REGULAR ARTICLE

blood advances

First-in-human phase 1 trial of induced regulatory T cells for graft-versus-host disease prophylaxis in HLA-matched siblings

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Key Points

- A 3 × 10^8/kg iTreg phase 1 dose was safely infused into adults receiving HLA-identical sibling PBSC transplantation.
- iTregs circulated up to 2 weeks without evidence of GVHD modulation in patients receiving CSA/MMF prophylaxis.

Human $CD4^+25^-$ T cells cultured in interleukin 2 (IL-2), rapamycin, and transforming growth factor β (TGFβ) along with anti-CD3 monoclonal antibody–loaded artificial antigen-presenting cells generate $FoxP3^+$ induced regulatory T cells (iTregs) with potent suppressive function. We performed a phase 1, single-center, dose-escalation study to determine the safety profile of iTregs in adults with high-risk malignancy treated with reduced-intensity conditioning and mobilized peripheral blood stem cells (PBSCs) from HLA-identical sibling donors. Sixteen patients were enrolled and 14 were treated (2 productions failed to meet desired doses). One patient each received 3.0 × 10^6/kg, 3.0 × 10^7/kg, and 3.0 × 10^8/kg iTregs with corresponding T-conventional-to-iTreg ratios of 86:1, 8:1, and 1:2. After 3 patients received 3.0 × 10^8/kg in the presence of cyclosporine (CSA) and mycophenolate mofetil (MMF) with no dose-limiting toxicities, subsequent patients were to receive iTregs in the presence of sirolimus/MMF that favors Foxp3 stability based on preclinical modeling. However, 2 of 2 developed grade 3 acute graft-versus-host disease (GVHD), resulting in suspension of the sirolimus/MMF. An additional 7 patients received 3.0 × 10^8/kg iTregs with CSA/MMF. In the 14 patients treated with iTregs and CSA/MMF, there were no severe infusional toxicities with all achieving neutrophil recovery (median, day 13). Of 10 patients who received 3.0 × 10^8/kg iTregs and CSA/MMF, 7 had no aGVHD, 2 had grade 2, and 1 had grade 3. Circulating $Foxp3^+$ iTregs were detectable through day 14. In summary, iTregs in the context of CSA/MMF can be delivered safely at doses as high as 3 × 10^8/kg. This trial was registered at www.clinicaltrials.gov as #NCT01634217.

Introduction

Adoptive transfer of thymus-derived regulatory T cells (Tregs; tTregs) can ameliorate acute graft-versus-host disease (GVHD; aGVHD) and autoimmunity in mice.[1,2] However, high Treg doses (~1:1 with donor T cells) are required to reliably prevent disease.[3-5] Historically, clinical application has been substantially hampered by low tTreg frequency and relatively poorer proliferation than T-conventional cells (Tcons) in ex vivo culture.[6] Although 10-fold more tTregs can be isolated from peripheral blood (PB) than umbilical cord blood (UCB), magnetic cell separation–enriched PB frequently contains $CD25^+$ T-effector cells (Teffs) or memory T cells that preferentially expand over tTregs in culture, resulting in a product with reduced or lost suppressor function.[7]

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The full-text version of this article contains a data supplement.

PB $CD4^+$ Tcons can be driven to a Treg phenotype and function in vitro or in vivo in the context of low-dose or tolerogenic antigen exposure, as occurs after allogeneic hematopoietic cell transplantation (HCT; allo-HCT), leading to the development of induced Tregs (iTregs) that contribute to GVHD amelioration.[8,9] In preclinical murine models, polyclonal or antigen-specific $CD4^+$ iTregs suppress murine allogeneic[10,11] and human xenogeneic GVHD.[12] Previously, we showed that potently suppressive human iTregs could be reliably generated in high numbers from $CD4^+25^-$ T cells by culturing in interleukin 2 (IL-2), rapamycin (rapa), and transforming growth factor β (TGFβ), along with artificial antigen-presenting cells (APCs; aAPCs) expressing the high-affinity Fc receptor, CD64, loaded with a mitogenic anti-CD3 monoclonal antibody. The resulting iTregs provided comparable GVHD suppression to tTregs in a xenogeneic murine model.[12]

In adult patients, granulocyte colony-stimulating factor–mobilized PB stem cells (PBSCs) are often the preferred graft source. Relative to UCB transplants used in our prior studies,[6,13] mobilized PBSCs contain 10- to 12-fold more $CD3^+$ T cells. Therefore, an alternate approach to UCB-derived tTregs was needed. Hence, we developed a manufacturing method that reliably produced large numbers of iTregs with potent suppressive function for testing in recipients of PBSC transplantation (PBSCT). Our aim was to study the safety and efficacy of iTregs in a homogenous population of patients receiving matched sibling PBSCT with the graft and iTregs procured from the same donor using 1 conditioning regimen. We chose a reduced-intensity conditioning (RIC) approach as it is more commonly used at our institution than myeloablative for adult patients with hematological malignancies. Here, we present the results of a first-in-human phase 1 dose-escalation trial testing the safety of iTregs in the context of standard GVHD prophylaxis.

Patients and methods

Study design

This was a phase 1, single-center, dose-escalation study of iTregs with an extension phase at the maximal tolerable (or achievable) dose (MTD) in adult patients with high-risk malignancy treated with RIC and HLA-identical sibling donor PBSCT. aGVHD immunoprophylaxis consisted of cyclosporine (CSA)/mycophenolate mofetil (MMF) initially with the plan to switch sirolimus for CSA based on preclinical data, suggesting a more permissive effect of sirolimus. The study used a fast-track design with 1 patient per iTreg dose cohort (3.0×10^6/kg, 3.0×10^7/kg, 3.0×10^8/kg, and 10.0×10^8/kg). An extension phase aimed to enroll a total of 10 patients at the MTD.

The primary objective of the phase 1 component was to determine the MTD. The primary objective of the extension phase was to determine the safety profile of iTregs at the MTD or highest achievable dose and sample size estimates for a phase 2 iTreg efficacy trial for prevention of grade 3-4 aGVHD by day 100. Outcomes were compared with a cohort of similar patients treated in the same manner but without iTreg infusion. Clinical and laboratory data were prospectively collected in the University of Minnesota Blood and Marrow Transplant Database and systematically analyzed as of April 2020.

The protocol was approved by the institutional review board at the University of Minnesota. All patients and donors signed institutional review board–approved informed consent in accordance with the Declaration of Helsinki.

Patient-inclusion criteria

Eligible patients were 18 to 75 years of age with high-risk malignancies (acute myelogenous leukemia, acute lymphocytic leukemia, chronic myelogenous leukemia, myelodysplastic syndrome, non-Hodgkin or Hodgkin lymphoma), requiring allo-HCT between June 2014 and September 2016, and deemed eligible to undergo RIC HLA-identical sibling PBSCT with acceptable organ function and Karnofsky ≥60%.[14] Patients were excluded for active uncontrolled infection, pregnancy or breastfeeding, or prior myeloablative HCT within 3 months of study enrollment.

Donor-inclusion criteria

Donors were eligible if they met the following criteria: 12 to 75 years of age, >40-kg body weight and in good health, and HLA-A, -B, -DRB1 identical sibling match to recipient. Donors were required to undergo a nonmobilized apheresis procedure on day −14 for iTreg production and a separate granulocyte colony-stimulating factor–mobilized apheresis collection for PBSCs on day 0 (and on day +1 if needed).

