

Origin of Enriched Regulatory T Cells in Patients Receiving Combined Kidney–Bone Marrow Transplantation to Induce Transplantation Tolerance

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We examined tolerance mechanisms in patients receiving HLA-mismatched combined kidney–bone marrow transplantation (CKBMT) that led to transient chimerism under a previously published nonmyeloablative conditioning regimen (Immune Tolerance Network study 036). Polychromatic flow cytometry and high-throughput sequencing of T cell receptor-β hypervariable regions of DNA from peripheral blood regulatory T cells (Tregs) and CD4 non-Tregs revealed marked early enrichment of Tregs (CD3⁺CD4⁺CD25^{high}

CD127^{low}Foxp3⁺) in blood that resulted from peripheral proliferation (Ki67⁺), possibly new thymic emigration (CD31⁺), and, in one tolerant subject, conversion from non-Tregs. Among recovering conventional T cells, central memory CD4⁺ and CD8⁺ cells predominated. A large proportion of the T cell clones detected in posttransplantation biopsy specimens by T cell receptor sequencing were detected in the peripheral blood and were not donor-reactive. Our results suggest that enrichment of Tregs by new thymic emigration and lymphopenia-driven peripheral proliferation in the early posttransplantation period may contribute to tolerance after CKBMT. Further, most conventional T cell clones detected in immunologically quiescent posttransplantation biopsy specimens appear to be circulating cells in the microvasculature rather than infiltrating T cells.

Abbreviations: BMT, bone marrow transplantation; CKBMT, combined kidney–bone marrow transplantation; FCM, flow cytometry; IL, interleukin; PBMC, peripheral blood mononuclear cell; RTE, recent thymic emigrant; TCR, T cell receptor; Treg, regulatory T cell; TSDR, regulatory T cell–specific demethylated region

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Introduction

Induction of mixed chimerism by nonmyeloablative conditioning followed by bone marrow transplantation (BMT) is a powerful means of inducing allograft tolerance in animal models (1). Our group has previously demonstrated long-term acceptance of HLA-mismatched renal allografts without maintenance immunosuppression (>5 years) in seven of 10 patients after combined kidney–bone marrow transplantation (CKBMT). Four subjects remain free of immunosuppression for longer than 6–13 years, while three required reinstitution of immunosuppression after 6–7 years because of recurrent disease or chronic rejection (2). Transplantation tolerance induced with durable mixed chimerism is associated with central deletion of donor-reactive T cells during thymic maturation (1). However, in the patients undergoing CKBMT, multilineage donor chimerism was short-lived (≤ 21 days) (3,4), suggesting a role for other tolerance mechanisms. In these patients, we previously observed a striking enrichment of CD4⁺CD25⁺

cells and a significant increase in CD4⁺CD25^{high}CD127^{low} cells at 6 months (5). Here, we demonstrate marked enrichment of CD3⁺CD4⁺CD25^{high}CD127^{low}Foxp3⁺ regulatory T cells (Tregs) very early after CKBMT and provide evidence that these cells originate both from the thymus and from peripheral expansion. T cell receptor (TCR) sequencing of circulating Tregs reflected the early enrichment identified via flow cytometry (FCM) and enabled tracking of Treg clones pretransplantation and posttransplantation. TCR sequencing demonstrated that many T cell clones detected within allograft biopsy specimens were also detectable in the peripheral blood and very few were donor-reactive. CKBMT is thus associated with marked changes in both regulatory and effector cells, which might be involved in the tolerance-inducing potential and complications of CKBMT, respectively.

Patients and methods

Study protocol

All studies were performed with institutional review board approval. Five patients treated according to the Immune Tolerance Network study 036 regimen were included in this study; the protocol and results have been reported (2). Patients 1, 2, and 4 successfully discontinued immunosuppression in the first year, and allograft function has remained stable for >6 years. Patient 3 lost the allograft due to thrombotic microangiopathy. In patient 5, the graft was rejected after discontinuation of immunosuppression (2, 6).

Flow cytometry

Polychromatic FCM was performed by using an LSR II flow cytometer (BD Biosciences, San Jose, CA), as described earlier (4) and detailed in Supplemental Methods.

Analysis of demethylation status of Treg-specific demethylated region

Genomic DNA was isolated with use of the DNeasy Blood & Tissue Kit (Qiagen, Germantown, MD). The protocol for cultured cells was followed. Bisulfite treatment (7) and analysis of demethylation status (8) were performed at Epiontis GmbH (Berlin, Germany) as described (see Supplemental Methods for details). In female subjects, an adjustment by a factor of 2 was made to compensate for obligate Barr body X chromosome methylation.

T cell culturing from renal allograft biopsy specimens

Protocol kidney biopsy specimens were obtained at 6, 12, and 24 months, and one-half of a core was digested to a single-cell suspension and cultured with anti-CD3 antibody (OKT3, 90 ng/mL), interleukin (IL)-2 (100 U/mL), irradiated allogeneic Epstein-Barr virus-transformed B cells from a healthy donor, and peripheral blood mononuclear cells (PBMCs) from multiple healthy donors, with or without rapamycin (1 μg/mL), to prevent the expansion of non-Tregs while allowing the expansion of Tregs (9). Expanded cell lines were further cultured with IL-2 alone. Expanded lines were phenotyped (CD4, CD8, CD25, CD127, Foxp3) and cryopreserved.

T cell repertoire β-chain length distribution analysis

Methods for RNA isolation, RT-PCR, spectratyping, and data analysis are detailed in the Supplemental Methods section.

High-throughput TCR β-chain sequence analysis

Genomic DNA was isolated from sorted Tregs, CD4 non-Tregs, CD8s, available biopsy specimens, and cell lines pooled from each subject by time point using the Qiagen DNeasy Blood & Tissue Kit. DNA was frozen at -20°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 (Adaptive Biotechnology, Seattle, WA) and analyzed as described (10).

For analysis of circulating Tregs, CD3⁺CD4⁺CD127^{low}CD25^{high} Tregs were sorted from frozen PBMCs after thawing and overnight resting in 200 units/mL IL-2 (Figure 5A). Simultaneously, CD4 non-Treg and CD3⁺CD4⁻ (CD8) populations were sorted. We defined Treg clones as those detected in sorted Treg populations at more than twice the frequency of those in both the CD8 and CD4 non-Treg sorted populations. Clones detected in sorted Treg populations that did not meet these criteria were considered to reflect sorting error. All sorted Treg clones from all time points were pooled to define the Treg clone set for which samples from a given patient were interrogated for the analyses shown in Figure 5B and Table S1.

Genomic DNA was extracted from protocol biopsy specimens frozen in Optimal Cutting Temperature compound, after thawing and extensive washing with PBS, by using the Qiagen DNeasy Blood & Tissue Kit and subjected to the Adaptive Immunoseq TCR sequencing platform. Clone sets were compared with sorted CD4 and CD8 blood samples and Treg clone sets. TCR clones identified from the pooled cell lines and biopsy specimens were compared with the previously defined TCR sequences of donor-reactive clones (10). Computational and statistical analyses were performed as described previously (10).

Statistical analysis

Statistical analysis was performed by using Microsoft Excel (Microsoft Corporation, Redmond, WA) and GraphPad Prism (GraphPad Software, La Jolla, CA). A two-tailed paired t-test was applied for comparisons of data at different time points, except for analysis of natural killer cells, where a two-tailed, two-sample equal variance t-test was applied, because pretransplantation data were not available for patient 1.

