

## TRANSPLANTATION

## Tracking donor-reactive T cells: Evidence for clonal deletion in tolerant kidney transplant patients

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T cell responses to allogeneic major histocompatibility complex antigens present a formidable barrier to organ transplantation, necessitating long-term immunosuppression to minimize rejection. Chronic rejection and drug-induced morbidities are major limitations that could be overcome by allograft tolerance induction. Tolerance was first intentionally induced in humans via combined kidney and bone marrow transplantation (CKBMT), but the mechanisms of tolerance in these patients are incompletely understood. We now establish an assay to identify donor-reactive T cells and test the role of deletion in tolerance after CKBMT. Using high-throughput sequencing of the T cell receptor B chain CDR3 region, we define a fingerprint of the donor-reactive T cell repertoire before transplantation and track those clones after transplant. We observed posttransplant reductions in donor-reactive T cell clones in three tolerant CKBMT patients; such reductions were not observed in a fourth, nontolerant, CKBMT patient or in two conventional kidney transplant recipients on standard immunosuppressive regimens. T cell repertoire turnover due to lymphocyte-depleting conditioning only partially accounted for the observed reductions in tolerant patients; in fact, conventional transplant recipients showed expansion of circulating donor-reactive clones, despite extensive repertoire turnover. Moreover, loss of donor-reactive T cell clones more closely associated with tolerance induction than *in vitro* functional assays. Our analysis supports clonal deletion as a mechanism of allograft tolerance in CKBMT patients. The results validate the contribution of donor-reactive T cell clones identified before transplant by our method, supporting further exploration as a potential biomarker of transplant outcomes.

## INTRODUCTION

Chronic immunosuppression in kidney transplantation is associated with morbidities including nephrotoxicity, metabolic abnormalities, and increased risk of infection and malignancy (1). Moreover, despite recent improvements in 1-year kidney allograft survival, late rejection rates remain high (2). Immune tolerance in organ transplantation, defined as the absence of rejection without immunosuppression, would avoid these morbidities. Spontaneous tolerance is rare in conventional renal transplant recipients, with frequencies estimated at less than 5% (3, 4).

Tolerance was first intentionally induced in humans via combined kidney and nonmyeloablative bone marrow transplantation (CKBMT), a protocol designed to induce a mixed chimeric state in which hematopoietic elements are composed of a mixture of host and donor cells (5, 6). Among 10 patients who received CKBMT [5 subjects in Immune Tolerance Network (ITN) study NKDO3; 5 subjects in the study ITN 036ST], 7 have tolerated their allograft off immunosuppression for 4 to 12 years (6–8).

In the rodent regimens that led to the development of this protocol, mixed chimerism was durable and tolerance involved long-term intrathymic deletion of donor-reactive T cells (that is, “central tolerance”) (9–11). In human CKBMT patients, however, mixed chimerism was transient (6, 12), suggesting that additional, likely peripheral, mechanisms are involved in maintaining long-term tolerance. Functional mechanistic studies in these CKBMT patients suggested a role for early

suppression and long-term deletion of donor-reactive T cells in maintaining tolerance (6, 13). *In vitro* assays, however, cannot reliably distinguish anergy from deletion as mechanisms of unresponsiveness. We now establish an assay to specifically track donor-reactive T cells and test the role of deletion in maintaining long-term tolerance after CKBMT.

Tracking of donor-reactive clones in transplant patients is hampered by the large proportion (up to 10%) of T cells directly recognizing a set of major histocompatibility complex (MHC) alloantigens (14, 15), presumably involving many specificities. We devised a deep sequencing approach to identify and track the donor-reactive T cell repertoire. With immunoSEQ (Adaptive), T cell receptor (TCR)  $\beta$  (*TRB*) CDR3 regions are amplified with primers for all 54 known expressed V $\beta$  and all 13 J $\beta$  regions adapted for solid-phase polymerase chain reaction (PCR) and high-throughput sequencing (16–18). Each individual T cell clone has a distinct *TRB* CDR3 sequence. We hypothesized that CDR3 sequencing of a transplant recipient’s donor-reactive T cells, as identified by their proliferation in an anti-donor mixed lymphocyte reaction (MLR) before transplant, would identify donor-specific TCR sequences that could then be physically tracked in the recipient’s posttransplant peripheral blood samples to differentiate between anergy and deletion of donor-specific T cells. Using this analysis in four CKBMT and two conventional renal allograft recipients, we obtained evidence for clonal deletion as a mechanism of allograft tolerance in humans.

## RESULTS

## Defining a “fingerprint” of the anti-donor T cell repertoire

Figure 1 illustrates our strategy for defining the fingerprint of the alloreactive repertoire for any responder-stimulator (recipient-donor) pair.

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An allostimulated population was generated via carboxyfluorescein diacetate succinimidyl ester (CFSE) MLR. MLR responders, and irradiated stimulator peripheral blood mononuclear cells (PBMCs) were labeled with CFSE and violet dye, respectively, cocultured for 6 days, and then FACS (fluorescence-activated cell sorting)-sorted for violet-negative, CD3-positive, CFSE-low CD4<sup>+</sup> and, in separate tubes, CD8<sup>+</sup> cells (Fig. 1A). Deep sequencing was then performed on the genomic DNA extracted from these sorted T cell populations that had divided in response to donor antigens. To permit identification of clones expanding in the MLR, we also performed deep sequencing on unstimulated CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> FACS-sorted T cells from the same pretransplant peripheral blood sample. To be considered donor-reactive, a clone defined by the unique nucleotide sequence of its *TRB* CDR3 region must have been detected above a minimum frequency threshold of  $10^{-4}$  in the CFSE-low population in the stimulated (MLR) sample and have expanded at least fivefold relative to its frequency in an unstimulated sample from the same time point, thereby excluding highly abundant but not specifically donor-reactive clones (Fig. 1B). We could thus define a fingerprint of the pretransplant donor-reactive T cell

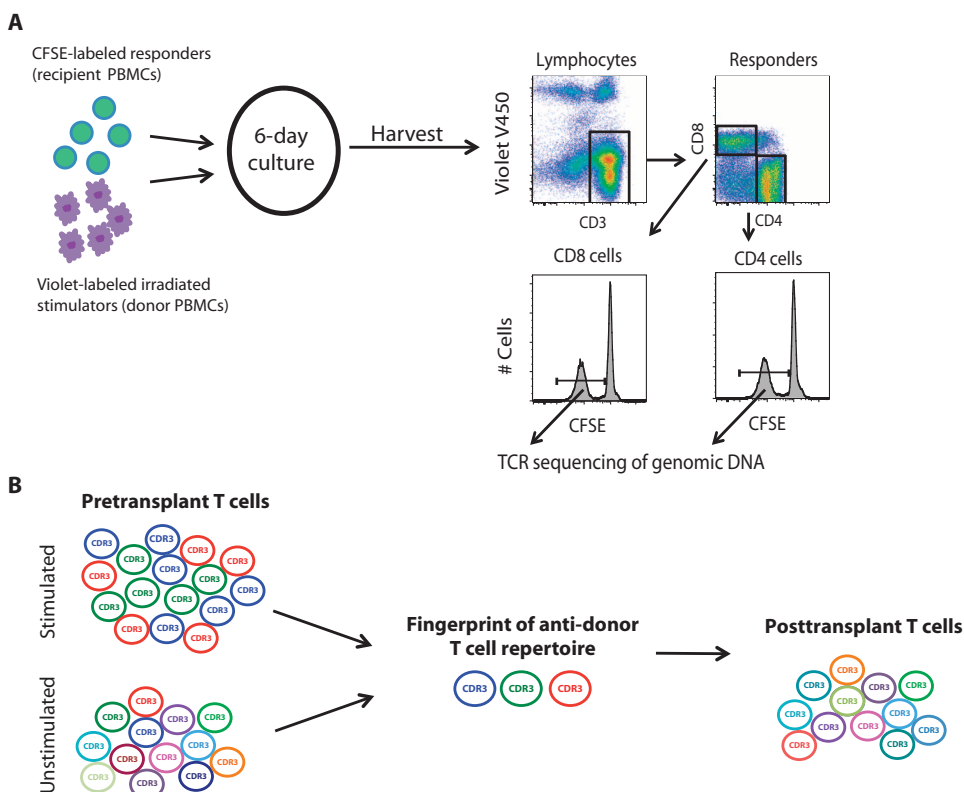
repertoire for each donor-recipient pair, and these clones could be tracked in unstimulated posttransplant samples.