Transplant procedure

All patients received an RIC regimen consisting of cyclophosphamide 50 mg/kg IV on day −6, fludarabine 30 mg/m^2 IV on days −6 to −2, and a single fraction of total body irradiation 200 cGy on day −1.[14]

The iTregs were administered IV over 15 to 60 minutes on day 0 at least 4 hours before the PBSC infusion. MMF was discontinued on day +30 or 7 days after engraftment, whichever was later, if no active GVHD. Targeted CSA levels were 200 to 400 mg/L and target sirolimus levels were 3 to 12 mg/mL. CSA or sirolimus taper began at day 100 if no aGVHD occurred. Patients received standard supportive disease and transplant-related care including antibacterial, antiviral, and antifungal prophylaxis.[14]

iTreg manufacture

A nonmobilized apheresis product was obtained from an HLA-identical sibling donor on day −14. Enrichment of $CD4^+25^-$ cells was performed by a 2-step method using the CliniMACS device (Miltenyi Biotec, Auburn, CA). First, $CD25^+$ cells (including tTregs) were depleted from peripheral blood mononuclear cells (PBMCs) with directly conjugated anti-CD25 magnetic microbeads (Miltenyi Biotec) and the CliniMACS device (depl05). $CD4^+$ cells were purified from $CD25^-$ PBMCs using directly conjugated anti-CD4 magnetic microbeads (Miltenyi Biotec) and the CliniMACS device (possel). Gammagard S/D (immunoglobulin [human] therapy; Baxter, Deerfield, IL) was used to prevent nonspecific binding.

Purified $CD4^+25^-$ T cells were resuspended at 0.25×10^6 to 0.50×10^6 viable cells per mL in X-VIVO 15 (Lonza, Walkersville, MD) supplemented with 10% human AB serum, heat-inactivated (Valley Biomedical Products and Services, Inc, Winchester, VA), GlutaMAX (2 mM), and *N*-acetylcysteine (2 mg/mL) in a tissue-culture flask (37°C/5% CO_2). $CD4^+25^-$ T cells were stimulated with anti-CD3 monoclonal antibody (MACS GMP CD3 Pure; Miltenyi Biotec) loaded, irradiated, and K562 transduced with CD64 and CD86 (KT64/86) aAPCs[7,12,13] (produced and tested according to a Biologics master file submitted to the US Food and Drug Administration) at an aAPC-to-Treg ratio of 1:1. Cultures were supplemented with 300 IU/mL IL-2 (Proleukin; Novartis

Corporation, East Hanover, NJ) on day 0 and maintained at that concentration (based on total medium) for culture duration. rapa (Rapamune 100 ng/mL; Wyeth Pharmaceuticals, Collegeville, PA) and TGFβ1 (3 ng/mL; R&D Systems, Minneapolis, MN) were added on day 0 and with each culture feed. IL-2 was supplemented for the entire volume whereas sirolimus and TGFβ were supplemented only with new (higher) culture volume. Culture density was adjusted every 2 to 3 days to maintain 0.25×10^6 to 0.50×10^6 viable cells per mL On day 7, rapa/TGFβ iTregs were restimulated with KT64/86 cells at 1:1 (KT64/86 to viable cell). Samples were removed 2 to 3 days prior to final product processing for sterility testing. On day 14 ± 1, cultures were harvested, washed, and resuspended with a 5% human serum albumin solution for patient infusion. Lot-release criteria included: 7-aminoactinomycin D viability ≥70%, $CD4^+CD25^+$ ≥60%, $CD4^-/CD8^+$ cells ≤10%, Gram stain with "no organisms seen," and endotoxin <5 EU/kg patient weight. These studies, along with our previously published work,[12] adhere to the minimum information on Treg manufacture as previously described, specifically, expression of CD4, CD25, CD127, Foxp3, Helios, and methylation status of the *Foxp3* gene.[15]

Flow cytometry

Flow cytometric studies were performed by the use of standard procedures. In brief, human-specific antibodies for CD4 (RPA-T4), CD8 (RPA-T8), CD14 (MSE2), CD19 (HIB19), CD25 (M-A251), CD45RA (HI100), IL-10 (JES3-9D7), and IL-17 (eBio64CAP17) were purchased from eBioscience. Antibodies to interferon γ (4S.B3), tumor necrosis factor α (Mab11), IL-2 (MQ1-17H12), IL-4 (MP425D2), TIGIT (VSTM3), Lag-3 (11C3C65), CTLA4 (BNI3), HLA-DR (L243), programmed cell death 1 (PD-1; EH12.2H7), and inducible costimulator (ICOS; C398.4A) were from BioLegend. Staining for Foxp3 was performed with an anti-human Foxp3 Flow Kit (clone 249D; BioLegend) following the manufacturer's instructions. Flow data were acquired on an LSRII flow cytometer (BD Biosciences), and the analysis was performed with FlowJo software (TreeStar, Inc). To establish gates for $Foxp3^+$ and $Foxp3^{++}$ staining, as well CD39 staining, a single control source of known rapa/TGFβ iTregs, expanded CD4 T cells (non-Tregs), and UCB tTregs was generated prior to analysis of clinical products, which were then thawed and assayed concomitant with each clinical product.

Suppression assay

A carboxyfluorescein diacetate succinimidyl ester–based suppression assay was used in which human PBMCs were labeled with carboxyfluorescein diacetate succinimidyl ester according to the manufacturer's instructions (Invitrogen) and plated at 10^5 per well in 96-well U-bottom plates with graded titrations of expanded rapa/TGFβ iTregs (1:2 to 1:32 iTreg:PBMCs) in 200 μL of X-VIVO15 culture media (without rapa or TGFβ). Cells were stimulated with anti-CD3–conjugated Dynal magnetic beads (Invitrogen) at a 1:1 bead-to-PBMC ratio. On day 4, cells were harvested and stained with antibodies to CD8. Acquired data were analyzed by the use of the proliferation platform in FlowJo software and the percentage of inhibition of division index (a product of the percentage divided and proliferation index) is presented. As with the flow controls, a single banked sample of research-expanded rapa/TGFβ iTregs were run as a positive control for suppressive function. Suppression is reported for only 6 product samples, the remaining 8 were not adequately cryopreserved, resulting in suppression assays that were unevaluable.

End points and definitions

The primary end point was the incidence of grade 3-5 infusional toxicity within 48 hours after iTreg infusions. Adverse events were classified according to the National Cancer Institute's Common Terminology Criteria for Adverse Events, version 4. A dose-limiting toxicity (DLT) was defined as a grade 3 adverse event of >24-hour duration that occurred within 48 hours of infusion from the following categories: cardiac, immune disorders (including allergic reactions); respiratory, thoracic, and mediastinal disorders (with the exception of mucositis); renal disorders; or central nervous disorders. DLTs also included any grade 4 or 5 adverse event of >24-hour duration with the exception of hematological toxicities or fever. DLT events were captured at 1 to 4 hours after iTreg infusion (and before PBSCT on day 0), and at 24 hours, 48 hours, and 7 days (if DLTs occurred at 48 hours). Secondary end points included the cumulative incidence of grade 2-4 aGVHD at day 100, and the cumulative incidence of chronic GVHD, relapse, and survival at 1 year as previously defined.[14]

Statistical analysis

The best available dose or the MTD was determined by design. The experimental design for this study was a fast-track 3+3 trial of a semi-log escalation of iTregs.[16] The trial was conducted in 2 steps with the addition of an extension arm to help confirm safety and provide preliminary estimates of efficacy. The first step enrolled 1 patient per increasing iTreg dose until encountering a DLT. Because there were no DLTs as expected, the second step was initiated in which the cohort size was increased to 3 patients, enrolling 6 patients using the 3+3 design at the highest dose with a regimen of CSA/MMF. After 6 patients were enrolled using CSA/MMF, 4 patients were continuously enrolled at this best available dose to complete the extension arm. A modification of Pocock stopping boundaries was used to continuously monitor for excessive infusional toxicity, primary graft failure, grade 3-4 acute GVHD, transplant-related mortality, and chronic GVHD during study accrual. Given the small numbers, events were primarily summarized using descriptive statistics such as frequencies and proportions. Among the 10 patients at the best available dose, GVHD was estimated using cumulative incidence, treating non-GVHD mortality as a competing risk. Engraftment, relapse, and infection were also estimated with cumulative incidence treating nonevent death as a competing risk. Overall survival was estimated by Kaplan-Meier curves. All analyses were performed using SAS 9.4 (SAS Institute, Inc, Cary, NC).