Results

Enrichment for effector/memory T cells early posttransplantation

Lymphocyte recovery in these subjects has been reported (4), including marked T cell lymphopenia immediately after transplantation (0–12 cells/μL by day 10), a transient increase in counts between weeks 2 and 3, and a subsequent decline that was followed by gradual T cell recovery in all patients (4). There was an initial increase in CD4:CD8 ratio that peaked on day 7, followed by inverted CD4:CD8 ratios by day 14 (4).

We further analyzed expression of CD45RA, CD45RO, and CD62L on CD4⁺ and CD8⁺ cells (Figures 1A–B and 1C–E, respectively). CD4⁺ cells showed a relative reduction of naïve cells on day 14 compared with pretransplantation (defined as CD45RA⁺, $p = 0.028$) (Figure 1A) with a corresponding increase in CD45RO⁺CD4⁺ cells ($p = 0.016$) (Figure 1B). Changes in proportions of CD8⁺ naïve (CD45RA⁺CD62L⁺) (Figure 1C), effector

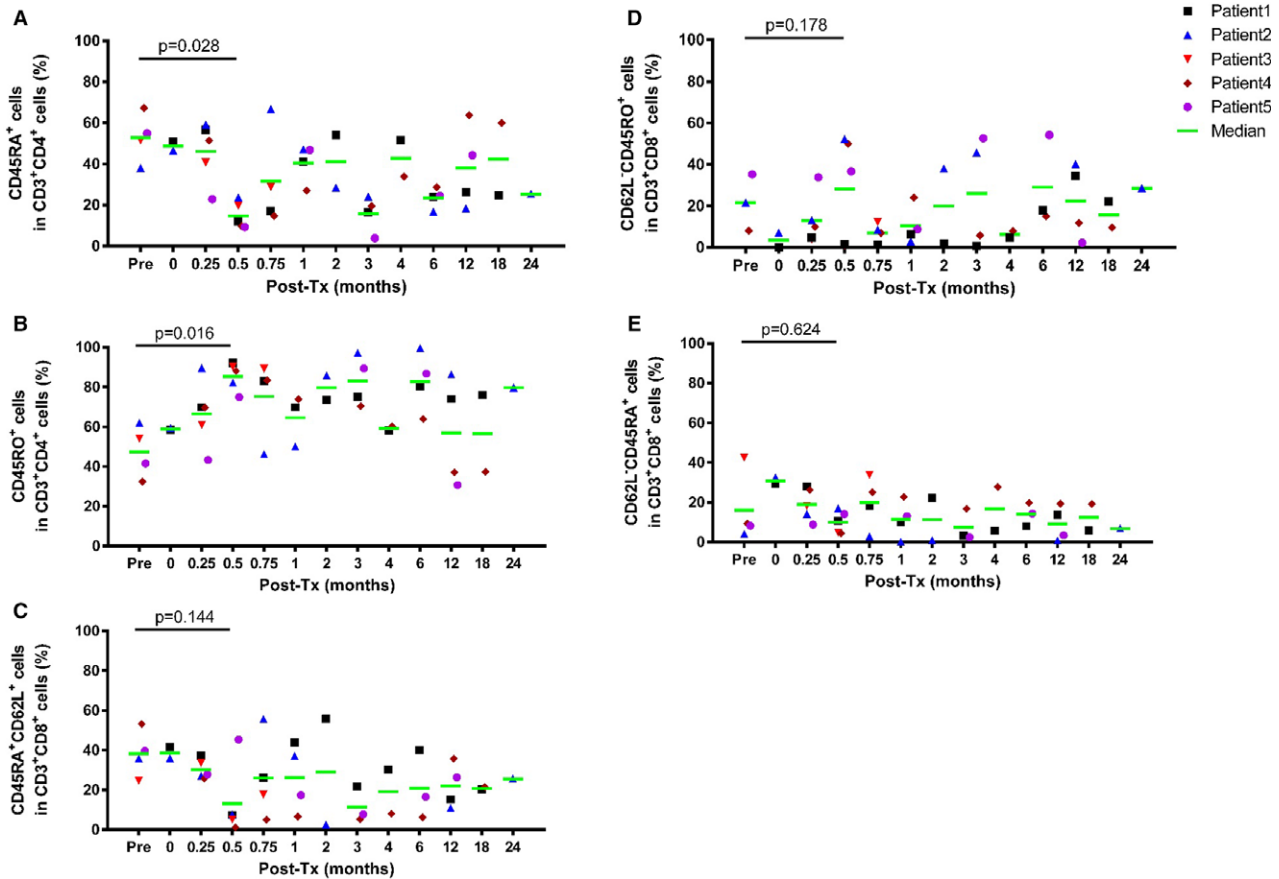


Figure 1: Evolution of naive and memory CD3⁺CD4⁺ and CD3⁺CD8⁺ subsets following CKBMT. (A) The percentages of CD45RA⁺ (naive CD4⁺ T cells) and (B) CD45RO⁺ (memory CD4⁺ T cells) in CD3⁺CD4⁺ cells. (C) CD45RA⁺CD62L⁺ (naive CD8⁺ T cells), (D) CD45RO⁺CD62L⁺ (effector memory CD8⁺ T cells), (E) CD45RA⁺CD62L⁻ (effector memory RA CD8⁺ T cells) in CD3⁺CD8⁺ cells are depicted for patients 1–5.

memory (CD62L⁻CD45RO⁺) (Figure 1D) and effector memory RA cells (defined as CD62L⁻CD45RA⁺) (Figure 1E) were variable and did not achieve statistical significance.

Activation of CD4 and CD8 lymphocytes early posttransplantation

Both CD4⁺ and CD8⁺ populations showed evidence for activation early after CKBMT. CD4⁺ cells upregulated HLA-DR and CD95 at day 14 ($p = 0.007$ and $p = 0.006$, respectively) (Figure 2A,B), and CD8⁺ cells upregulated CD25 at day 7 ($p = 0.031$) (Figure 2C). Duration and peak levels of HLA-DR (among CD4⁺ cells) and CD25 upregulation (in CD8⁺ cells) varied substantially between patients and persisted for up to 6 months (Figure 2A,C).

Enrichment for Tregs

Percentages of CD25^{high}CD127^{low}Foxp3⁺ T cells were dramatically increased as a proportion of CD3⁺CD4⁺ cells in the blood by 1–3 weeks after transplantation compared with pretransplantation levels ($4.9 \pm 1.4\%$). Percentages of CD25^{high}CD127^{low}Foxp3⁺ cells peaked at

71.8% (day 14), 52.7% (day 58), 42.9% (day 14), 70.0% (day 14), and 15.9% (day 14) for patients 1–5, respectively ($p = 0.028$ at day 14) (Figure 3A). The enrichment persisted for 12, 6, 1, 4, and 6 months in patients 1–5, respectively (Figure 3A). Although absolute numbers declined initially from the conditioning treatment, there was a partial recovery or even an increase in absolute numbers of CD3⁺CD4⁺CD25^{high}CD127^{low}Foxp3⁺ T cells in the blood of all CKBMT recipients compared with pretransplantation values by 2–3 weeks posttransplantation (Figure 3B). In contrast to this early recovery of Tregs, conventional CD3⁺CD4⁺ cells recovered slowly (Figure 3C). These data suggest that there was either selective sparing, expansion, or generation of CD3⁺CD4⁺CD25^{high}CD127^{low}Foxp3⁺ Tregs early after conditioning and CKBMT.