### Reproducible detection of alloreactive TCRs in blood samples obtained at different times

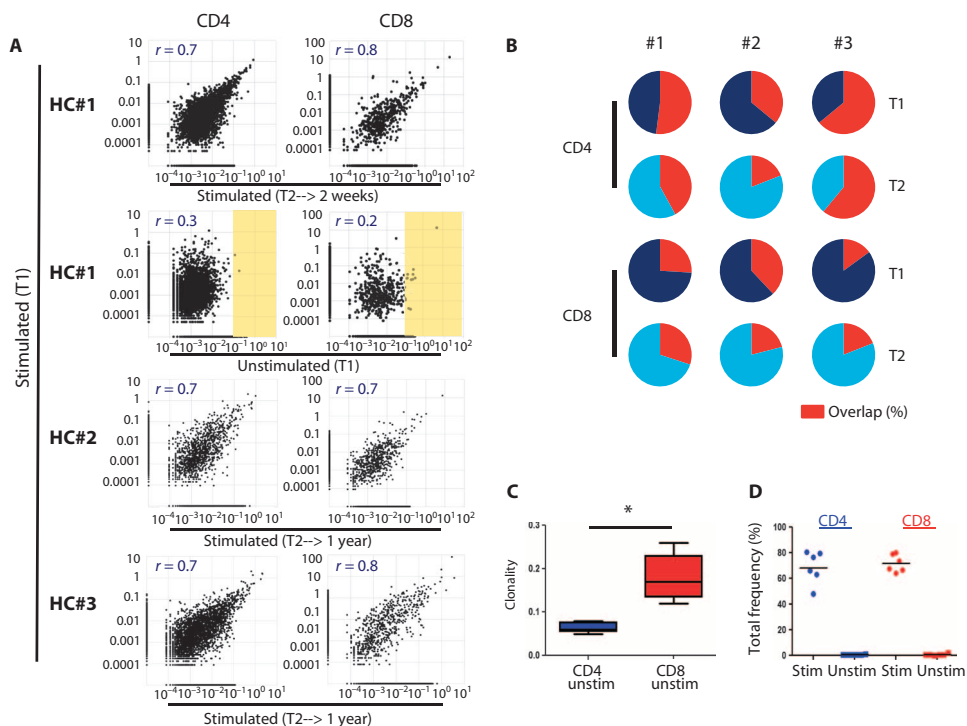
To validate the approach of tracking a set of alloreactive *TRBs* over time, we tested whether individual alloreactive T cell clones could be reproducibly detected in blood samples drawn at multiple times. Using PBMCs obtained from three healthy controls at different time points separated by 2-week or 1-year intervals, we set up parallel MLRs with the same responder-stimulator pairs for each time point. We then performed deep sequencing on the dividing T cells in the MLR, as well as on unstimulated T cells (fig. S1, B and C). Deep CDR3 sequencing identified fewer unique clones in allostimulated versus unstimulated populations, resulting in decreased entropy and increased clonality, a trend that was most striking in the CD4 compartment (table S1).

A comparison of the log clonal frequencies from the MLRs (stimulated samples) separated by 2 weeks showed strong linear correlations among CD4 ( $r = 0.7$ ) and CD8 ( $r = 0.8$ ) clones, but minimal correlation

with the unstimulated repertoire (CD4:  $r = 0.3$ ; CD8:  $r = 0.2$ ); the linear correlation persisted in the clonal frequencies identified in MLRs from blood draws separated by a year-long interval (Fig. 2A). Figure 2B shows the relative frequency overlap of the alloreactive clones (defined in Fig. 1) between the two time points for each healthy control. The degree of overlap was comparable across the 2-week (HC#1) and year-long (HC#2 and HC#3) time intervals. There was less overlap in alloreactive CD8 than CD4 T cell clones detected at different time points. This may reflect the presence of a few very high frequency clones in the unstimulated CD8 repertoires (Fig. 2A), resulting in fewer unique CD8 clones being sampled in each blood draw (table S1). Indeed, the diversity of the unstimulated CD8 repertoires was less than that of CD4s: the clonality of a pool of identical clones is “1,” whereas that of a pool of all unique clones is “0”; the significantly increased clonality of CD8 compared to CD4 cells in the same unstimulated samples is shown in Fig. 2C ( $n = 5$ ;  $P = 0.0062$ , two-tailed paired *t* test). Cumulatively, all alloreactive CD4 clones constituted ~48 to 80% of clones in each MLR and less than 1.1% of the unstimulated CD4 repertoire (table S2). For the CD8s, the alloreactive clones accounted for approximately 64 to 80% of the stimulated population and less than 2.5% of the unstimulated population (Fig. 2D). Together, our healthy control studies showed that the alloreactive T cell populations identified via CFSE MLR that recognized a given set of alloantigens were



**Fig. 1. MLR experimental design and schematic of TCR sequencing analysis strategy to identify and track donor-reactive T cells.** (A) CD3<sup>+</sup>violet<sup>-</sup> cells, representing the responder T cells, were selected and further separated into CD4<sup>+</sup> and CD8<sup>+</sup> subgroups. Within each subgroup, the CFSE-low cells were isolated for DNA extraction and TCRβ CDR3 deep sequencing. Sorting strategy is indicated with boxes on the dot plots and bars on the histograms; CFSE staining in unstimulated control sample shown in fig. S1A. (B) Pretransplant fingerprint of anti-donor T cell repertoire defined as all clones detected at a frequency greater than  $10^{-4}$  in the stimulated condition (CFSE-low cells in MLR) that have expanded at least fivefold relative to their frequency in the unstimulated T cell population (unstimulated repertoire defined via TCR sequencing of CD3<sup>+</sup>CD4<sup>+</sup> or CD3<sup>+</sup>CD8<sup>+</sup> T cells isolated via FACS sorting of PBMCs from the same sample used for the CFSE MLR). Each donor-reactive clone identified by its unique CDR3 nucleotide sequence could then be tracked in posttransplant unstimulated peripheral blood samples.



**Fig. 2. Overlap of the alloreactive T cell repertoire via TCR sequencing in repeat MLR assays in healthy controls.** (A) Top panel: Linear correlation of log frequencies of alloreactive clones detected in MLRs performed at time point 1 [stimulated (T1)] and time point 2 [stimulated (T2)] for healthy control #1 (HC#1) from blood draws separated by a 2-week interval. Second panel from top: No such correlation is observed when clonal frequencies in the stimulated populations are plotted against frequencies in the unstimulated population [unstimulated (T1)]; high-frequency clones in the unstimulated populations highlighted in yellow. These results are representative of similar analyses in HC#2 and HC#3 (fig. S1D). Bottom two panels: Linear correlation persists in MLRs performed from blood draws separated by a year interval (HC#2, HC#3). Same allogeneic stimulator for each HC at T1 and T2. Overlapping number of sequences detected: HC#1 T1 and T2 stim CD4 = 2944, CD8 = 465; HC#1 T1 stim and T1 unstim: CD4 = 3011, CD8 = 478; HC#2 T1 and T2 stim: CD4 = 1162, CD8 = 642; HC#3 T1 and T2 stim: CD4 = 2850, CD8 = 652. (B) Pie charts showing relative overlap of the summed frequencies of alloreactive clones (fingerprint as defined in Fig. 1) over time in three healthy controls. Each circle represents the cumulative frequency of all alloreactive clones identified in the sample; red segment shows the percentage of that total frequency arising from alloreactive clones identified at both T1 and T2 (tabulated values table S2). (C) Boxplot comparing the clonality of the unstimulated CD4 and CD8 T cell repertoires ( $n = 5$ ;  $*P = 0.0062$ , two-tailed paired  $t$  test; tabulated values in table S1). (D) Cumulative frequencies of all alloreactive clones for each pair of stimulated and unstimulated HC at all time points.

reproducibly detectable in separate peripheral blood samples over highly disparate time intervals ranging from 2 weeks to 1 year.