Results

iTreg product characteristics

In total, 55 donor/recipient pairs were screened to enroll 16 donor/recipients. Of the 39 screening failures, 15 were the decision of the treating physician who thought the patient's disease was too high risk to delay admission for 8 days awaiting iTreg culture. In addition, 11 patients did not meet eligibility criteria, and 13 donors were unable to donate PBSCs twice.

Sixteen patients were enrolled and 16 iTreg products were manufactured. All products met lot-release criteria for CD8 contamination

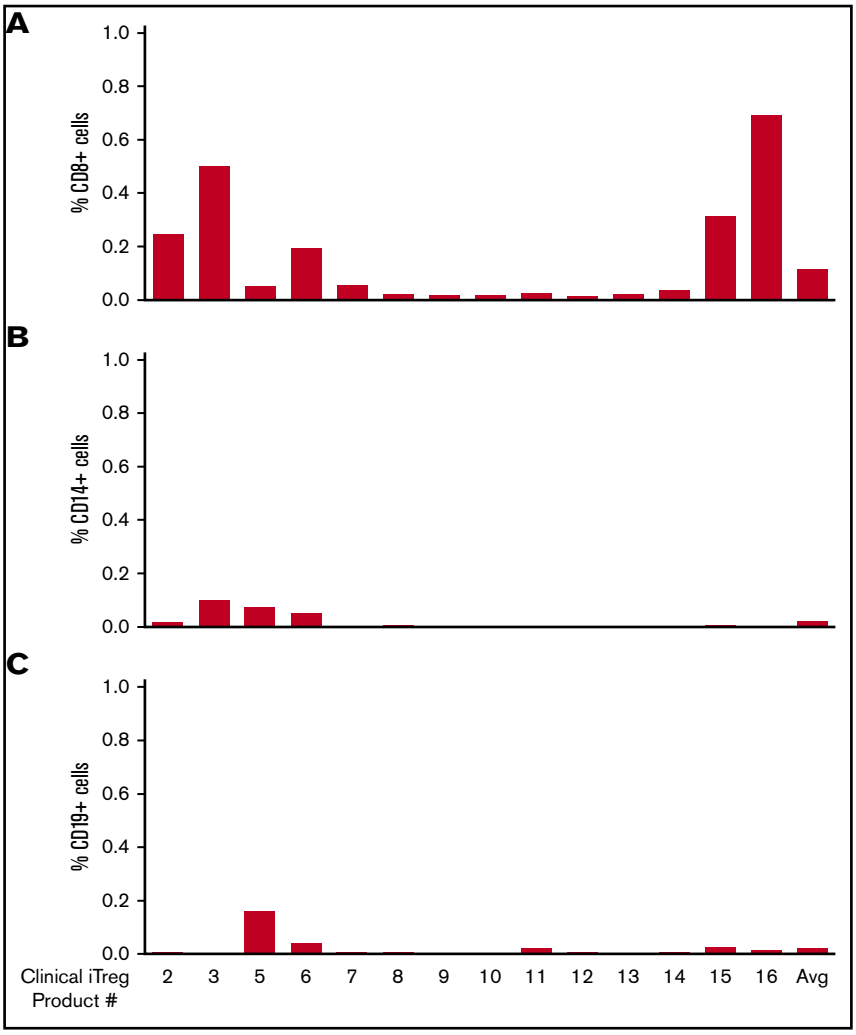

Figure 1. Cells other than iTregs present in infused products. To assess the cell purity of clinical rapa/TGF iTreg products, aliquots were taken postprocessing and frozen. Frozen aliquots were then thawed and batch-analyzed by flow cytometry for CD8 T cells (CD8; A), monocytes (CD14; B), and B cells (CD19; C).

(Figure 1; range, 0.0% to 0.8%), sterility (culture, Gram stain, and mycoplasma), endotoxin, and viability. However, 2 iTreg products (#1 and #4) failed to meet the lot release because of the low percentage of $CD4^+25^+$ and were not infused. The mean theoretical yield of the 14 infused products was 1.6×10^{12} cells (range, 0.3×10^{12} to 8.1×10^{12}). Cell numbers for individual products at different time points in product manufacture are shown (Table 1). For the 14 infused products, the mean total number of nucleated cells (TNCs) at the end of expansion culture was 47×10^9 (range, 15×10^9 to 110×10^9) corresponding to a mean fold-expansion of 624-fold (range,165-fold to 2252-fold) (Table 1). The mean proportion of $CD4^+CD25^+$ cells in products was 95% (range, 86% to 99%). Cell viability of purified $CD4^+25^-$ cells was 97.8% ± 0.6% (range, 92.9% to 99.6%), and at time of infusion was 96.9% ± 0.7% (range, 89.2% to 99.0%).

Approximately 60% of clinical iTreg products expressed Foxp3 with ~30% $Foxp3^{bright}$ (Figure 2A-B), with 83% ± 4% (range, 49% to 100%) of $CD4^+$ cells expressing the Treg-mediated suppressor antigen, CTLA4 (Figure 2C). The iTregs in the product were potent suppressor cells with a median of 64% ± 5% (range, 48% to 75%) suppression of CD3-stimulated T cells at a 1:2 iTreg-to-PBMC ratio (Figure 2D) in the 6 product samples where there was sufficient iTreg recovery following thaw and wash. Stable Foxp3 expression in tTregs is controlled through the Treg-specific demethylated region in the *Foxp3* gene.[17] To date, no method to induce Tregs in murine or human cells has resulted in Treg-specific demethylated region methylation, and we previously found no evidence for rapa/TGFβ iTreg-induced demethylation.[12] Very few cells in the rapa/TGFβ iTreg products expressed Helios (supplemental Figure 1A), a transcription factor involved in maintaining Foxp3 expression.[18] rapa/TGFβ iTreg products had uniformly high expression of CD45RO, HLA-DR, and ICOS (83% ± 4%, 93% ± 1%, and 96% ± 2%, respectively). Consistent with prior data, rapa/TGFβ iTreg expressed CD39, an enzyme involved in the production of the suppressive nucleoside adenosine, on >60% (Figure 3A), and CD103, a component of the α4β7 receptor required for intestinal homing, on >11% of cells in the clinical iTreg products (Figure 3B).

Upon antigen exposure, $CD4^+$ T cells differentiate from naive ($CCR7^+CD27^+CD45RA^+$, $CD95^-$), to central memory ($CCR7^+CD27^+CD45RA^-$, $CD95^+$), and/or effector memory ($CCR7^-CD27^{+/-}CD45RA^-$, $CD95^+$) and to short-lived effector T cells ($CCR7^-CD27^-CD45RA^+$).[19-21] With continued antigen exposure, T cells can acquire a senescent phenotype, characterized by the graded loss of costimulatory molecules (eg, CD27 and CD28)

Table 1. Product characteristics

Patient UPN	Product no.	Target iTreg dose, $\times 10^6$ cells/kg	Target iTreg: T-cell dose	Before expansion			After expansion					
				Pre-CD25 selection	Post-CD25 selection							
				TNC, $\times 10^{10}$	TNC, $\times 10^9$	% $CD4^+$ $CD25^+$	Expansion, fold	Theoretical TNC, $\times 10^{12}$	Actual TNC,* $\times 10^{10}$	% $CD4^+$ $CD25^+$	Actual iTreg dose, $\times 10^6$ cells per kg	Actual iTreg: T-cell dose
Infused products												
6352	2	3	N/A	2.4	3.6	15	2252	8.1	2.7	98	3	1:86
6354	3	30	N/A	2.0	2.0	10	878	1.8	1.5	94	30	1:8
6473	5	300	1:1	2.4	1.9	8	878	1.7	1.8	97	262	2:1
6492	6	300	1:1	2.4	2.3	10	745	1.7	4.5	94	303	1:1
6609	7	300	1:1	3.4	1.0	3	705	0.7	11	98	300	1:1
6214†	8	300	1:1	0.7	0.6	8	399	0.2	4.8	99	300	1:1
6374	9	300	1:1	3.4	2.5	7	565	1.4	4.8	99	300	2:1
6817	10	300	1:1	2.6	3.2	12	898	2.8	8.2	96	300	1:1
6864	11	300	1:1	1.6	1.9	12	252	0.5	5.5	97	295	2:1
6869	12	300	1:1	3.0	1.8	6	165	0.3	5.1	93	299	1:1
6876	13	300	1:1	2.3	2.5	11	301	0.8	4.7	86	301	1:1
6884	14	300	1:1	3.3	2.6	8	195	0.5	3.6	91	300	1:1
6911	15	300	1:1	2.0	3.8	18	181	0.7	2.9	89	292	1:1
6929	16	300	1:1	2.5	2.8	11	324	0.9	3.7	94	300	1:1
Mean‡				2.4	2.3	10	624	1.6	4.6	95	256	1:1
SEM				0.21	0.25	1.1	151	0.6	0.7	1		
Noninfused products												
6287	1	3		1	0.17	14	4	0.74	0.7	32	NA	
6372	4	300		3.1	0.37	12	333	120	0.9	34	NA	

N/A, not applicable; TNC, total nucleated cell; UPN, unique patient no.