As the stability of regulatory function is related to the demethylation status of the Treg-specific demethylated region (TSDR) of Foxp3 (TSDR-Foxp3), we determined methylation status of TSDR-Foxp3 before and after CKBMT (Figure 3D). In patients 1–3, the marked enrichment in CD3⁺CD4⁺CD25^{high}CD127^{low}Foxp3⁺ T cells at 6 months

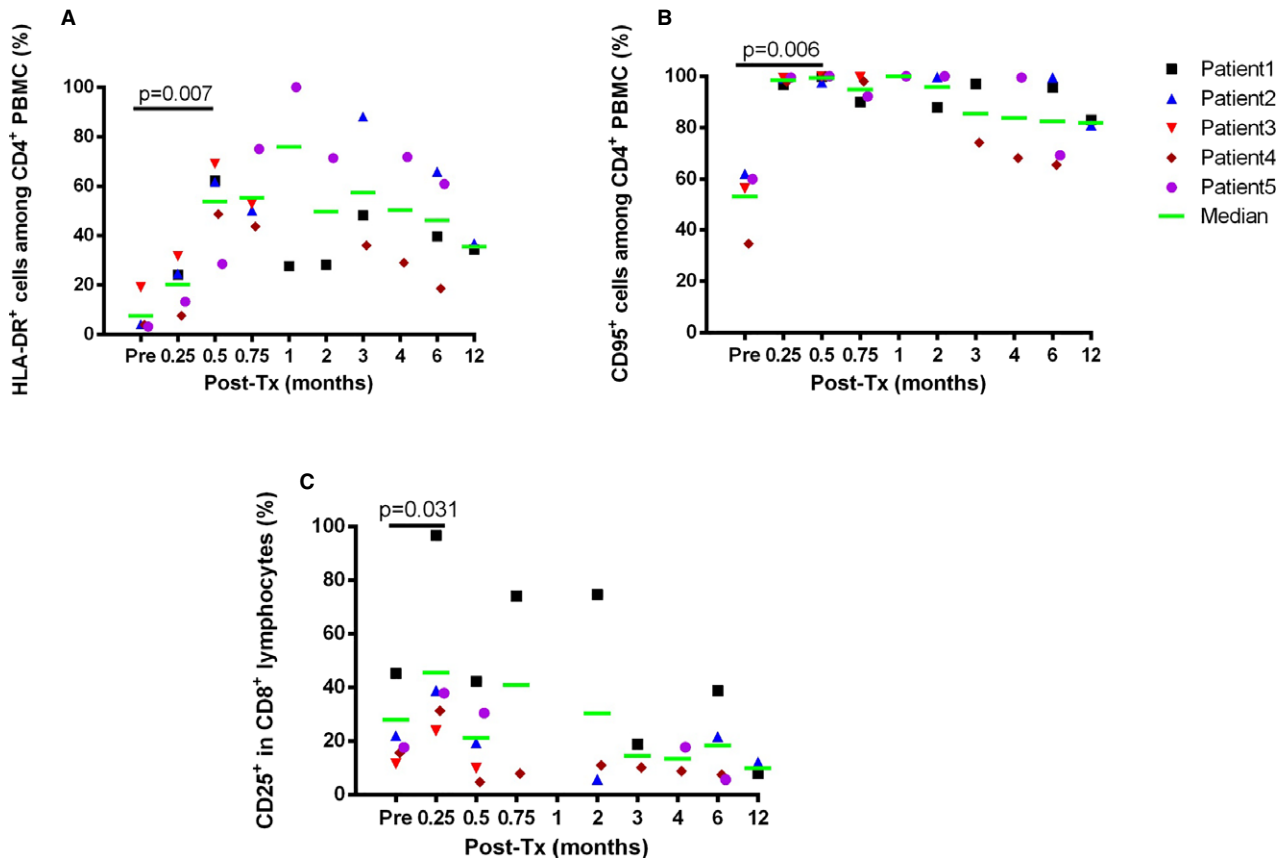


Figure 2: Evolution of activation markers in CD4 and CD8 cells following CKBMT. (A) The percentages of HLA-DR⁺ cells among total CD4⁺ cells are depicted for patients 1–5. (B) The percentages CD95⁺ in total CD4⁺ cells are depicted for patients 1–5. (C) The percentages of CD25⁺ cells among total CD8⁺ cells are depicted for patients 1–5.

(patients 1 and 2) and 3 weeks (patient 3), respectively (Figure 3A), was accompanied by an increased percentage of demethylated TSDR-Foxp3 (Figure 3D). In contrast, patients 4 and 5 no longer showed enrichment of CD3⁺CD4⁺CD25^{high}CD127^{low}Foxp3⁺ Tregs at 6 months post-CKBMT (Figure 3A) and, consistently, had no increase in percentage of demethylated TSDR-Foxp3 (Figure 3D).

Phenotypic analysis of Tregs

Further phenotypic analysis was performed using markers for activation and naïve/memory phenotype. In all three tolerant patients, patients 1, 2, and 4, there was an increase in the proportion of CD45RA⁺ (naïve or resting) Tregs at 1 week compared with pretransplantation (Figure 4A), whereas patients 3 and 5 showed reduced percentages of CD45RA⁺ Tregs in the same period. By 2 weeks posttransplantation, most Tregs were CD45RA⁻ in all subjects, and this phenotype predominated thereafter.

We evaluated the expression of CD31, a marker of recent thymic emigrants (RTEs) (11), and Ki-67, a marker of proliferating cells (12) (Figure 4B,C, respectively). At 7 days posttransplantation, RTEs increased among Tregs compared with pretransplantation in three of five patients (patients 1, 2, and 3)

($p = 0.189$) (Figure 4B). At the same time, percentages of Ki-67⁺ Tregs also peaked and increased compared with pretransplantation levels in five of five patients ($p = 0.032$) (Figure 4C). Among non-Treg CD4⁺ T cells, CD31 expression increased compared with pretransplantation in two of five patients and did not differ strikingly from percentages among Tregs (Figure S1a, Figure 4B). A marked early increase in Ki-67 expression was also detected among CD8 cells (defined as CD3⁺CD4⁻ PBMCs) and in non-Treg CD4⁺ T cells (defined as CD3⁺CD4⁺Foxp3⁻ PBMCs (Figure S1b and S1c, respectively).

Further, there was a significant increase in the expression of HLA-DR among CD3⁺CD4⁺CD25^{high}CD127^{low}Foxp3⁺ T cells at 2 weeks posttransplantation ($p = 0.011$) (Figure 4D). Collectively, these data suggest that the early recovery of Tregs in patients receiving this regimen reflected a combination of Treg expansion with a possible contribution of migration from the thymus.