### Reduced circulating donor-reactive T cell clones in tolerant CKBMT patients

Our studies of alloreactive T cells in healthy controls showed that donor-reactive clones identified in MLRs could be consistently detected in peripheral blood at disparate time points. We therefore used this approach to identify donor-reactive T cells before transplant and track them over time after transplant in six subjects: four CKBMT recipients (subjects 1, 2, 4, and 5 from ITN trial ITN036ST) who were removed from immunosuppression 8 months after transplant and two kidney transplant recipients receiving conventional immunosuppression (IS#1 and IS#2). Sequencing statistics are summarized in table S3. For each transplant recipient, we defined a fingerprint of the anti-donor T cell repertoire using pretransplant PBMCs. The

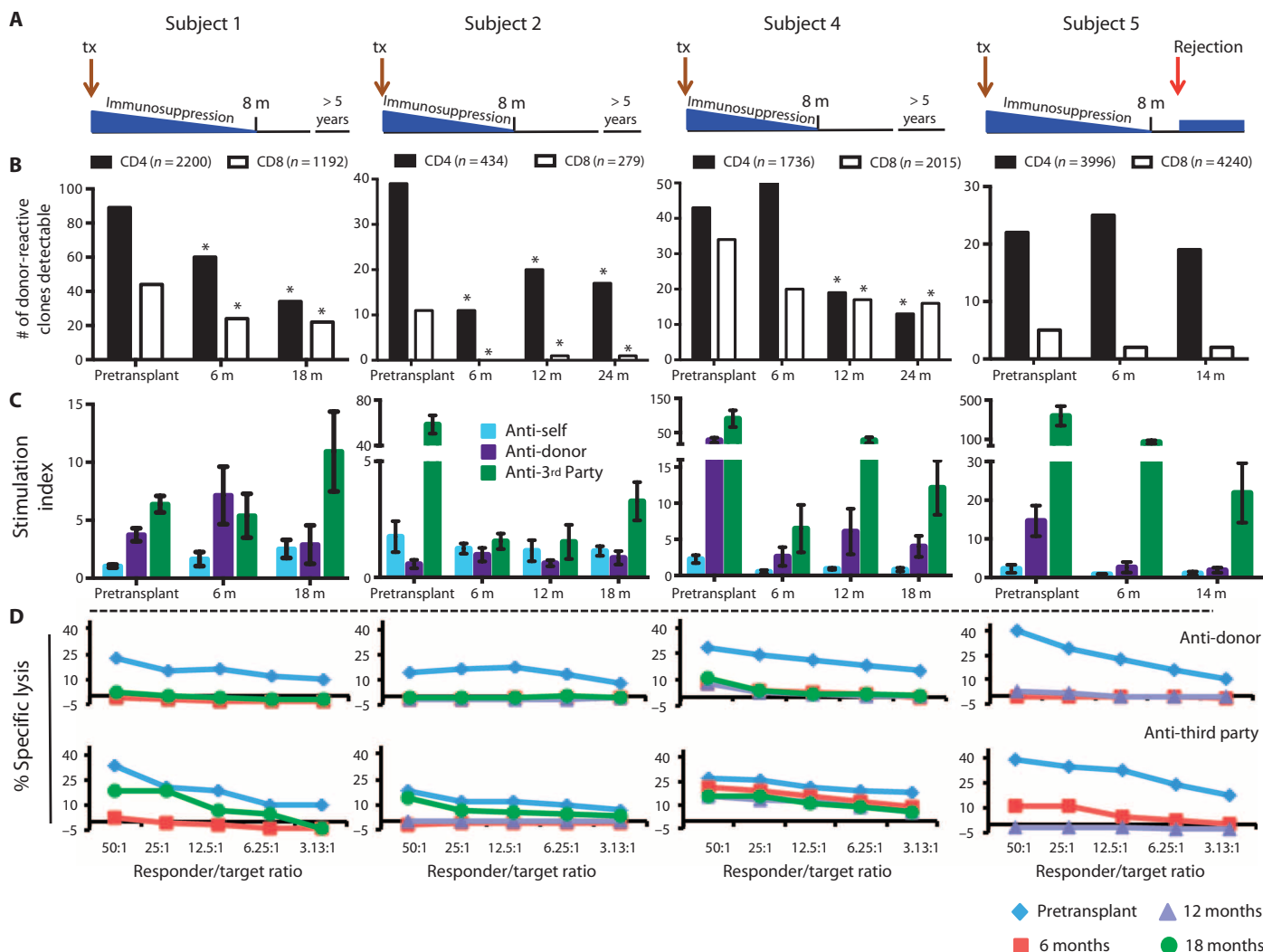
limit of detection of T cell clones for tracking in the unstimulated pre- and post-transplant samples was determined with a power calculation that took into account the cell number and the number of reads obtained from each sample.

CKBMT subject 1 has stable allograft function >5 years after stopping immunosuppression at 8 months after transplant (Fig. 3A). All CD4 and CD8 samples permitted clonal detection at  $\geq 10^{-5}$  frequency. We identified 2200 donor-reactive CD4<sup>+</sup> clones and 1192 CD8<sup>+</sup> clones as defined by our approach in Fig. 1B. We then compared the number of these donor-reactive clones that were detectable in unstimulated pre- and posttransplant blood samples. A significant reduction in the number of circulating donor-reactive CD4<sup>+</sup> and CD8<sup>+</sup> clones was observed at both 6 and 18 months posttransplant compared to pretransplant blood (Fig. 3B, figs. S3 and S4, and table S4). Results were robust to changes in definition of donor-reactive clones ranging from 5- to 10-fold for all subjects; the 5-fold expansion criterion included the largest number of clones while excluding clones with minimal expansion that might reflect “bystander” effects (fig. S2).

Functional MLR assays in subject 1 (Fig. 3C) showed persistent anti-donor reactivity (greater than anti-self) at 6 months that was markedly reduced at 1.5 years (comparable to anti-self), whereas cell-mediated lympholysis (CML) assays (Fig. 3D) revealed donor unresponsiveness at both times; responses to extensively human leukocyte antigen (HLA)-mismatched (from donor and recipient) third party donors recovered between 6 and 18 months after transplant, demonstrating that im-

mune unresponsiveness was specific to the donor. Limiting dilution analyses (LDAs) quantify functional cytotoxic T lymphocyte precursors (CTLps) and interleukin-2-producing helper T lymphocytes (HTLs) (fig. S5). Donor-reactive CTLps were undetectable by 6 months, suggesting that the remaining donor-reactive cells were anergic, because only partial deletion of donor-reactive CD8 cells was apparent (Fig. 3B). Anti-donor HTLs were measurable at 6 months but not at 18 months.

We performed similar analyses in two additional tolerant CKBMT recipients. Subject 2 is >5 years after CKBMT with no rejection. Her pretransplant MLR was weak, perhaps due to extensive HLA sharing with the donor (table S3 legend). Significantly fewer CD4<sup>+</sup> and CD8<sup>+</sup> donor-reactive clones were detected at 6, 12, and 24 months after CKBMT than before (Fig. 3B, figs. S3 and S4, and table S4). Although TCR analysis revealed residual donor-reactive CD4 clones, functional assays (Fig. 3, C and D, and fig. S5) showed minimal to no posttransplant



**Fig. 3. Clinical course, tracking of donor-reactive T cell clones, MLR, and CML results in CKBMT subjects.** (A) Overview of clinical course. (B) Number of donor-reactive *TRB* CDR3 clones (y axis) detected in the unstimulated CD4 (black) and CD8 (white) repertoire at the indicated time points (x axis). The fingerprint of the anti-donor T cell repertoire was defined for each subject as clones in the pretransplant MLR with  $\geq 10^{-4}$  frequency that were expanded at least fivefold relative to their frequency in the pretransplant unstimulated sample (total number indicated above the relevant panel). Sufficient power was obtained to consider a

frequency of  $10^{-5}$  as detectable in all unstimulated populations for subjects 1, 2, and 4, and  $5 \times 10^{-5}$  for subject 5. \* $P < 0.05$  compared to pretransplant ( $P$  values in table S4, two-sided Fisher's exact test). (C) MLR: proliferative responses to recipient (anti-self), donor (anti-donor), and third party (anti-3rd party) relative to proliferation of unstimulated PBMCs are shown at the indicated time points. Each bar represents the mean  $\pm$  SD stimulation index of triplicate cultures. (D) CML: responses at the indicated time points are shown. MLR and CML data have been previously summarized by Kawai *et al.* (8).

responses to donor, with recovery of third party responses at 18 months in MLR and CML (8).