*This represents the actual number of cells expanded for patient use. Not all cells were restimulated on day 7.

†Patient 6214 developed zoster before conditioning, necessarily delaying the PBSC infusion and scheduled infusion of iTregs. Therefore, the cultured iTregs were cryopreserved on day 7 of culture and were later thawed, restimulated as per the other products for 7 days and infused.

‡Mean and range for infused products only.

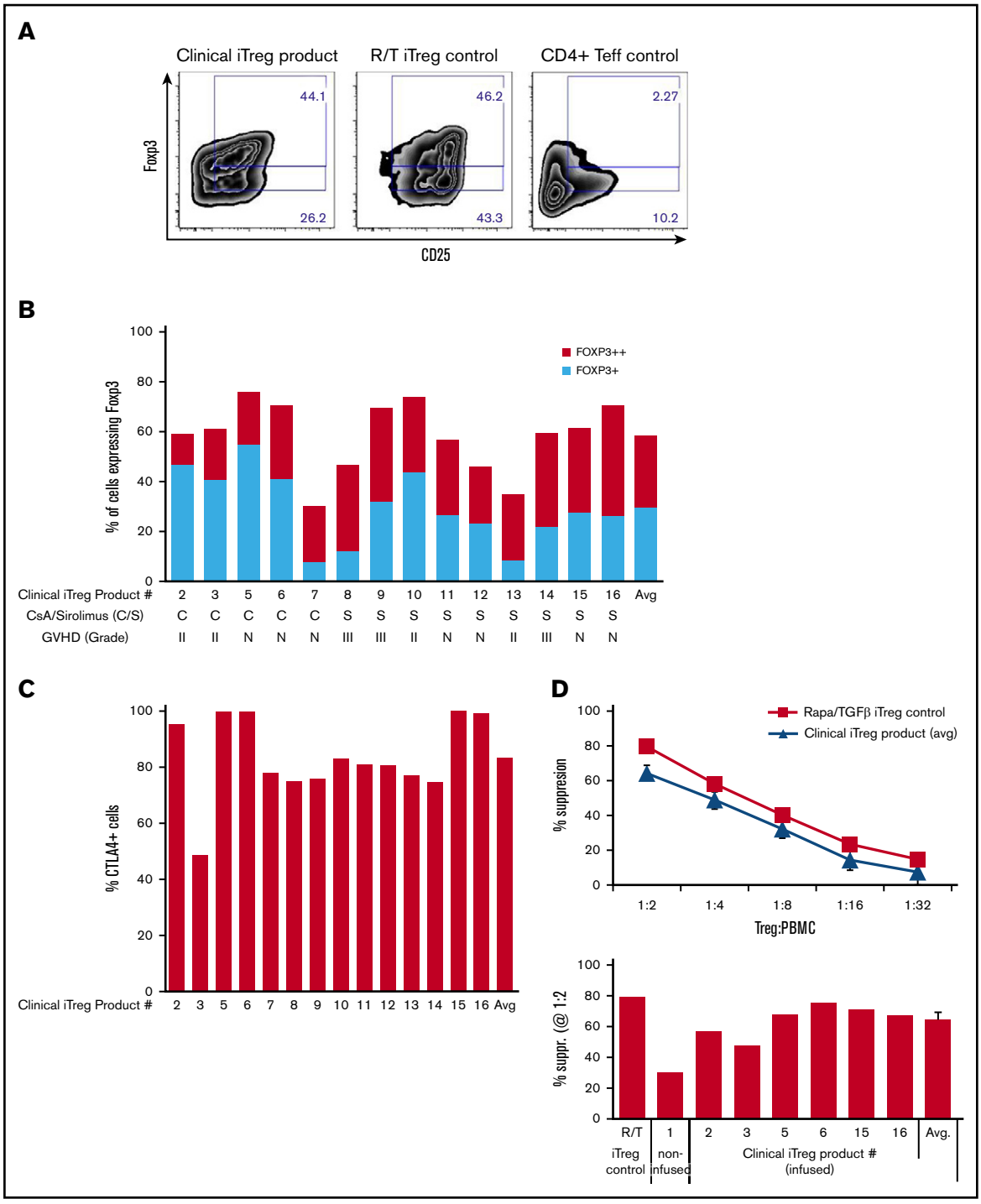

Figure 2. Assessing the phenotype and function of infused products. To assess the phenotype and function of clinical rapa/TGFβ iTreg products, aliquots were taken postprocessing and frozen. Frozen aliquots were then batch-analyzed for phenotype and function. Representative example (A) and summary (B) of Foxp3 expression in clinical iTreg products. (C) Expression of the suppressive molecule CTLA4 on clinical iTreg products. (D) Representative example and summary of 1:2 (Treg-to-PBMC) ratio for the indicated 9 clinical products. N, none; R/T, rapa/TGFβ.

and increasing expression of inhibitory molecules (eg, PD-1, Lag-3, and TIGIT).[19] Because large-scale in vitro expansion of CD4 and CD8 T cells for adoptive immunotherapy has been linked with senescence and decreased in vivo function,[22-24] we sought to determine the differentiative state and inhibitory receptor expression of expanded rapa/TGFβ iTreg clinical products (supplemental Figure 1). rapa/TGFβ iTreg clinical products were uniformly of effector memory phenotype (CCR7$^-$, CD45RA$^-$, CD95$^+$),[25] and maintained high expression of CD27 and CD28, consistent with their potent immune-suppressive function.[19] rapa/TGFβ iTreg clinical

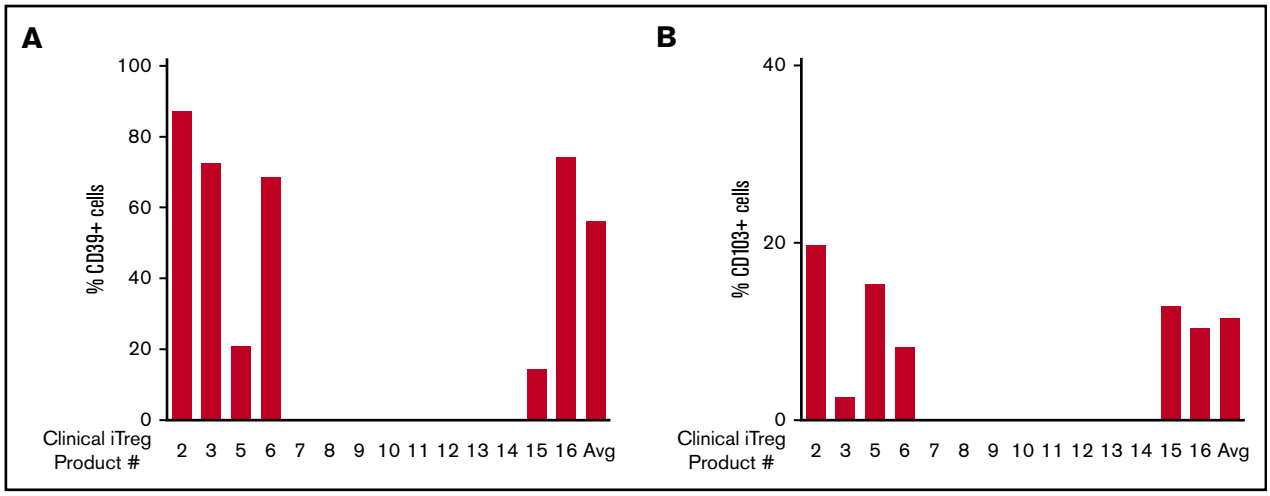

Figure 3. Expression of suppression-related molecules on clinical iTreg products compared with rapa/TGFβ iTregs and UCB tTregs expanded on a research scale with clinically relevant protocols. To assess the expression of suppression-related molecules, clinical rapa/TGFβ iTreg products, aliquots were taken postprocessing and frozen. Frozen aliquots were then thawed and batch-analyzed with rapa/TGFβ iTregs and UCB tTregs expanded on a research scale with clinically relevant protocols for the expression of: CD39 (A), CD73 (B), and (CD103).