High-throughput TCR sequencing of Tregs

We performed high-throughput TCR β CDR3 sequencing on genomic DNA extracted from sorted pretransplantation and posttransplantation (CD3⁺CD4⁺CD127^{low}CD25^{high}) Tregs (Figure 5A). Because of the small size of the Treg

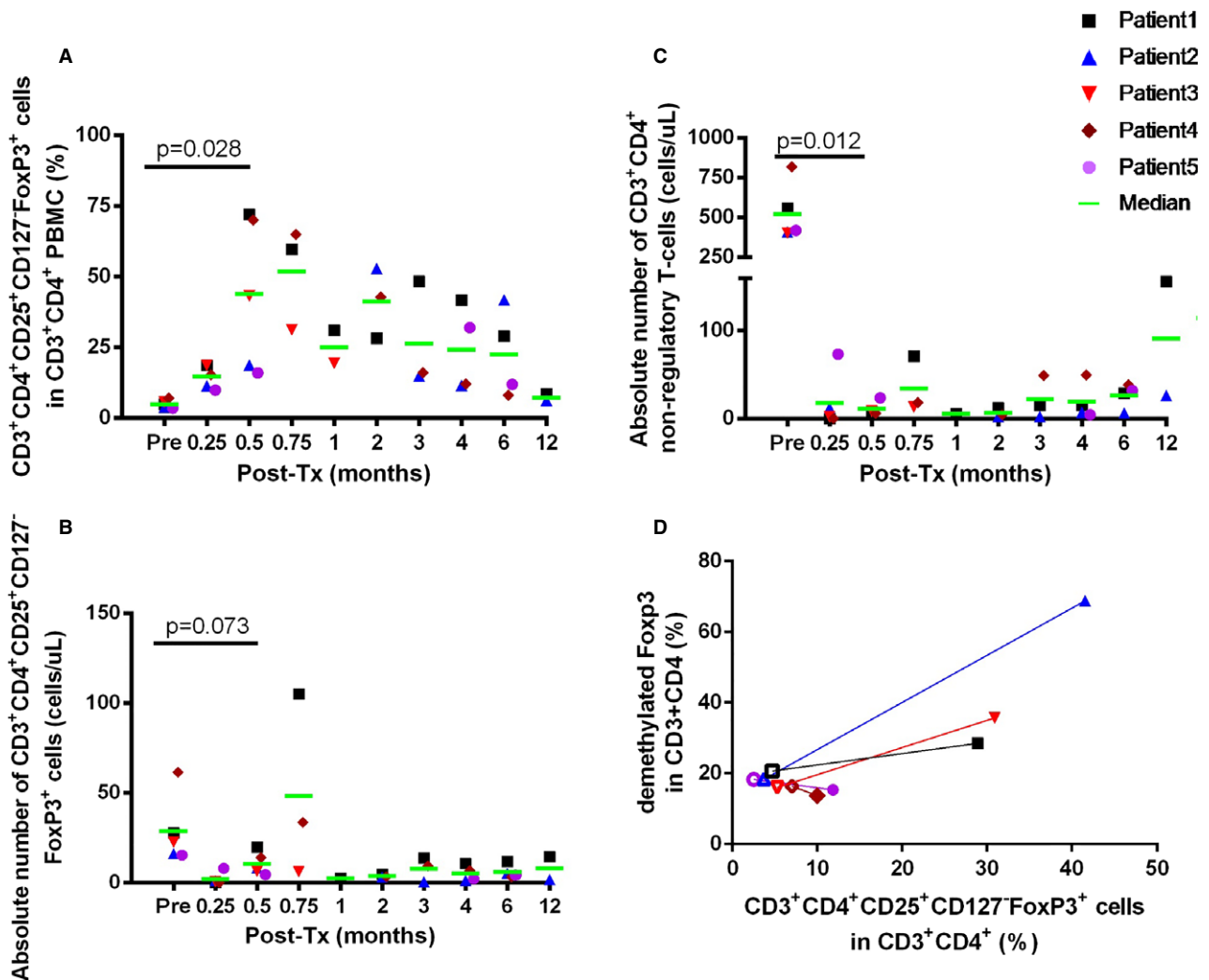


Figure 3: Enrichment of Tregs following CKBMT. (A) Percentages of CD4⁺CD25⁺CD127⁻Foxp3⁺ cells in CD3⁺CD4⁺ cells are shown for patients 1–5. (B) Absolute number of CD4⁺CD25⁺CD127⁻Foxp3⁺ cells in the peripheral blood (cells/μL) are shown for patients 1–5. (C) Absolute number of nonregulatory CD3⁺CD4⁺ cells in the peripheral blood (cells/μL) are shown for patients 1–5. (D) Correlation plot for CD4⁺CD25⁺CD127⁻Foxp3⁺ cells in CD3⁺CD4⁺ cells and demethylated Foxp3 in CD3⁺CD4⁺ cells are depicted for patients 1–5. Open symbols are values pretransplantation (before conditioning) for patients 1–5, while closed symbols represent values at time day 21 for patient 3 and day 180 for patients 1, 2, 4, and 5.

population, sufficient cell numbers were limited to a few time points in patients 1, 2, 4, and 5; however, hundreds or thousands of unique clones were identified in most samples (Table 1). We then interrogated circulating CD4 T cells at various times for these Treg clones. Figure 5B shows the cumulative frequency of all Treg clones identified in the circulating total CD4 T cell populations: these mirror the FCM findings, showing enrichment of Tregs in all patients at 6 months posttransplantation compared with pretransplantation, most strikingly in patient 2. Treg enrichment in tolerant patients 1, 2, and 4 did not persist after 12 months posttransplantation, consistent with the FCM results shown in Figure 3.

TCR sequencing enabled interrogation of pretransplantation cell populations for the Treg clones detected

posttransplantation (Figure 5C). Some, but not all, of the Treg clones found at 6 months posttransplantation were detected in the CD4 pretransplantation population for each of the patients, with the greatest overlap seen in patient 4's 6-month sample. While small numbers of Treg clones were identified as non-Tregs pretransplantation for patients 1, 4, and 5, the overall low numbers of such clones do not point to prominent induction of Tregs from non-Tregs present before transplantation (Figure 5C). For patient 2, a similar pattern was seen at 6 months posttransplantation; however, a remarkably high number of Treg clones detected at 12 months in this patient appeared to be induced Tregs that were detectable in the non-Treg population pretransplantation (Figure 5C).