Subject 4 also shows allograft tolerance >4.5 years after CKBMT. Figure 3B shows a progressive reduction in CD8<sup>+</sup> donor-reactive clones reaching significance at 12 months after transplant; donor-reactive CD4<sup>+</sup> clones showed an initial nonsignificant increase at 6 months after transplant followed by significant reductions at 12 and 24 months (Fig. 3B, figs. S3 and S4, and table S4). Several functional assays, including the MLR, revealed persistent anti-donor responsiveness at 12 and 18 months (Fig. 3, C and D, and fig. S5), unlike subjects 1 and 2 and NKDO3 subjects (13). To test whether the same clones responded to the donor in post- and pretransplant

MLRs, we performed clonal analysis on 1-year CD4<sup>+</sup> and CD8<sup>+</sup> T cells dividing in anti-donor CFSE MLR. The overlap of alloreactive clones over 1 year was markedly less than that observed in healthy controls over the same interval (fig. S6A and Fig. 2B, respectively). The number of posttransplant donor-reactive CD4 and CD8 T cells defined by the 12-month MLR (clones with frequencies  $\geq 10^{-4}$  in the 12-month posttransplant MLR expanded at least fivefold compared to the unstimulated 12-month posttransplant sample) remained relatively constant in unstimulated pre- and posttransplant blood samples (fig. S6B). Collectively, these data suggest that the persistent MLR in subject 4 was largely mediated by a subset of clones that did not lead to rejection, of which some may have developed after transplant, whereas

others did not expand sufficiently in the pretransplant MLR to be defined as alloreactive, escaped deletion by conditioning treatment, and were neither expanded nor deleted in the presence of the donor graft.

### No reduction in donor-reactive clones in a CKBMT recipient who failed tolerance induction

One month after discontinuing immunosuppression, CKBMT subject 5 developed acute rejection that culminated in graft loss despite anti-rejection therapy (Fig. 3A). Of the four CKBMT recipients, only subject 5 showed no significant reduction in numbers of circulating donor-reactive CD4 and CD8 clones after transplantation (Fig. 3B, figs. S3 and S4, and table S4). Remarkably, MLR, CML, and CTLp assays showed donor-specific unresponsiveness at 6 and 12 months (Fig. 3, C and D, and fig. S5). Thus, the functional assays did not distinguish the lack of clinical tolerance, whereas the clonal analysis showed a distinct pattern compared to the three tolerant subjects.

### Lack of deletion of donor-reactive clones in conventional kidney transplant recipients

To provide a further comparison to tolerant CKBMT recipients, we studied two kidney transplant recipients receiving conventional immunosuppression. Subject IS#1 received a living unrelated donor kidney. Subject IS#2 received a living unrelated donor kidney after a failed previous living-related transplant and had Banff grade 1B acute cellular rejection (ACR) at 3 months after transplant. In contrast to tolerant patients, donor-reactive CD4<sup>+</sup> clones were significantly enriched in the posttransplant compared to pretransplant peripheral blood in IS#1 and IS#2. Donor-reactive CD8<sup>+</sup> clones were not significantly changed (Fig. 4A, fig. S7, and table S4).

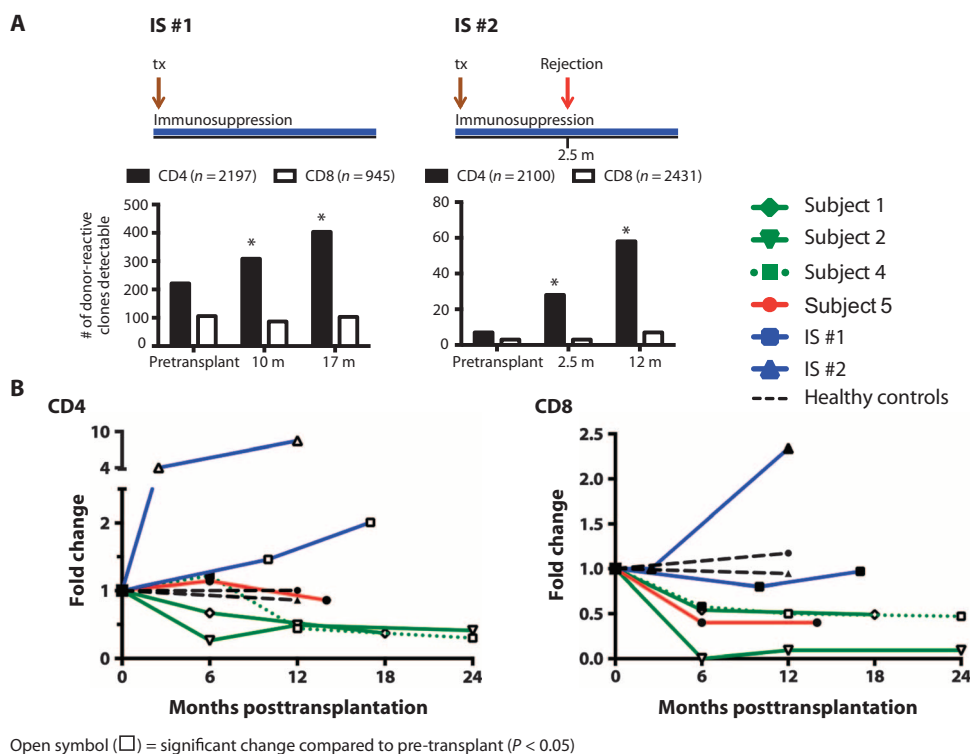
Figure 4B summarizes the fold change in the number of donor-reactive clones detected in posttransplant compared to pretransplant blood in all six transplant patients. In contrast to the three tolerant CKBMT subjects, there was no significant reduction in either of the two “conventional” kidney transplant subjects or the nontolerant CKBMT subject in circulating donor-reactive CD4 and CD8 T cell clones. Notably, the limited number of donor-reactive CD8 clones tracked in subject 5 and IS#2 limited the ability to evaluate changes over time. For nontolerant subjects, observed expansion of donor-reactive CD4 cells was greater when the definition of donor reactivity required greater expansion in the pretransplant MLR (fig. S2), suggesting that the donor-reactive pretransplant clones responding most strongly in MLR were most likely to expand after transplant.

As additional controls, an identical analysis was performed in healthy controls in

whom clones defined as alloreactive in a particular MLR were tracked in an unstimulated sample 1 year later. For both CD4 and CD8 T cells, there was no significant change in the number of alloreactive T cells, in contrast to the reduction seen in the tolerant CKBMT subjects and the expansion in donor-reactive CD4 cells in the conventional transplant patients (Fig. 4B, details in table S5).

### T cell repertoire turnover

The loss of donor-specific clones in tolerant subjects might reflect global T cell depletion due to conditioning, leading to repertoire turnover as T cells developed de novo from thymic recovery. To quantify changes in T cell repertoire over time, we calculated the Jensen-Shannon divergence (JSD) of the top 1000 nucleotide sequences before and after transplant (Fig. 5A). A JSD of 1 reflects complete divergence of two repertoires, whereas the JSD of two identical repertoires is 0. For reference, we determined JSD of pairs of samples of T cells isolated from peripheral blood 1 year apart from two healthy controls. Although all transplant recipients showed greater repertoire divergence than healthy controls, JSD values were higher for CD4<sup>+</sup> populations of CKBMT recipients compared to CD8<sup>+</sup> T cells. Among the conventional transplant recipients, IS#2 showed repertoire



**Fig. 4. Tracking of donor-reactive T cell clones in conventional kidney transplant recipients and summary of clonal analysis results for all six subjects.** (A) Clinical course and number of donor-reactive *TRB* CDR3 clones (y axis) detected in the unstimulated CD4 (black) and CD8 (white) repertoire at the indicated time points (x axis). Sufficient power was obtained to consider a frequency of  $10^{-5}$  as detectable in all unstimulated populations for IS#1 and  $5 \times 10^{-5}$  for IS#2. \* $P < 0.05$  compared to pretransplant ( $P$  values in table S4, two-sided Fisher's exact test). (B) Change over time in detection of unstimulated T cell populations of donor-reactive CD4 and CD8 clones (defined in Fig. 3B legend). Fold change is the odds ratio of the number of donor-reactive clones detected in unstimulated posttransplant CD4 and CD8 populations relative to the number detected in unstimulated pretransplant populations (pretransplant = 1). Open symbol, statistically significant reduction or increase ( $P < 0.05$ ). Tabulated results are shown in tables S4 and S5.

turnover close to that of the CKBMT patients, whereas that of IS#1 was lower. The nontolerant CKBMT recipient subject 5 showed the highest JSD, with almost complete turnover of CD4 and CD8 repertoires (Fig. 5A).