products also had low inhibitory receptor expression, suggesting maintenance of function in vivo (supplemental Figure 1G).

The 2 products that failed had post-CD25 depletion recoveries of only 0.17×10^9 and 0.37×10^9 TNCs vs $2.3 \times 10^9 \pm 0.25 \times 10^9$ TNCs for the other 14 products. One expanded only fourfold, suggesting that the nonmobilized apheresis product was of poor quality as both recovery and expansion were compromised. The other product had a fold expansion on the lower end of the range for the 14 other units (fourfold vs an average of 624- ± 151-fold for the other 14 products), pointing to the CD25 selection step as being suboptimal. Importantly, even though these products failed lot release, they did not secrete effector cytokines when restimulated (supplemental Figure 1H).

Patient characteristics

Fourteen patients were treated (7 male,7 female; median age, 63 years) with underlying diagnoses of acute myeloid leukemia (n = 8), myelodysplastic syndrome (n = 4), and non-Hodgkin lymphoma (n = 2). As planned, 1 patient each received 3.0×10^6/kg, 3.0×10^7/kg, 3.0×10^8/kg iTregs with a corresponding Tcon-to-iTreg ratio of 86:1, 8:1, and 1:2 (Table 2). As a Tcon-to-tTreg ratio of 1:1 given concurrently was required to optimally suppress GVHD in mice,[26] dose escalation was halted at 3.0×10^8/kg, corresponding to a Tcon-to-iTreg of 0.9 ± 0.1 for the patients treated and representing a reproducibly achievable maximum dose after 1 stimulation.

After 3 patients received 3.0×10^8/kg with CSA/MMF immunoprophylaxis with no DLTs, 2 patients received 3.0×10^8/kg iTregs using sirolimus/MMF as planned. However, as both patients developed grade 3 aGVHD, a stopping rule was met for excessive GVHD and the sirolimus/MMF arm was suspended. An additional 7 patients (for a total of 10) received 3.0×10^8/kg iTregs along with CSA/MMF.

iTreg kinetics, toxicity profile

Patients receiving iTregs had an increased percentage of circulating $Foxp3^+CD25^+$ among $CD4^+$ on days 3 and 7, and the degree of increase was higher in patients receiving 3.0×10^8/kg vs $\leq 30 \times 10^6$/kg iTregs (Figure 4). The percentage of $CD4^+$ cells in circulation with a phenotype ($Foxp3^+CD25^+$) consistent with iTregs or activated $CD4^+$ T cells returned to basal levels at or before day 15. As predicted, iTreg persistence was increased in patients receiving immunosuppression with sirolimus, compared with CsA, although this increase did not correlate to increased efficacy. To assess CD8 T-cell expansion and effector phenotype in patients receiving CsA vs sirolimus immunosuppression, PBMCs isolated on days 3, 7, 14, and 28 was also stained with antibodies to perforin and granzyme B following phorbol myristate acetate/ionomycin restimulation (Figure 4). Consistent with increased GVHD, patients receiving sirolimus immunosuppression had an increase in percentage of $CD8^+$ cells in the periphery on day 28, and an increased percentage of these $CD8^+$ cells expressed both perforin and granzyme B.

Infusional toxicities were monitored at baseline before the iTreg infusion, and then at 4 (prior to the PBSC infusion), 24, and 48 hours as well as 7 days. No severe infusional toxicities (grade 4-5) were associated with the administration of iTregs at 1 to 4 hours and none at subsequent time points, as summarized in Table 3.

Transplant outcomes

Transplant outcomes are shown in Table 2. All 14 patients achieved neutrophil engraftment at a median of 7 days (range, 6-13 days) and a platelet engraftment at a median of 16 days (range, 0-34 days). All surviving patients achieved full donor bone marrow and blood $CD33^+$ chimerism at 100 days after PBSCT. Full donor blood $CD3^+$ chimerism at day 100 was achieved in all surviving patients except for 2 who had 96% and 98% $CD3^+$ donor chimerism. By day 180, all patients had full donor blood $CD3^+$ chimerism.

Of 10 patients who received 3.0×10^8/kg iTregs using CSA/MMF, 2 developed grade 2 and 1 grade 3 aGVHD by day 100. All 3 had stage III skin involvement with 1 patient also having grade 4 lower gastrointestinal involvement. Five developed chronic GVHD requiring systemic immune suppression. Four of these 5 patients had de novo chronic GVHD. Six patients were alive at 1 year and 3 were

Table 2. Transplant outcomes

UPN	Product no.	iTreg dose level targe per kg	Actual no. of iTregs infused per kg	T effector: iTreg	GVHD prophylaxis	Days after iTreg/PBSCT: Engraftment Neutrophil	Engraftment Platelet	Grade II-IV aGVHD at onset, d	Chronic GVHD by 1 y, d	Infections by 1 y	Relapse	Survival at analysis
6352	2	3.0×10^6	2.9×10^6	86:1	CSA/MMF	9	16	II, 42		CMV viremia	No	Alive
6354	3	3.0×10^7	3.05×10^7	8:1	CSA/MMF	4	12	II, 165	350		No	Alive
6473*	5	3.0×10^8	2.62×10^8	1:2	CSA/MMF	12	0		245	*C difficile* colitis	No	Alive
6492*	6	3.0×10^8	3.03×10^8	1:1	CSA/MMF	12	19		218	Lemierre syndrome, gram-negative bacterium	Yes (97)	Dead day 1047 relapse
6609*	7	3.0×10^8	3.0×10^8	1:1	CSA/MMF	6	0		162	*S epidermidis* bacteremia	No	Alive
6374	9	3.0×10^8	3.0×10^8	1:1	Sirolimus/MMF	9	21	III, 27	222	Pneumonia	No	Alive
6214	8	3.0×10^8	3.0×10^8	1:2	Sirolimus/MMF	12	24	III, 15	247	Pneumonia, UTI	No	Alive
6817*	10	3.0×10^8	3.0×10^8	1:1	CSA/MMF	7	0	II, 47	244		No	Alive
6864*	11	3.0×10^8	3.0×10^8	1:1	CSA/MMF	13	34			HHV6 viremia *C difficile* colitis	No	Dead (59) bacterial infection
6869*	12	3.0×10^8	3.0×10^8	1:1	CSA/MMF	10	21			*C difficile* colitis	No	Dead (206) viral infection
6876*	13	3.0×10^8	3.0×10^8	1:1	CSA/MMF	7	0	II, 41		*C difficile* colitis	No	Dead (74) aGVHD
6884*	14	3.0×10^8	3.0×10^8	1:1	CSA/MMF	7	0	III,156		*C difficile* colitis CMV viremia EBV viremia	Yes (103)	Alive
6911*	15	3.0×10^8	3.0×10^8	1:1	CSA/MMF	7	16		328	Sinusitis	No	Alive
6929*	16	3.0×10^8	3.0×10^8	2:1	CSA/MMF	7	30				Yes (21)	Dead (191) complications of heart surgery

C difficile, *Clostridium difficile*; CMV, cytomegalovirus; EBV, Epstein-Barr virus; HHV6, human herpesvirus 6; *S epidermidis*, *Staphylococcus epidermidis*; UTI, urinary tract infection. See Table 1 for expansion of other abbreviations.
*The 10 patients who received 3.0×10^8/kg iTregs per kg with CSA/MMF.

