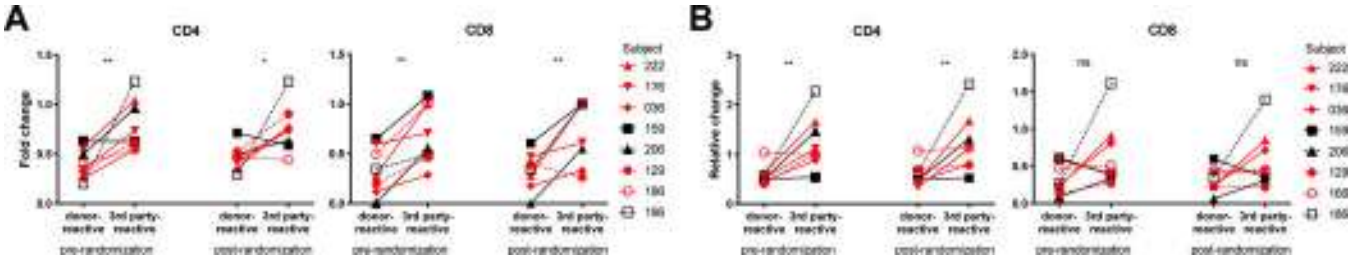

Figure 5. Reduced detection of donor-reactive relative to 3rd party-reactive TCRβ sequences.

At pre-randomization and post-randomization time points, comparison of (A) fold change in detection of donor-reactive sequences to fold change in detection of 3rd party-reactive sequences and (B) change in detection relative to pre-transplant unstimulated sequences of donor-reactive sequences compared to 3rd party-reactive sequences. *p<0.05, **p<0.01, p-value calculated from two-sided paired t-test.