We next compared the likelihood, after transplantation, of detecting any clones detected before transplant compared to those defined as donor-reactive. Among tolerant subjects, no significant decrease in donor-reactive compared to all pretransplant CD4 clones was observed after transplant. Subject 2 showed a significant relative increase in donor-reactive CD4 cells by 1 year after transplant, and subject 4 showed a significant relative increase only at 6 months after transplant (Fig. 5B). A significant and sustained reduction in the detection of donor-reactive compared to all CD8<sup>+</sup> clones was observed in subjects 2 and 4, suggesting antigen-driven loss of donor-reactive CD8 clones (Fig. 5B). The notion that some, but not all, deletion of donor-reactive clones in tolerant subjects may have reflected repertoire turnover was supported by analysis of third party-reactive T cells identified before transplant (fig. S8), some of which showed posttransplant reductions.

The determination of relative loss of donor-reactive compared to all pretransplant clones was affected by the definition of “donor-reactive” in some subjects. For example, for subject 1, when this definition required increasing levels of MLR expansion, greater relative loss of donor-reactive CD4 and CD8 clones was seen (Fig. 5C). This result suggests that more strongly donor-reactive clones were more likely to show deletion after CKBMT. Overall, posttransplant reductions in donor-reactive clones in tolerant subjects may reflect a mixture of repertoire turnover and specific deletion of donor-reactive T cells, possibly after initial antigen-driven expansion.

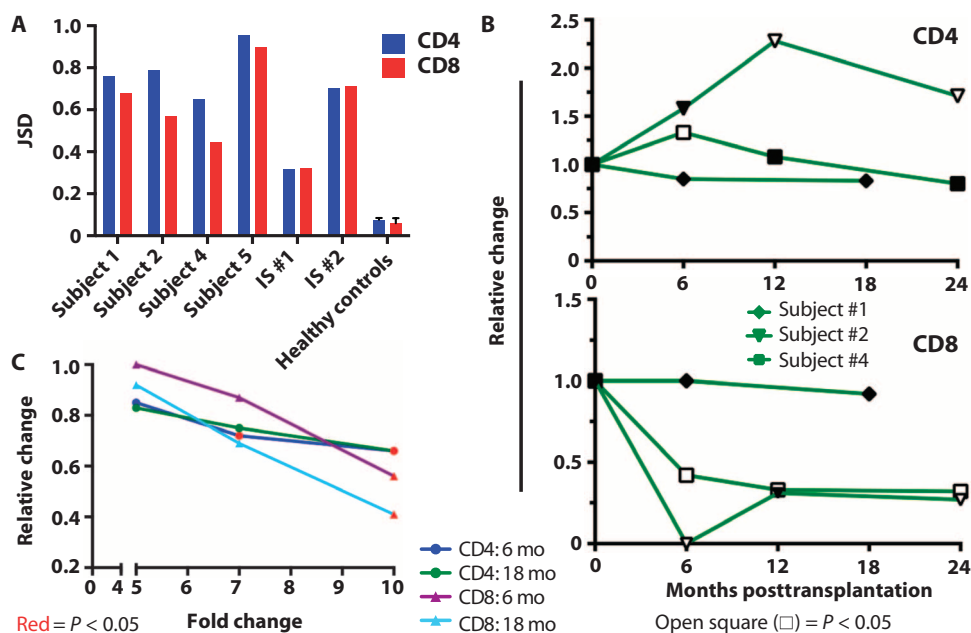
### Reduced TCR diversity in nontolerant compared to tolerant subjects

Because lymphopenia-driven proliferation (LIP) in a T cell-deficient environment (19) may reduce the repertoire diversity associated with T cell reconstitution after lymphoablative conditioning (20), we compared overall clonal diversity (by Simpson's index *D*) of posttransplant T cell populations (Fig. 6A). In contrast to tolerant subjects, in whom the CD4 T cell diversity returned to pretransplant values, nontolerant subjects showed persistently decreased clonal diversity (increased *D*) after transplantation ( $P = 0.017$ , Student's *t* test, comparing two groups at time point nearest 1 year after transplant) (Fig. 6B). No difference was seen between the groups in the diversity of CD8 repertoires.

## DISCUSSION

T cell responses to allogeneic MHC molecules are orders of magnitude stronger than other responses (14, 15, 21, 22), presumably involving myriads of T cell receptors (TCRs). We have developed an approach using deep TCR sequencing to identify, before transplant, and track, after transplantation, human transplant recipients' donor-reactive T cell repertoires. We demonstrate the feasibility of this approach and use it to address mechanisms of tolerance in CKBMT recipients.

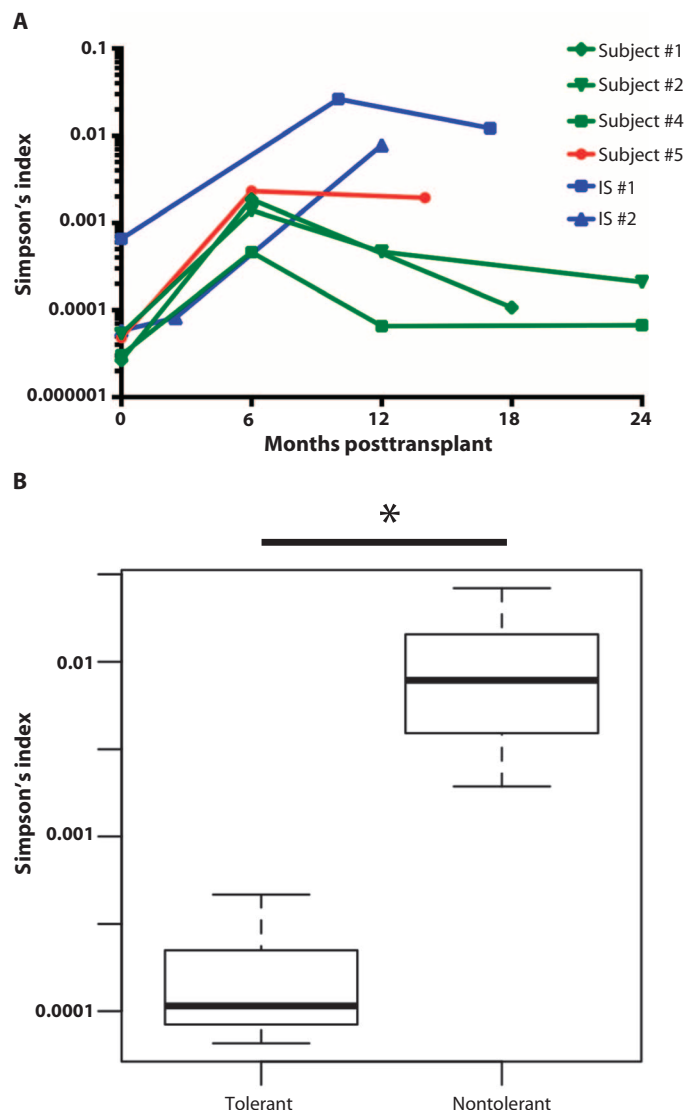
Previous studies in ITN CKBMT trial NKDO3 (6, 13) could not distinguish between anergy and deletion of donor-reactive T cells in maintaining long-term tolerance. Our new method allowed specific assessment of clonal deletion. Circulating donor-reactive CD4<sup>+</sup> and CD8<sup>+</sup> clones, identified before transplantation by CFSE MLR, decreased after transplantation in all tolerant subjects. Deletion was either partial or not apparent at 6 months and frequently evolved over time. In the only CKBMT subject who failed to achieve operational tolerance, significant