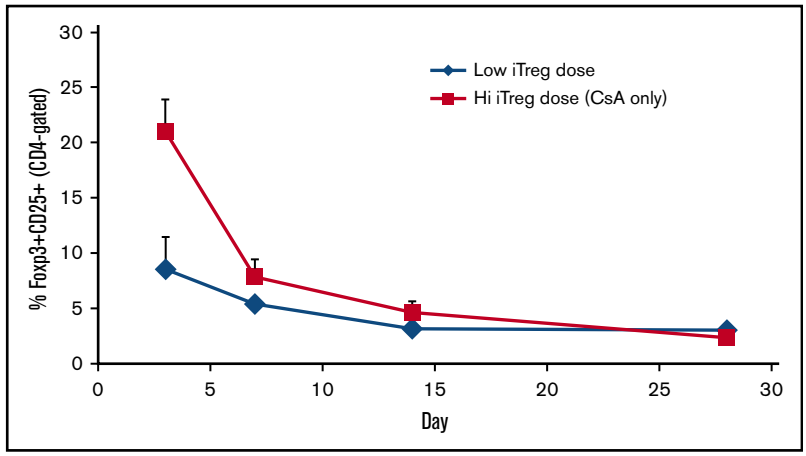

Figure 4. Patients receiving high doses of iTregs have higher levels of Foxp3$^+$ CD25$^+$ cells in PB. PB was collected from patients on days 3, 7, 14, and 28 following adoptive transfer of rapa/TGFβ iTregs. After Ficoll, the percentage of of CD4$^+$ cells that were Foxp3$^+$CD25$^+$ Tregs present in PBMCs was determined by flow cytometry. Low iTreg samples (n = 2) were from patients receiving ≤30 × 10^6/kg, high iTreg dose (CSA only) was from patients receiving 300 × 10^6 iTreg with CsA immunosuppression (n = 10) and high iTreg dose (sirolimus only) was from patients receiving 300 × 10^6 iTreg with sirolimus immunosuppression (n = 2).

alive at 3 years. Cause of death included aGVHD (n = 1), chronic GHVD (n = 1), relapse (n = 3), and infection (n = 2).

Outcomes were compared with those of a contemporary institutional cohort of 203 adult patients with hematological malignancies receiving the same RIC and GVHD prophylaxis, but without iTregs. Neutrophil engraftment was similar between iTreg patients (100% at a median of 7 days) and the contemporary controls (100% at a median of 9 days; $P = .22$). The cumulative incidence of grade 2-4 aGVHD was 30% (95% confidence interval [CI], 3% to 48%) for iTreg recipients vs 41% (95% CI, 34% to 48%; $P = .51$) for the contemporary control group (supplemental Figure 2) and 10% (95% CI, 0% to 28%) vs 25% (95% CI, 19% to 31%; $P = .29$) for grades 3-4 aGVHD.

To determine whether iTreg infusion influenced lymphocyte recovery, we compared lymphocyte recovery in the 10 patients who received the highest dose of iTregs of 3.0 × 10^8/kg iTregs per kg with CSA/MMF to 69 contemporary controls for whom samples were available. As illustrated in Figure 5, recipients of iTregs had significantly higher absolute lymphocyte counts ($P < .01$) and higher CD4$^+$ cells for the first 6 months after transplant ($P < .01$). Additionally, absolute CD4$^+$Foxp3$^+$CD25^{hi} numbers at day 28 after PBSCT were significantly higher in iTreg recipients than controls (median, 0.0336/μL vs 0.0130/μL; $P < .01$).

There was no significant difference between the iTreg cohort and the contemporary controls in the incidence of bacteremia at day 100 (60% [95% CI, 26% to 88%] vs 53% [95% CI, 46% to 60%]; $P = .75$), chronic GVHD at 2 years (50% [95% CI,18% to 82%] vs 45% [95% CI, 37% to 53%]; $P = .83$), or relapse at 2 years (30% [95% CI, 3% to 57%] vs 36% [95% CI, 29% to 43%]; $P = .95$). One-year GVHD-free relapse-free survival (grade III/IV GVHD-free, chronic GVHD requiring systemic immunosuppression free, relapse free, survival) was 20% (95% CI, 1% to 45%) for the 10 iTreg patients who received 3.0 × 10^8/kg iTregs using CSA/MMF and 18% (95% CI, 13% to 23%) for the 203 contemporary control patients ($P = .81$). Three-year disease-free survival was 30% (95% CI, 7% to 58%) for iTreg patients, and 25% (95% CI, 17% to 32%) for contemporary controls.

Discussion

This is the first-in-human clinical trial in allo-HCT patients to determine safety and potency of infusing large numbers of iTregs produced in a good manufacturing practice facility. HLA-identical sibling donor iTregs were safely administered at a maximum biological dose of 3 × 10^8/kg iTregs per kg achieving a Tcon-to-iTreg ratio of ∼1:1, our target ratio to suppress GVHD with CSA/MMF immunoprophylaxis. Although the use of CSA may have negatively affected the iTregs, we observed higher absolute CD3$^+$ T cells, CD4$^+$ cells, and CD4$^+$Foxp3$^+$CD25^{hi} cells at day 28 along with faster lymphocyte recovery in iTreg patients vs contemporary controls. More patients and consideration for further dose escalation will be needed before reaching an efficacy conclusion. Although only 2 patients received sirolimus/MMF, it was ineffective as GVHD prophylaxis in the context of this iTreg clinical trial.

The colon and small intestine are primal GVHD target organs. The α4β7 integrin pair interacts specifically with mucosal addressin cell adhesion molecule-1 (MAdCAM-1) on high endothelial venules in the Peyer patches and intestinal lamina propria and is critical for immune cell homing to these tissues.[27-29] Disruption of α4β7 and MAdCAM-1, either through antibody-mediated blocking of the α4 subunit or gene disruption of the β7 subunit, can reduce graft rejection and GVHD.[30] TGFβ induces integrin α4 (CD103) expression and gut homing in T cells. Even though almost no UCB tTreg express CD103,[31] >11% of PB rapa/TGFβ iTregs were CD103$^+$, potentially enhancing the gut-protective effects of iTregs.

Our current good manufacturing practice–compatible preclinical studies indicated that up to 100-fold higher doses of aAPC-expanded iTregs (yield, ≥240 × 10^9) than bead-expanded UCB tTregs could

Table 3. Infusional toxicities after iTreg products in 14 patients

Adverse event	Baseline prior to iTreg infusion (day 0)	1-4 h after iTreg infusion and before PBSCT (day 0)	Time after iTreg infusion		
			24 h (±2 h)	48 h (±2 h)	Day +7
Dyspnea grade 2	0	1	0	0	0
Hypoxia grade 2	0	1	0	1	0
Chills grade 1	0	3	1	0	0
Chills grade 2	0	1	0	0	0
Hypertension grade 2	0	3	0	0	0
Hypertension grade 3	0	1	0	0	0
Hypotension grade 1	0	0	1	0	0
Sinus tachycardia grade 1	0	3	0	0	0