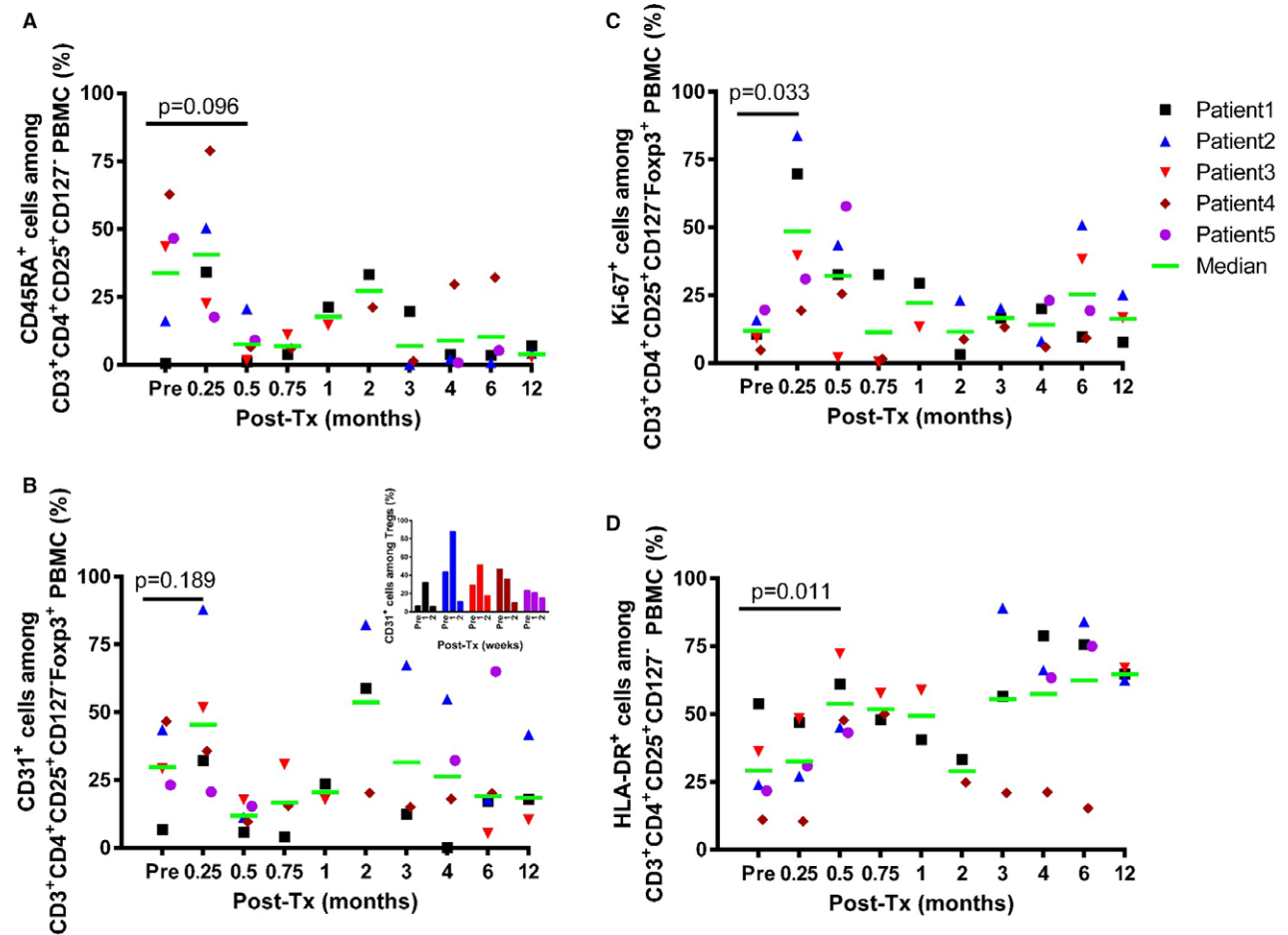


Figure 4: Treg phenotype following CKBMT. (A) The percentages of CD45RA⁺ cells in CD4⁺CD25⁺CD127⁻Foxp3⁺ cells in CD3⁺CD4⁺ cells are shown for patients 1–5. (B) The percentages of CD31⁺ cells in CD4⁺CD25⁺CD127⁻Foxp3⁺ cells in CD3⁺CD4⁺ cells are shown for patients 1–5. The inset shows the percentage of CD31⁺ cells in CD4⁺CD25⁺CD127⁻Foxp3⁺ cells from the pretransplantation time point until week 2 posttransplantation. (C) The percentages of Ki-67⁺ cells in CD4⁺CD25⁺CD127⁻Foxp3⁺ cells in CD3⁺CD4⁺ cells are shown for patients 1–5. (D) The percentages of HLA-DR⁺ cells in CD4⁺CD25⁺CD127⁻Foxp3⁺ cells in CD3⁺CD4⁺ cells are shown for patients 1–5.

Analysis of intra-graft T cells

Consistent with the detection of FoxP3 in long-term protocol biopsy specimens (3), we hypothesized that intra-graft Tregs might play a role in long-term tolerance. Therefore, we analyzed TCRs in serial posttransplantation biopsy specimens. The pathologic biopsy data are summarized in Table S2: in general, lymphocyte infiltrates were absent or scant, except in patient 3 at the time of graft loss and in patient 5 at 12 months, whose biopsy contained infiltrates consistent with treated acute cellular rejection (Table S2). We initially performed spectratyping on biopsy specimens from patient 2 at day 0 and at 6, 12, and 24 months posttransplantation. While few Vβ families were identified on day 0, the posttransplantation biopsy specimens revealed polyclonal repertoires (Figure S2). The absence of most Vβ in the naïve donor graft biopsy specimens may reflect the *ex vivo* perfusion performed before biopsy specimens were obtained, which preceded

reperfusion, resulting in purging of T cells present in the microcirculation at the time of biopsy.

To investigate the T cell repertoire of intra-graft T cells in greater depth, we performed high-throughput TCRβ CDR3 sequencing on the remaining biopsy specimens for patients 1, 4, and 5 and compared the data with previously generated sequence databases of pretransplantation and posttransplantation peripheral blood CD4 and CD8 T cells and donor-specific clones as described in Morris et al (10) and with the TCR sequences of circulating Tregs. Hundreds or thousands of unique T cell clones were identified in each biopsy specimen, including a mixture of CD4 and CD8 clones, as defined by their identification in a sorted circulating CD4 or CD8 T cell population (Table 2). In patients 1 and 4, who were tolerant, a high proportion (20–45% in CD4s, 11–35% in CD8s) of clones in the biopsy specimens were also detected in the pretransplantation