**Fig. 5. TCR repertoire turnover in kidney allograft recipients.** (A) JSD comparing pre- and posttransplantation (last posttransplantation time point) TCR repertoires. JSD on top 1000 nucleotide clones ranked by frequency (0 denotes identical repertoires; 1 denotes complete repertoire divergence). Healthy controls: average JSD on top 1000 nucleotide clones of two healthy controls in whom TCR sequencing was performed at two time points 1 year apart. (B) Anti-donor CD4 and CD8 clonal analysis relative to overall repertoire turnover. Relative numbers of donor-reactive (as defined in Fig. 3B legend) versus non-donor-reactive clones (all other clones detected in unstimulated pretransplant sample) detected at any level (threshold detection of assay  $10^{-6}$ ) in posttransplant samples. Relative change is the odds ratio of the relative (post/pre) number of donor-reactive clones divided by the relative (post/pre) number of non-donor-reactive clones in unstimulated samples at the same time. A value of 1 indicates that the proportion of donor-reactive clones (defined before transplant as above) detected at a given time point was equal to that of all clones (detected before transplant) detected at the same time point. A value  $<1$  indicates lower rate of detection of donor-reactive versus all clones, and a value  $>1$  indicates greater rate of detection of donor-reactive versus all clones. Open symbols, statistically significant reduction or increase ( $P < 0.05$ , two-sided Fisher's exact test); tabulated data and *P* values in table S7. (C) Anti-donor CD4 and CD8 clonal analysis relative to overall repertoire turnover in subject 1: effect of varying definition of donor reactivity by different fold expansion criteria (frequency in pretransplant anti-donor MLR/frequency in unstimulated pretransplant sample  $>5$ , 7, or 10). Red points are statistically significant ( $P < 0.05$ ); tabulated data and *P* values in table S9.

reductions of donor-specific clones were not observed. These studies provide evidence for a role for deletion in the maintenance of allograft tolerance in humans. Although we cannot exclude the possibility that donor-reactive clones moved from the circulation into the allograft, this would be inconsistent with protocol biopsies showing no rejection and minimal cellular infiltrates, which were enriched for Foxp3<sup>+</sup> cells, in tolerant CKBMT recipients (6, 7).

Conventional transplant recipients showed persistent expansion of donor-reactive T cell clones after transplant, despite considerable repertoire turnover, suggesting that expansion was allograft-driven and



**Fig. 6. Recovery of CD4 repertoire diversity in tolerant recipients. (A)** CD4 T cell repertoire diversity (measured by Simpson's index  $D$ ) over time after transplantation ( $D$  of 1 indicates that all clones are identical; smaller  $D$  indicates clones are more unique and therefore the repertoire is more diverse). **(B)** Comparison of CD4 Simpson's index ( $D$ ) in tolerant (subjects 1, 2, and 4) and nontolerant (subjects 5, IS#1, and IS2) subjects near 1 year after transplant (10 months: IS#2; 12 months: subjects 2, 4, and IS#1; 14 months: subject 5; 18 months: subject 1). \* $P = 0.017$ , two-sided Student's  $t$  test on logarithm of  $D$  ( $n = 6$ ).

showing that pretransplant MLRs identify biologically relevant donor-reactive clones. A role in graft-versus-host disease was identified for a CD4 clone recognizing a recipient minor histocompatibility antigen in MLR after HLA-identical hematopoietic cell transplantation (23), but our studies examine the entire alloreactive repertoire against HLA antigens. Further evidence from our studies that biologically significant clones are identified in pretransplant MLR includes the following: (i) the donor-reactive clones expanding most strongly in pretransplant MLR were most likely to be expanded in posttransplant blood in nontolerant subjects (fig. S2); (ii) the donor-reactive clones expanding most strongly in pretransplant MLR were more likely than other clones to be deleted in posttransplant blood of tolerant subjects (Fig. 5C); (iii) donor-reactive clones that were insufficiently dominant to be identified as donor-reactive in pretransplant MLR persisted in constant numbers and contributed to a posttransplant MLR in subject 4, but did not cause rejection. In contrast, subject 5 failed to show deletion of donor-reactive clones identified in pretransplant MLR and rejected the graft. These data suggest that non-dominant donor-reactive clones that can produce a posttransplant MLR when dominant clones are deleted may be of minimal biological significance, whereas those that dominate in the pretransplant MLR are of major importance.

Deletion of donor-reactive clones in CKBMT recipients was partially explained by global T cell depletion induced by conditioning (6, 7, 12, 13). The initial recovery of T cells in CKBMT recipients is most likely driven by LIP because most T cells express an effector/memory phenotype in the first 3 to 6 months after transplant (12), as observed for rapid LIP (19), which is largely antigen-driven (24, 25). Therefore, residual donor-reactive clones may expand initially in response to bone marrow and/or kidney alloantigens. However, donor-reactive T cell deletion is unlikely to reflect only repertoire turnover, given the similar, high level of repertoire turnover in all CKBMT recipients and lack of significant donor-reactive clonal deletion only in the subject who failed tolerance. The persistence of some preexisting donor-reactive clones along with the sometimes progressive deletion observed over time in tolerant patients is consistent with initial LIP/antigen-driven expansion of surviving donor-reactive clones. Indeed, two tolerant subjects, despite showing an absolute loss of donor-reactive CD4 clones, showed an increase in these clones relative to all preexisting clones, one at 6 months and another persisting longer after transplant (Fig. 5B). Thus, the relative numbers of persisting donor-reactive and non-donor-reactive clones may be the net effect of T cell-depleting conditioning (affecting any clone), antigen-driven expansion (donor-reactive clones), and antigen-driven deletion (donor-reactive clones). During rejection, entry of donor-reactive T cells, especially CD8 clones, into the graft may also reduce circulating clonal frequencies, perhaps mitigating the detection of expanded anti-donor clones in the rejecting CKBMT subject 5. Ultimately, the relative loss of donor-reactive T cell clones was greater than that of all clones in several instances in the tolerant subjects. Thus, our data suggest that expanded donor-reactive clones are gradually deleted in tolerant subjects, resulting in specific clonal reduction, especially among clones with strongest anti-donor reactivity.

The reduction of donor-reactive clones in tolerant subjects is consistent with our hypothesis that donor-reactive T cells are slowly deleted in response to repeated encounter with donor antigens on quiescent, accepted allografts (13). Late deletion seems less likely to occur intrathymically, because hematopoietic chimerism was short-lived (12), making long-term intrathymic antigen-presenting cell (APC) chimerism unlikely. However, peripheral APCs presenting intact or processed

donor antigen might migrate to the thymus (26) and mediate ongoing deletion.

Clonal analysis detected persistent donor-reactive clones with greater sensitivity than *in vitro* assays that revealed donor-specific unresponsiveness in CKBMT subjects 1, 2, and 5. Donor-specific unresponsiveness was particularly surprising in the rejector, subject 5. Unlike the functional assays, TCR clonal analysis is not affected by anergy or suppression. The absence of a posttransplant MLR in subject 5 despite the lack of deletion of dominant donor-reactive clones suggests that these clones may have been anergic and that anergy could have been broken by the renal infection that preceded rejection (8). The poor predictive value of MLR and CML with respect to graft outcomes is consistent with previous studies in animals (27, 28), patients receiving conventional transplants (29–33), and patients receiving a different CKBMT protocol for HLA-mismatched kidney allograft tolerance induction (34). Overall, clonal analysis distinguished between tolerance and nontolerance among the six subjects, suggesting a new and specific method of assessing transplantation tolerance.