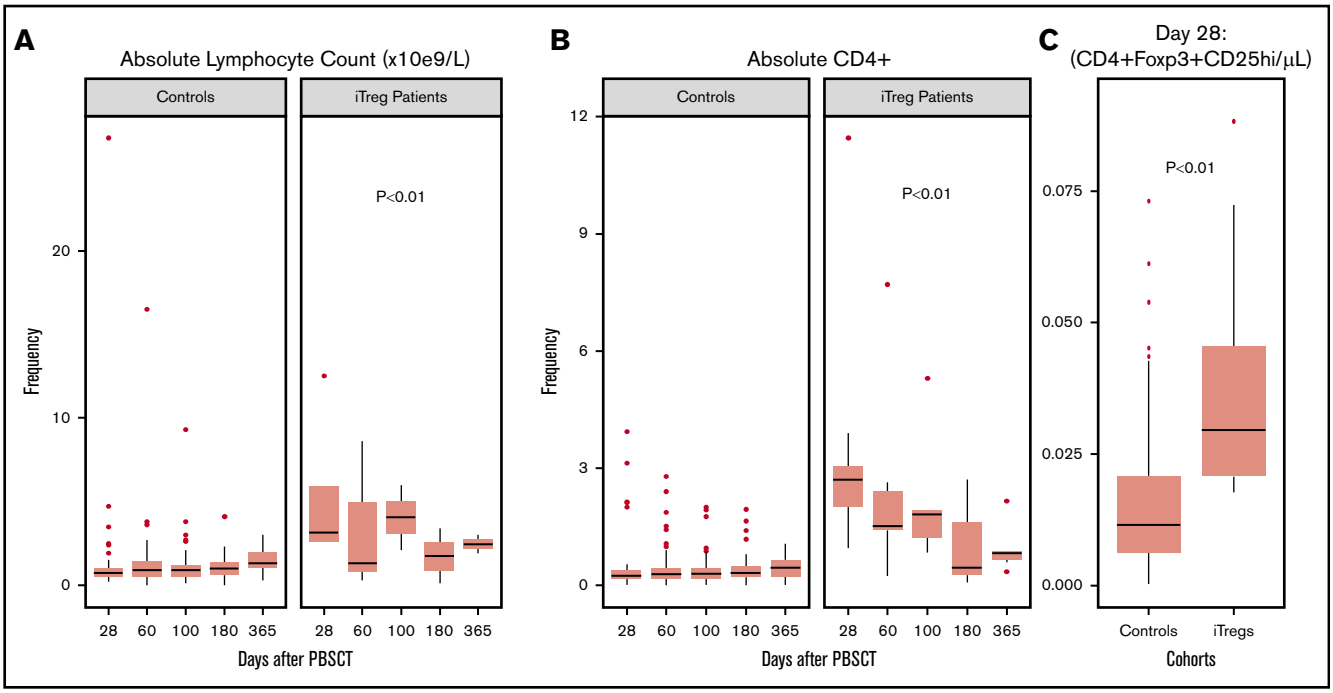

Figure 5. Absolute numbers of $CD4^+$ T cells, absolute lymphocyte counts, and $CD4^+Foxp3^+CD25^{hi}$ recovery in contemporary controls vs iTreg patients. (A) Absolute lymphocyte count. (B) Absolute $CD4^+$ cells. (C) Absolute $CD4^+Foxp3^+CD25^{hi}$. ALC, absolute lymphocyte count.

be achieved even with a shorter culture duration of 12 days vs 18 days. Notably, rapa/TGFβ iTregs and expanded PB tTregs had a similar in vivo potency when directly compared in a xenogenic GVHD model[12] that served as foundational data for the iTreg trial described here. Murine studies of autoimmunity and GVHD have shown that T-cell receptor–transgenic, antigen-specific Tregs are more effective than polyclonal Tregs at suppressing disease[10,11,32] but this effect is very specific, and HY-Tg iTregs did not suppress GVHD in female mice lacking the HY antigen.[11] Although polyclonal iTregs may be less effective on a per-cell basis, GVHD is a multiepitope disease and our production platform generated enough cells to meet the ~1:1 iTreg-to-Teff ratio that was sufficient to suppress disease in our xenogeneic model of GVHD.

Dose escalation was halted at 3.0×10^8/kg (which equated to ~1:1 iTregs to Tcons), despite no observed DLTs, as manufacturing costs became prohibitive. As seen with UCB tTreg trials,[6,13] iTregs could be detected for only 14 days. Unexpectedly, significant increases occured in day 28 absolute lymphocyte counts and numbers of $CD3^+$ cells, $CD4^+$ cells, and $CD4^+25^{hi}FoxP3^+$ cells. Because neither aGVHD rates nor severity were reduced by iTregs, the mechanism(s) for improved cell recoveries is unknown. Because the major difference between this trial and the historical controls is whether iTregs were infused, it is tempting to speculate that iTregs reduced thymus injury, decreased negative regulatory effects in the host that limited $CD4^+$ and $CD4^+25^+FoxP3^+$ cell recovery, or released soluble molecules that stimulated expansion of the above cell types.

During the dose-escalation phase of the trial, all patients received CSA/MMF as GVHD prophylaxis as standard of care at our institution for over the past decade. Because CSA would not be the optimal GVHD prophylaxis agent as it inhibits IL-2 production and hence may interfere with iTreg survival and function,[33] and rapamycin in the iTreg cultures has been shown to promote selective expansion of tTregs[34-39] and stabilization of Fox3 expression, sirolimus replaced CSA once the maximum dose of 3.0×10^8/kg iTregs was achieved. Although sirolimus/MMF has been shown to be inferior to sirolimus/tacrolimus in preventing aGVHD,[40] we anticipated it would be sufficient prophylaxis in the setting of matched sibling donor PBSCT with iTreg infusion. However, both patients who received sirolimus/MMF as GVHD prophylaxis developed grade 3 aGVHD within a month of iTreg infusion. Despite the fact that both patients were successfully treated for aGVHD and were alive at a year after transplant, sirolimus/MMF was deemed ineffective as GVHD prophylaxis in this iTreg clinical trial and therefore, GVHD prophylaxis returned to CSA/MMF. Whether the ineffectiveness of sirolimus/MMF as GVHD prophylaxis was due to the regimen per se, independent of iTregs or an adverse effect of sirolimus/MMF on iTregs due to their known antiproliferative effects, is unknown.

A major concern in the field is the potential for instability of iTregs under inflammatory conditions, such as would be present in aGVHD. However, neither rodent[12] nor these human studies have shown any evidence of increased aGVHD severity or accelerated time to onset when iTregs are infused at the time of allotransplant. In fact, preclinical data have suggested that iTregs should not increase but rather reduce aGVHD risk and potentially promote engraftment; a major concern remained the possibility that iTregs under the highly inflammatory conditions of aGVHD would become Teffs, increasing the incidence and/or severity of aGVHD. Fortunately, this did not occur. In the 10 patients who received 3.0×10^8/kg iTregs using CSA/MMF, the incidence of aGVHD and chronic GVHD, aGVHD organ involvement, and response to therapy did not significantly differ from contemporary controls. Given the timing, logistics of 2 donor

aphereses, and costs of manufacturing single-patient Tregs (up to $100 000 per product), the use of third-party Tregs in the future would be a more feasible approach and should be explored in future studies. Further studies also are warranted to evaluate higher Tcon-to-iTreg ratios using a restimulation approach[7] and testing combined iTreg and tTreg infusion, which synergistically cooperate to treat murine colitis.[41]

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Authorship

Contribution: T.E.D. performed the statistical analysis; M.L.M. had primary responsibility for drafting the manuscript; and all authors contributed equally to the conception, design, and interpretation of data, and reviewed, edited, and approved the manuscript.