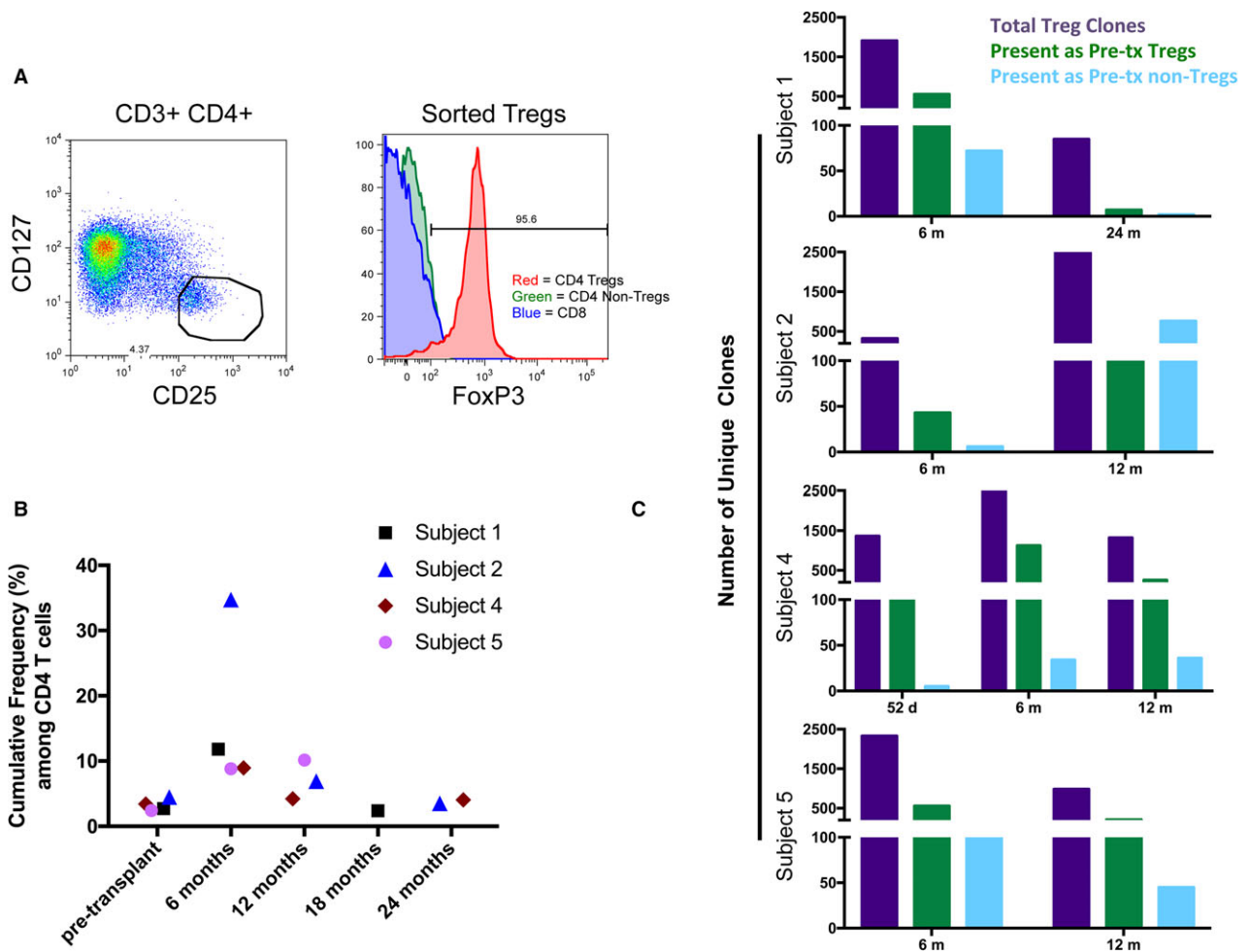


Figure 5: Analysis of Treg repertoire following CKBMT. (A) Illustrative sorting gates for isolation of regulatory T cells (Tregs) ($CD3^+CD4^+CD25^{high}CD127^+$). Intracellular FoxP3 staining confirms high percentage of FoxP3 expression among sorted Tregs not seen in CD4 non-Tregs and CD8 T cells from the same sample. (B) Cumulative frequency of Treg clones pooled from all Treg samples for a given patient found in the unstimulated CD4 samples. Clones defined by amino acid sequence of CDR3 with associated V β and J β genes. Unique clone numbers shown in Table S1. (C) Assessment of pretransplantation sorted non-Treg CD4 cells (blue bars) and Tregs (green bars) for TCRs detected in the indicated sorted posttransplantation Treg populations (purple bars) (nucleotide level analysis). Number of non-Treg:Treg CD4 clones identified pretransplantation: patient 1, 123 266:6,592; patient 2, 114 011:5,891; patient 4, 130 542:107 540; and patient 5, 179 754:24 925.

and/or posttransplantation circulating CD4 and CD8 T cell populations (Table 2). Further, the cumulative frequency within the peripheral circulation of clones also detected in the biopsy specimens was strikingly high, particularly among CD8s, accounting for >40% of the circulating CD8 population for at least one posttransplantation time point for each patient (Figure 6). The numbers of overlapping clones shared between biopsy specimens and PBMCs at the same time point were comparable to the overlap between PBMC populations sampled at different time points (Table S3), consistent with the possibility that T cells in biopsy specimens were largely within the microcirculation. More than 200 clones in each biopsy were identifiable as Tregs on the basis of sorted circulating Treg

populations (Table 2), by frequency, within three of four of the biopsies, accounting for 1.76%, 7.12%, and 1.46% of clones for subjects 1 (6 months posttransplantation), 4 (6 months posttransplantation), and 5 (12 months posttransplantation), respectively.

The availability of alloreactive TCR sequencing data on our subjects allowed us to examine the biopsy data for previously defined donor-reactive TCRs (10). Although some donor-reactive CD4 and CD8 clones were identified in each of the biopsy specimens (Table 2), they accounted for <1.5% by frequency of the biopsy TCR clones. Additionally, while the CD4:CD8 ratios of pretransplantation-detected clones found both in the

Table 1: T cell clones from sorted peripheral blood T regulatory cells

	Template count	Unique clones
Patient 1, pretransplantation	10 184	6592
Patient 1, 6 months posttransplantation	3243	1909
Patient 1, 24 months posttransplantation	107	85
Patient 2, pretransplantation	7425	5891
Patient 2, 6 months posttransplantation	466	322
Patient 2, 12 months posttransplantation	10 681	4292
Patient 4, pretransplantation	114 915	107 540
Patient 4, 52 days posttransplantation	1526	1365
Patient 4, 6 months posttransplantation	8928	5695
Patient 4, 12 months posttransplantation	1749	1323
Patient 5, pretransplantation	36 170	24 925
Patient 5, 6 months posttransplantation	4196	2329
Patient 5, 12 months posttransplantation	1647	983

Template count refers to the calculated number of productive T cell receptors sequenced.

circulation and within the biopsy specimen were similar (>1:1 for all patients), this ratio was markedly altered for donor-reactive clones found only within the 12-month posttransplantation biopsy specimen of the rejector patient 5 (9 CD4:17 CD8) (Table 2). This finding is consistent with the notion that clones in tolerated grafts largely represented circulating T cells, whereas infiltrating donor-reactive T cells, enriched for CD8 clones, may have persisted despite treatment and contributed to the clones detected in patient 5's allograft, which was ultimately rejected.

Table 2: Overlapping T cell clones from biopsy samples compared with peripheral blood mononuclear cells (PBMCs) pretransplantation and posttransplantation, donor-reactive T cell repertoire as described in Morris et al 2015 (10), and circulating T regulatory cell repertoires

Biopsy specimens	Patient 1		Patient 4	Patient 5
	6 months	12 months	6 months	12 months
Total unique clones	13 492 (<i>10 751</i>)	464 (<i>357</i>)	3020	15 459 (<i>12 375</i>)
Pretransplantation CD4 PBMCs (in blood)	<i>1164</i>	<i>45</i>	667	<i>257</i>
Pretransplantation CD8 PBMCs (in blood)	<i>750</i>	<i>39</i>	594	<i>176</i>
All posttransplantation CD4 PBMCs (in blood)	<i>1620</i>	<i>91</i>	1237	<i>1523</i>
All posttransplantation CD8 PBMCs (in blood)	<i>959</i>	<i>65</i>	1009	<i>463</i>
Pretransplantation or posttransplantation CD4 PBMCs(in blood) (% of total unique clones)	<i>2170 (20.18%)</i>	<i>107 (29.97%)</i>	1359 (45%)	<i>1649 (13.3%)</i>
pretransplantation or posttransplantation CD8 PBMCs(in blood) (% of total unique clones)	<i>1219 (11.34%)</i>	<i>69 (19.33%)</i>	1067 (35.33%)	<i>554 (4.48%)</i>
donor-reactive CD4s (defined pretransplantation) (% = sum frequency)	<i>126 (1.2%)</i>	<i>1 (0.28%)</i>	42 (1.39%)	<i>9 (0.06%)</i>
Donor-reactive CD8s (defined pretransplantation) (% = sum frequency)	<i>30 (0.3%)</i>	<i>0</i>	21 (0.69%)	<i>17 (0.11%)</i>
T regulatory clones (pooled peripheral blood Treg clones) (% = sum frequency)	237 (1.76%)	5 (1.08%)	215 (7.12%)	225 (1.46%)
Cell line overlap (same time point)	9	61	7	6

Available posttransplantation time point samples for each patient are pooled in the "all posttransplantation" rows. Italics indicate samples analyzed on level of amino acid plus V β and J β genes; all others analyzed on nucleotide level.

Because lymphocytes were sparse in protocol biopsy specimens of tolerated allografts (3) (Table S2), we initially developed a technique for polyclonally expanding T cells from small pieces of core biopsy specimens, generating numerous cell lines. Expression of host-specific class I HLA antigens revealed that these were of recipient origin (Figure S3). In addition, these cell lines were characterized by FCM for expression of CD3, CD4, and CD8 (Table S4), with some further analyzed for Treg markers. The phenotype of some lines was consistent with a Treg lineage (Table S4, Figure S4). TCR sequencing results from pooled cell lines demonstrated oligoclonality (Table S5), and there was little overlap with clones detected in biopsy specimens or the donor-reactive repertoire (Table 2).