The similar clonal expansion in two conventional kidney transplant recipients despite disparate clinical outcomes (IS#2 but not IS#1 had a rejection) may reflect the limited cell numbers available for IS#2, resulting in a higher threshold frequency to declare a clone “present.” Moreover, important differences in donor-reactive T cell clone numbers might be present in the kidney graft and not the circulation. Indirect anti-donor alloreactivity may also contribute to rejection (35–37).

Repertoire turnover was greater for CKBMT patients compared to the nonrejecting conventional transplant recipient, consistent with more potent T cell–depleting treatments in conditioning for CKBMT. However, both conventional transplant recipients also showed markedly greater TCR turnover than healthy controls over 1 year, demonstrating the potential of our approach to provide insight into the effects of immunosuppressive regimens. In a recent report (38) on TCR repertoire in multiple sclerosis (MS) patients receiving conditioning and autologous hematopoietic cell transplantation, T cell diversity recovered more quickly in MS patients who responded to treatment compared to nonresponders. It is interesting, therefore, that CD4 T cell diversity returned to baseline levels more rapidly in tolerant than in nontolerant subjects (Fig. 6A). This return of diversity did not correlate with recovery of naive-type CD4 cells, which was more rapid in CKBMT subjects 4 and 5 than in subjects 1 and 2 (12).

Our study is limited by the small number of these tolerant patients available and will require further validation in larger cohorts as transient chimerism protocols are evaluated in additional subjects. Although differences in immunosuppression between the CKBMT and conventional patients may have affected the observed clonal behavior, reestablishment of a new T cell repertoire due to T cell–depleting conditioning is insufficient to account for deletion of donor-reactive clones in tolerant patients. Further exploration of how various induction and maintenance immunosuppression regimens differentially affect donor-reactive clones after transplant will be of interest.

In conclusion, we have described a method whereby donor-reactive recipient T cell clones are identified before transplant, then tracked after transplantation. We obtained evidence for a role for deletion of donor-reactive CD4 and CD8 T cells in maintaining tolerance in CKBMT recipients with transient chimerism. A recent study (39) used high-throughput CDR3 sequencing to detect donor-reactive T cells in the graft and urine of a patient with allograft dysfunction. However, that study relied on a

measurable posttransplant MLR to identify donor-reactive clones and did not assess the fate of preexisting donor-reactive T cells. Our approach of identifying donor-reactive clones before transplantation and tracking them prospectively avoids dependence on functional assays after transplant, which correlate poorly with outcomes. This strategy has revealed the biological importance of donor-reactive T cell clones detected in a pretransplant MLR, demonstrating posttransplant expansion of these clones even in the face of global T cell repertoire turnover and has provided new mechanistic insights into tolerance achieved via transient chimerism, implicating eventual peripheral deletion of donor-reactive T cells in response to an accepted renal allograft.

## MATERIALS AND METHODS

### Study design

The object of this study was to study the fate of pretransplant donor-reactive T cells in transplant patients and to provide insight into the mechanisms of long-term tolerance in CKBMT. Laboratory investigations were performed on four of five CKBMT patients (subjects 1, 2, 4, and 5) in study ITN036ST, which included for-protocol PBMC collections before and after transplant, and two conventional transplant patients from Columbia’s Center for Translational Immunology Biobank Core of transplant recipient clinical specimens. CKBMT subject 3 was removed from ITN036T after early allograft loss due to thrombotic microangiopathy, and posttransplant PBMCs were therefore not available for analysis. The conventional transplant patients studied were those for whom sufficient posttransplant PBMCs were available to perform the required *in vitro* assays and for whom one or more posttransplant kidney transplant biopsies, indicating rejection or lack thereof, were available. There was no randomization or blinding.

### Subjects

CKBMT subjects: clinical outcomes in five CKBMT patients in study ITN036ST have been described (7, 8). Conventional transplant recipients: subject IS#1 had end-stage renal disease (ESRD) secondary to focal segmental glomerulosclerosis (FSGS) and received a renal transplant from a living related donor. Subject IS#1 received thymoglobulin and methylprednisolone for induction therapy and was subsequently maintained on tacrolimus and mycophenolate. Allograft biopsies performed at 10 and 17 months after transplantation to evaluate acute increases in serum creatinine showed no evidence of cellular or antibody-mediated rejection, and were consistent with calcineurin inhibitor toxicity. Subject IS#2 also had ESRD due to FSGS and received a renal transplant from a living unrelated donor several years after a previous living-related transplant had failed. Because the patient was highly sensitized, he received plasmapheresis and intravenous immunoglobulin preoperatively and received rituximab, basiliximab, and methylprednisolone as induction therapy. A 3-month protocol biopsy revealed Banff grade 1B ACR, which was treated with thymoglobulin and corticosteroids; subsequent protocol biopsies at 6 months and 1 year were suspicious for ongoing rejection. Informed consent was obtained from all subjects. The study protocols were approved by the Massachusetts General Hospital and Columbia University Medical Center Institutional Review Boards.

### Mixed lymphocyte reactions

**Preparation of CFSE-labeled responders.** For HC#1 (T1- and T2-stimulated samples) and CKBMT subjects 1 and 2, MLRs were set



up using purified T cells as responders. Previously frozen pretransplant PBMCs were thawed, washed, and resuspended in MACS buffer. MACS beads (Pan T Cell Isolation Kit II, Miltenyi Biotec, catalog no. 130-091-156) were used to generate “untouched” T cells. These T cells were resuspended in phosphate-buffered saline (PBS) at  $1 \times 10^6$  cells/ml, labeled with CFSE at a concentration of 0.2 to 0.5  $\mu$ M (CellTrace CFSE Proliferation Kit, Molecular Probes, catalog no. C34554), washed three times, and resuspended in MLR medium (AIM-V supplemented with 5% AB heat-inactivated human serum, 0.01 M HEPES, and 50  $\mu$ M 2-mercaptoethanol at a concentration of  $2 \times 10^6$  cells/ml). For HC#2 and HC#3, CKBMT subjects 4 and 5, the two conventional transplant recipients, and the anti-third party responses, whole PBMCs were used as responders instead of purified T cells. PBMCs were labeled with CFSE as above and resuspended at  $2 \times 10^6$  cells/ml.

**Preparation of violet dye-labeled stimulators.** Cryopreserved donor (or healthy control) PBMCs were thawed, washed, resuspended in PBS, and labeled with BD Horizon Violet Proliferation Dye 450 (catalog no. 562158). After labeling, cells were washed twice, resuspended in MLR medium at  $2 \times 10^6$  cells/ml, and irradiated at 30 to 35 Gy.

**Plating of cells.** One million CFSE-labeled pretransplant responder cells and 1 million violet dye-labeled irradiated stimulators were plated in each well of a 24-well plate (total well volume, 1 ml). For HC#2, HC#3, IS#1, IS#2, and the anti-third party MLRs, we used 96-well plates with each well containing 200,000 responder PBMCs and 200,000 stimulators (total well volume, 200  $\mu$ l). MLR cultures were incubated at 37°C for 5 to 6 days.

### Flow cytometry

MLR wells were harvested after 6 days of culture. Cells were resuspended in FACS buffer; stained for 30 min with fluorochrome-conjugated antibodies against CD3 (BD Pharmingen clone SP34-2, catalog no. 552852), CD4 (BioLegend clone OKT4, catalog no. 317426), and CD8 (BD Pharmingen clone DK1, catalog no. 557834); washed; and filtered before FACS sorting on a BD Influx cell sorter to isolate two discrete cell populations (violet<sup>-</sup>CD3<sup>+</sup>CD4<sup>+</sup>CFSE<sup>lo</sup> and violet<sup>-</sup>CD3<sup>+</sup>CD8<sup>+</sup>CFSE<sup>lo</sup>) representing the CD4<sup>+</sup> and CD8<sup>+</sup> recipient-derived donor-reactive T cells. For unstimulated cell populations, PBMCs were thawed and stained with anti-CD3, anti-CD4, and anti-CD8, and then FACS-sorted into CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> populations.

### DNA isolation and sequencing

Genomic DNA was isolated from sorted cell populations using the Qiagen DNeasy Blood and Tissue Kit. DNA was frozen at  $-20^\circ\text{C}$  and shipped on dry ice to Adaptive Biotechnologies for high-throughput TCRB CDR3 sequencing. The TCR sequencing data were retrieved from Adaptive’s immunoseq software.