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References

1. Bluestone JA, Thomson AW, Shevach EM, Weiner HL. What does the future hold for cell-based tolerogenic therapy? *Nat Rev Immunol*. 2007;7(8):650-654.
2. Riley JL, June CH, Blazar BR. Human T regulatory cell therapy: take a billion or so and call me in the morning. *Immunity*. 2009;30(5):656-665.
3. Bluestone JA, Tang Q, Sedwick CE. T regulatory cells in autoimmune diabetes: past challenges, future prospects. *J Clin Immunol*. 2008;28(6):677-684.
4. Hoffmann P, Ermann J, Edinger M, Fathman CG, Strober S. Donor-type CD4(+)CD25(+) regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. *J Exp Med*. 2002;196(3):389-399.
5. Taylor PA, Lees CJ, Blazar BR. The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. *Blood*. 2002;99(10):3493-3499.
6. Brunstein CG, Miller JS, Cao Q, et al. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood*. 2011;117(3):1061-1070.
7. Hippen KL, Merkel SC, Schirm DK, et al. Massive ex vivo expansion of human natural regulatory T cells (T(regs)) with minimal loss of in vivo functional activity. *Sci Transl Med*. 2011;3(83):83ra41.
8. Sawamukai N, Satake A, Schmidt AM, et al. Cell-autonomous role of TGFβ and IL-2 receptors in CD4+ and CD8+ inducible regulatory T-cell generation during GVHD. *Blood*. 2012;119(23):5575-5583.
9. Zhang P, Tey SK, Koyama M, et al. Induced regulatory T cells promote tolerance when stabilized by rapamycin and IL-2 in vivo. *J Immunol*. 2013;191(10):5291-5303.
10. Semple K, Yu Y, Wang D, Anasetti C, Yu XZ. Efficient and selective prevention of GVHD by antigen-specific induced Tregs via linked-suppression in mice. *Biol Blood Marrow Transplant*. 2011;17(3):309-318.
11. Li J, Heinrichs J, Haarberg K, et al. HY-specific induced regulatory T cells display high specificity and efficacy in the prevention of acute graft-versus-host disease. *J Immunol*. 2015;195(2):717-725.
12. Hippen KL, Merkel SC, Schirm DK, et al. Generation and large-scale expansion of human inducible regulatory T cells that suppress graft-versus-host disease. *Am J Transplant*. 2011;11(6):1148-1157.
13. Brunstein CG, Miller JS, McKenna DH, et al. Umbilical cord blood-derived T regulatory cells to prevent GVHD: kinetics, toxicity profile, and clinical effect. *Blood*. 2016;127(8):1044-1051.
14. Warlick ED, DeFor TE, Bejanyan N, et al. Reduced-intensity conditioning followed by related and unrelated allografts for hematologic malignancies: expanded analysis and long-term follow-up. *Biol Blood Marrow Transplant*. 2019;25(1):56-62.
15. Fuchs A, Gliwiński M, Grageda N, et al. Minimum information about T regulatory cells: a step toward reproducibility and standardization. *Front Immunol*. 2018;8:1844.
16. Le C. *Applied Categorical Data Analysis and Translational Research*. Hoboken, NJ: John Wiley & Sons; 2010.

17. Huehn J, Polansky JK, Hamann A. Epigenetic control of FOXP3 expression: the key to a stable regulatory T-cell lineage? *Nat Rev Immunol*. 2009;9(2):83-89.

18. Fu W, Ergun A, Lu T, et al. A multiply redundant genetic switch "locks in" the transcriptional signature of regulatory T cells. *Nat Immunol*. 2012;13(10):972-980.

19. Akbar AN, Henson SM. Are senescence and exhaustion intertwined or unrelated processes that compromise immunity? *Nat Rev Immunol*. 2011;11(4):289-295.

20. Gattinoni L, Klebanoff CA, Restifo NP. Paths to stemness: building the ultimate antitumour T cell. *Nat Rev Cancer*. 2012;12(10):671-684.

21. Restifo NP, Gattinoni L. Lineage relationship of effector and memory T cells. *Curr Opin Immunol*. 2013;25(5):556-563.

22. Crompton JG, Clever D, Vizcardo R, Rao M, Restifo NP. Reprogramming antitumor immunity. *Trends Immunol*. 2014;35(4):178-185.

23. Crompton JG, Sukumar M, Restifo NP. Uncoupling T-cell expansion from effector differentiation in cell-based immunotherapy. *Immunol Rev*. 2014;257(1):264-276.

24. Kamphorst AO, Ahmed R. CD4 T-cell immunotherapy for chronic viral infections and cancer. *Immunotherapy*. 2013;5(9):975-987.

25. Coghill JM, Carlson MJ, Panoskaltsis-Mortari A, et al. Separation of graft-versus-host disease from graft-versus-leukemia responses by targeting CC-chemokine receptor 7 on donor T cells. *Blood*. 2010;115(23):4914-4922.

26. Taylor PA, Panoskaltsis-Mortari A, Swedin JM, et al. L-Selectin(hi) but not the L-selectin(lo) CD4+25+ T-regulatory cells are potent inhibitors of GVHD and BM graft rejection. *Blood*. 2004;104(12):3804-3812.

27. Hill GR, Ferrara JL. The primacy of the gastrointestinal tract as a target organ of acute graft-versus-host disease: rationale for the use of cytokine shields in allogeneic bone marrow transplantation. *Blood*. 2000;95(9):2754-2759.

28. Fu YY, Egorova A, Sobieski C, et al. T cell recruitment to the intestinal stem cell compartment drives immune-mediated intestinal damage after allogeneic transplantation. *Immunity*. 2019;51(1):90-103.e3.

29. Takashima S, Martin ML, Jansen SA, et al. T cell-derived interferon-γ programs stem cell death in immune-mediated intestinal damage. *Sci Immunol*. 2019;4(42):eaay8556.

30. Waldman E, Lu SX, Hubbard VM, et al. Absence of beta7 integrin results in less graft-versus-host disease because of decreased homing of alloreactive T cells to intestine. *Blood*. 2006;107(4):1703-1711.

31. Hsu PS, Lai CL, Hu M, et al. IL-2 enhances gut homing potential of human naive regulatory T cells early in life. *J Immunol*. 2018;200(12):3970-3980.

32. Masteller EL, Warner MR, Tang Q, Tarbell KV, McDevitt H, Bluestone JA. Expansion of functional endogenous antigen-specific CD4+CD25+ regulatory T cells from nonobese diabetic mice. *J Immunol*. 2005;175(5):3053-3059.

33. Zeiser R, Nguyen VH, Beilhack A, et al. Inhibition of CD4+CD25+ regulatory T-cell function by calcineurin-dependent interleukin-2 production. *Blood*. 2006;108(1):390-399.

34. Hendrikx TK, Velthuis JH, Klepper M, et al. Monotherapy rapamycin allows an increase of CD4 CD25 FoxP3 T cells in renal recipients. *Transpl Int*. 2009;22(9):884-891.

35. Ma A, Qi S, Wang Z, et al. Combined therapy of CD4(+)CD25(+) regulatory T cells with low-dose sirolimus, but not calcineurin inhibitors, preserves suppressive function of regulatory T cells and prolongs allograft survival in mice. *Int Immunopharmacol*. 2009;9(5):553-563.

36. Schleuning M, Judith D, Jedlickova Z, et al. Calcineurin inhibitor-free GVHD prophylaxis with sirolimus, mycophenolate mofetil and ATG in Allo-SCT for leukemia patients with high relapse risk: an observational cohort study. *Bone Marrow Transplant*. 2009;43(9):717-723.

37. Singh AK, Horvath KA, Mohiuddin MM. Rapamycin promotes the enrichment of CD4(+)CD25(hi)FoxP3(+) T regulatory cells from naïve CD4(+) T cells of baboon that suppress antiporcine xenogenic response in vitro. *Transplant Proc*. 2009;41(1):418-421.

38. Battaglia M, Stabilini A, Roncarolo MG. Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood*. 2005;105(12):4743-4748.

39. Coenen JJ, Koenen HJ, van Rijssen E, et al. Rapamycin, not cyclosporine, permits thymic generation and peripheral preservation of CD4+ CD25+ FoxP3+ T cells. *Bone Marrow Transplant*. 2007;39(9):537-545.

40. Cutler C, Antin JH. Sirolimus immunosuppression for graft-versus-host disease prophylaxis and therapy: an update. *Curr Opin Hematol*. 2010;17(6):500-504.

41. Haribhai D, Williams JB, Jia S, et al. A requisite role for induced regulatory T cells in tolerance based on expanding antigen receptor diversity. *Immunity*. 2011;35(1):109-122.