Discussion

The mechanisms of tolerance achieved via HLA-haploidentical CKBMT associated with transient chimerism are not fully understood (2–5). In some, but not all, CKBMT recipients, suppressive tolerance mechanisms could be implicated in donor-specific hyporesponsiveness/unresponsiveness at 6 months to 1 year, but no evidence for suppressive mechanisms in the persisting donor-specific unresponsiveness was seen at later time points (≥ 18 months) (5). Using high-throughput sequencing of TCR β CDR3 regions and a pretransplantation mixed lymphocyte reaction to identify donor-reactive clones, we recently obtained evidence for gradual deletion of these clones over time only in tolerant CKBMT recipients and not

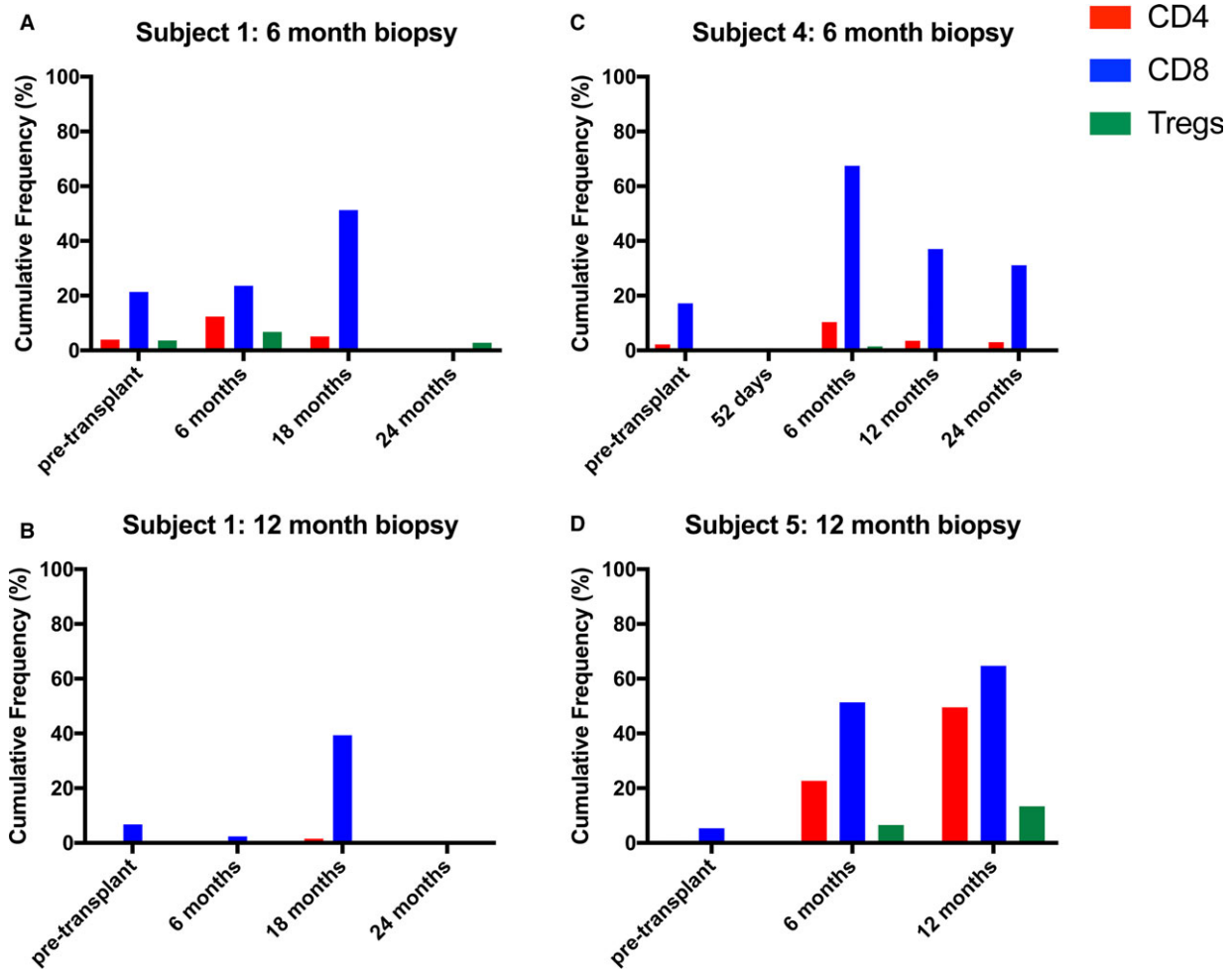


Figure 6: Cumulative frequency *in circulation* of clones found both in the indicated biopsy specimen and in unstimulated CD4 (red), CD8 (blue), and Treg (green) TCR populations in circulating peripheral blood mononuclear cells at the indicated times (clones defined by amino acid sequence of CDR3 with associated V β and J β genes).

in those who failed tolerance or received conventional transplants (10).

We have previously reported enrichment of Tregs in recipients of allogeneic BMT with similar nonmyeloablative conditioning (13) and in the first series of CKBMT patients (5). However, these studies were performed on frozen samples with only a limited number of time points available for evaluation. Tregs were also implicated by high levels of Foxp3 expression relative to effector cell RNA in the kidneys of CKBMT patients (3). Here, we report that after CKBMT, Tregs are relatively spared from siplizumab (MEDI-507)-based, T cell-depleting conditioning therapy and provide evidence that new emigrants from the thymus as well as peripheral expansion may contribute to marked Treg enrichment among peripheral CD4 T cells early posttransplantation. While both Treg and non-Treg CD4 populations included CD45RA⁺ and CD31⁺ cells in the first weeks posttransplantation,

consistent with a wave of new thymic emigration, we cannot rule out the possibility that RTE CD4 T cells are spared from depletion by the conditioning regimen and have not yet undergone sufficient lymphopenia-driven expansion to convert to the memory phenotype by 1–2 weeks. Notably, the period of Treg enrichment corresponds to the early period during which suppression could be implicated in the donor-specific unresponsiveness observed *in vitro* (5).

Although Foxp3 is a robust marker for murine Tregs, Foxp3 can be induced by activation in naïve human conventional CD4⁺ T cells (14). Tregs express high levels of CD25 (15, 16) and lack cell surface CD127 (IL-7 receptor α -chain) expression (17, 18). We used the combination of Foxp3, CD127, and CD25 to identify Tregs, which may be contaminated to a minor degree by nonregulatory Foxp3-expressing CD4⁺CD45RO⁺Foxp3^{low}CD127^{low/intermediate} effector cells (19). Stable and high Foxp3 expression is a

feature of natural (thymus-derived) Tregs and is required for suppressive function (20, 21), and its genetic locus is demethylated in stably Foxp3⁺ natural Tregs (19), in contrast to TGFβ-induced Tregs (22). Our TSDR demethylation analysis shows a strong correlation with the percentage of CD25⁺Foxp3⁺CD127^{low} cells among peripheral CD4⁺ cells, consistent with the interpretation that these represent expansions of natural Tregs.