### In vitro immunologic assays

Standard MLR, CML, and LDA assays were performed using the methods detailed previously (5, 40).

### Computational and statistical analysis

Mapping of the reads, identification of CDR3 regions and V/J genes, and bias adjustment were performed by Adaptive (16) through their proprietary software. We receive tabulated *TRB* sequencing data from Adaptive, including CDR3 nucleotide and amino acid sequences, raw copy number (read counts), adjusted copy number and frequency, V/J

genes and gene families, inferred insertions and deletions in V-D-J junctions, etc.

**Repertoire diversity.** We measured the diversity of each repertoire by two approaches: (i) entropy (41) ( $H \equiv \sum p_i \log_2 p_i$ , where  $p_i$  is the frequency of clone  $i$ ) and clonality ( $S \equiv 1 - H_{\text{obs}}/H_{\text{max}}$ ), where  $H_{\text{max}}$  is the entropy of a repertoire with the same number of clones, each having exactly the same frequency; (ii) Simpson’s index ( $D \equiv \sum p_i^2$ , where  $p_i$  is the frequency of clone  $i$ ). Compared to entropy (and clonality), Simpson’s index is more sensitive to changes in frequency of dominant clones.

**Comparison of repertoires.** We measured the difference between two repertoires using JSD (42) and Pearson correlation, both of which range from 0 to 1. We defined expanded clones in MLR by a minimum frequency in stimulated samples ( $f$ ;  $f$  is set at 0.01%) and fold change ( $C = \text{frequency in stimulated pretransplant samples/frequency in unstimulated pretransplant samples}$ ;  $C$  is conventionally set at 5). We define a clone as detectable if the frequency is larger than a threshold ( $m$ ;  $m$  is usually 0.001% for samples with  $\geq 10^6$  T cells sequenced with 2 million reads). This threshold was set on the basis of power estimation. We model the TCR sequencing procedure by two random processes: the first (P1) is a sample of T cells randomly taken from the entire repertoire; the second (P2) is multiplexed PCR cloning of CDR3 regions from the cells in a sample. Assuming the total number of cells from P1 is  $N$ , the total number of sequence reads is  $R$  (usually  $R > 2N$ ), and the “quantum efficiency” in P2 (defined as the average chance of a cell being cloned in PCR) is  $q$ , then the expected total number of cells cloned in P2 is  $Nq$ , and the number of reads per cell follows a Poisson distribution with  $\lambda = \frac{R}{Nq}$ . If  $q$  is in the order of 40%, then  $\lambda$  is usually larger than 5, which means that ~95% of sampled cells will be represented by at least two reads, a detection threshold in Adaptive’s analytical pipeline. For a clone with a frequency  $f$  in the entire repertoire, the number of such cells in P2 follows a Poisson distribution with  $\lambda' = Nfq$ . Any clone with  $\lambda' > 4$  will have a 90% of chance of detection. If  $N$  is 1 million and  $q$  is 40%, then to achieve 85% detection power requires  $f$  greater than 0.001%.

**Testing expansion and deletion.** To test expansion or deletion of clones, we first identified donor-reactive unique clones (defined as above) in pretransplant MLR, counted their number ( $N$ ), and tested whether these clones are equally likely to be detectable in unstimulated pre- and posttransplant samples. Specifically, we generated a  $2 \times 2$  contingency table:  $[D_{\text{pre}}, N - D_{\text{pre}}; D_{\text{post}}, N - D_{\text{post}}]$ , where  $D_{\text{pre}}$  is the number of detectable preidentified donor-reactive (or third party-reactive) clones in unstimulated samples before transplant and  $D_{\text{post}}$  is the number of detectable clones in unstimulated posttransplant samples. Two-sided Fisher’s exact test was performed, and  $P$  values and odds ratios were reported.

**Repertoire turnover analysis.** To test whether posttransplant reductions in donor-reactive clones were distinguishable from general repertoire turnover, we set the null hypothesis to be that a donor-reactive clone is equally likely to be present as any other pretransplant clones in the posttransplant samples. We defined donor-reactive clones as described above and set the threshold of detectability at  $10^{-6}$  (there is no need to adjust for detection power, because the comparison is internally controlled within each posttransplant sample). For each posttransplant sample, we generated a  $2 \times 2$  contingency table:  $[N_1, N_0; D_1, D_0]$ , where  $N_1$  is the number of detected pretransplant clones,  $N_0$  is the number of undetected pretransplant clones,  $D_1$  is the number of detected donor-reactive clones, and  $D_0$  is the number of undetected donor-reactive clones. We performed Fisher’s exact test to assess significance and report odds ratio as relative change.

## SUPPLEMENTARY MATERIALS

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Fig. S1. Validation study design and analysis details of the tracking of alloreactive clones in healthy controls.

Fig. S2. Anti-donor clonal analysis at increasing fold expansion criteria.

Fig. S3. Frequencies of donor-reactive clones in unstimulated PBMCs before and after transplant in CKBMT recipients (CD4).

Fig. S4. Frequencies of donor-reactive clones in unstimulated PBMCs before and after transplant in the CKBMT recipients (CD8).

Fig. S5. LDA (CTLp and HTL) for subjects 1, 2, 4, and 5.

Fig. S6. Comparison of pre- and posttransplant anti-donor MLR in subject 4.

Fig. S7. Frequencies of donor-reactive clones before and after transplant in the conventional kidney transplant recipients.

Fig. S8. Detection of third party-reactive TCRs before and after transplant.

Table S1. Cell counts, entropy, and clonality for healthy control experiments.

Table S2. Sum frequency of alloreactive clones in healthy controls (%).

Table S3. Cell numbers, number of unique clones, and total number of reads for each patient sample.

Table S4. Tabulated data from clonal analysis in subjects 1, 2, 4, 5, IS#1, and IS#2 (Figs. 3B and 4).

Table S5. Tabulated data from clonal analysis in healthy controls (Fig. 4B).

Table S6. Sum frequency of alloreactive clones in subject 4 (%).

Table S7. Relative turnover analysis in tolerant subjects (Fig. 5B).

Table S8. Cell numbers, number of unique clones, and total number of reads for anti-third party responses.

Table S9. Relative turnover analysis at increasing fold expansion criteria: Subject 1 (Fig. 5C).

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processed samples, performed experiments, and analyzed and interpreted data. H.R. and J.Z. supervised analysis and edited the manuscript. T.K. designed, supervised, and carried out the clinical protocol and care of the CKBMT patients and participated in sample procurement. W.W. identified donors and participated in sample procurement from conventional transplant patients. S.Y. participated in sample procurement for healthy control experiments. Y.S. analyzed and interpreted data and wrote the paper. M.S. participated in the design of the clinical protocol of the CKBMT patients, designed and oversaw the laboratory studies, interpreted the data, and wrote the paper. **Competing interests:** H.R. owns stock and receives consulting fees from Adaptive Biotechnologies. All other authors declare no competing financial interests. M.S. and H.R. have filed a patent, "Tracking donor-reactive TCR as a biomarker in transplantation," based on findings presented herein.

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## Tracking donor-reactive T cells: Evidence for clonal deletion in tolerant kidney transplant patients

Heather Morris, Susan DeWolf, Harlan Robins, Ben Sprangers, Samuel A. LoCascio, Brittany A. Shonts, Tatsuo Kawai, Waichi Wong, Suxiao Yang, Julien Zuber, Yufeng Shen and Megan Sykes

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### Tolerating transplant

Transplant rejection remains a formidable barrier to successful organ transplantation. Recent advances, such as combined kidney and bone marrow transplantation (CKBMT), hint that rejection can be overcome by the induction of immune tolerance. Now, Morris *et al.* have developed a way to track T cells to determine how this tolerance works. They used high-throughput T cell receptor sequencing to find donor-reactive T cells before transplant and then tracked these clones after CKBMT. These donor-reactive T cells were reduced in CKBMT patients who achieved tolerance but not in a CKBMT patient who failed to achieve tolerance or in recipients of conventional, nontolerizing transplant protocols. These data suggest that clonal deletion is a mechanism of graft tolerance after CKBMT in humans.

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