Tregs include several subsets distinguishable by their expression of CD45RA and CD45RO (23). Tolerant patients showed a significant increase in the proportion of CD45RA⁺ Tregs early posttransplantation, which may be naïve or resting Tregs, which express low levels of Foxp3 and express CD31, suggesting they include RTEs (19). Three of five CKBMT recipients showed an increase in CD31⁺ cells among Tregs in the early posttransplantation period, consistent with, but not definitively indicative, of a thymic origin. In mice, *de novo* intrathymic generation of antigen-specific Tregs occurred after peripheral injection of antigen under proinflammatory conditions (24). A similar phenomenon might occur in patients undergoing CKBMT in association with the inflammatory “engraftment syndrome” seen early posttransplantation, in which expanded recipient CD8⁺ T cells are detected in the glomeruli without a rejection pattern (25). The marked increase in Ki-67 expression suggests an important role for lymphopenia-driven peripheral expansion in the enrichment of Tregs in the immediate post-CKBMT period. It is possible that tissue-resident Tregs may also contribute to this enrichment, as suggested by studies in lymphopenic mice (26). The peripheral CD8⁺ T cells in these patients showed very early upregulation of CD25, even before BMT was administered, suggesting a marked effect of the conditioning itself. The studies presented here show that high levels of activated CD8⁺ T cells persisted for at least 1 week posttransplantation and in one case persisted for up to 6 months.

TCR sequencing enabled us to directly investigate whether the clones expanding posttransplantation were present pretransplantation. We validated that sorted CD4⁺CD127⁻CD25^{high} Tregs were FoxP3⁺ and, to ensure the clones we defined as Tregs were not sort contaminants, we removed from consideration clones that were detected at similar frequencies in sorted non-Treg populations. Strikingly, many of the Tregs found at 6 months posttransplantation were present pretransplantation. Our data did not suggest a strong component of Treg induction from non-Tregs detected pretransplantation, with the exception of patient 2 at 12 months. In all cases, Treg enrichment after CKBMT with this protocol appears to reflect relative sparing of preexisting Tregs compared with effector CD4 cells, combined with posttransplantation lymphopenia-driven expansion and *de novo* generation in the thymus. Human Tregs have been shown to be relatively resistant to cyclophosphamide (27), which,

along with siplizumab (13), may contribute to Treg enrichment. Rabbit antithymocyte globulin, which was used to treat engraftment syndrome in some patients, has been reported to enrich Tregs in humans (28).

Thymic irradiation in our conditioning regimen depletes preexisting thymocytes and makes space for *de novo* thymopoiesis. Consistent with the notion that an early wave of thymopoiesis may have followed this treatment, peripheral CD4⁺ T cells included substantial proportions of naïve (CD45RA⁺) cells at 1 week, and it was not until 2 weeks posttransplantation that these shifted largely to a CD45RO⁺ memory-type population, presumably due to lymphopenia-driven expansion. Studies in humanized mice have shown that naïve human T cells expand and convert to effector/memory phenotype when placed in a lymphopenic environment (29). Additionally, memory T cells may be relatively resistant to mAb-induced depletion, resulting in selective sparing and expansion from a small residual pool of peripheral T cells (30).

After antigenic stimulation, CD45RA⁺Foxp3^{low} Tregs become CD45RO⁺Foxp3^{high} “effector” Tregs, which are highly proliferative and strongly suppressive (31). HLA-DR identifies a terminally differentiated, highly suppressive subpopulation of effector Tregs (31, 32). The marked enrichment in CD4⁺CD45RO⁺Foxp3⁺CD127^{low}CD45RA⁻HLA-DR⁺ Tregs that we observed after CKBMT may reflect antigen- and/or lymphopenia-driven enrichment of these effector-type Tregs (33–39), consistent with the increased Ki67 expression among Tregs early posttransplantation. While we do not yet have an established method of identifying the donor-specific Treg repertoire to assess enrichment for these cells, our TCR tracking study suggested that antigenic pressure from the graft combined with lymphopenia promotes expansion of donor-specific T cells early after kidney transplantation (10).

High-throughput sequencing allowed assessment of the hypothesis that Tregs accumulated in the transplanted kidney, as preclinical and clinical studies leading to the CKBMT protocol suggested a role for the allograft itself in promoting tolerance (5, 13, 40). Long-term protocol biopsy specimens revealed absent or minimal lymphoid infiltrates (3). This absence of lymphoid infiltrates contrasts with observations in a murine model in which MHC-mismatched renal allografts are spontaneously accepted in a Treg-dependent fashion, where accumulations of Tregs is seen around the cortical arteries (41) (42). While some Treg clones were found within the biopsy specimens, the most abundant graft T cell clones were prominent in the circulation and were not donor-reactive; the extensive clonal overlap between biopsy specimens and circulating T cells is consistent with the degree of overlap expected from samples of the size tested from a single population. These findings and the contrasting absence of many Vβ families in pretransplantation biopsy specimens from avascular, perfused

kidneys support the notion that TCR sequences from the transplanted kidney largely reflect circulating cells within the kidney's microvasculature. However, our definition of donor-reactive clones only applies to those detected pretransplantation and our data do not rule out the possibility that new donor-specific clones that emerge from the thymus posttransplantation may be included in the biopsy specimens (10).

Our sequencing analysis suggested a lack of utility of generating T cell lines via polyclonal expansion to characterize graft-infiltrating T cells. Because of the paucity of lymphocytes in biopsy samples, we polyclonally expanded T cells from small biopsy specimens. However, TCR β sequencing of these cell lines revealed little overlap with clones identified directly from whole biopsy specimens and included many fewer clones, suggestive of selective clonal expansion rather than reflecting the diversity of the initial population.

In summary, our studies demonstrate enrichment for Tregs in the circulation early posttransplantation in CKBMT recipients and suggest that peripheral expansion, possibly in combination with early *de novo* generation in the thymus, may be largely responsible for this enrichment, which may play a significant role in the initial tolerance achieved with CKBMT and may contribute to the quiescent state that results in eventual deletion of donor-reactive T cells.

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Specific Contributions

B.Sprangers, S.D., T.S., and T.M. performed experiments, analyzed data, and wrote the manuscript. A.O. analyzed TCR sequencing data. S.L. coordinated sample acquisition, processed samples, and analyzed data. B.Shonts, J.Z., and R.S. performed analyses of the cell lines. H.M. analyzed data. F.I.P. and D.M.D. provided expert advice on and performed flow cytometry. E.Z.

participated in the design of the protocol for T cell expansion from protocol biopsies. S.O. performed TSDR analysis. V.S. performed and R.W. supervised and analyzed TCR spectratype analysis and sequencing. R.C. performed pathology analysis and provided specimens. T.K. designed and carried out the clinical protocol and patient care. L.T. participated in experimental design. M.S. participated in the design of the clinical protocol, participated in and oversaw design of all laboratory studies, data analysis and interpretation, and wrote the manuscript.

Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1: Expression of CD31 and Ki-67 in nonregulatory T cells.

Figure S2: Number of Vβ detected in biopsy samples at different time points.

Figure S3: Representative cell line sample for each patient.

Figure S4: Representative cell line with Treg phenotype.

Table S1: T cell clones defined as Tregs within pretransplantation and posttransplantation circulating sorted Treg T cell populations.

Table S2: Pathologic evaluation of allograft biopsy specimens in patients 1–5.

Table S3: Comparison of the number of unique clones shared between biopsy and PBMCs (same time point) versus PBMCs at disparate time points for patients 1, 4, and 5.

Table S4: CD4, CD8, and FoxP3 phenotyping of expanded intra-graft T cell line

Table S5: T cell clones from cultured cell lines compared to peripheral blood T cells pretransplantation and posttransplantation, donor-reactive TCR as described in Morris et al, 2015 (10), and circulating T regulatory cell repertoires.