Phase II Clinical Trial of Rapamycin-Resistant Donor CD4+ Th2/Th1 (T-Rapa) Cells After Low-Intensity Allogeneic Hematopoietic Cell Transplantation

Short Title: Clinical Trial of Allogeneic T-Rapa Cells

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KEY POINTS

- Donor T-Rapa cells were comprised of Th1 and Th2 effectors with a reproducible gene expression profile.
- Pre-emptive T-Rapa DLI was safe and associated with donor engraftment without excessive GVHD.

ABSTRACT

In experimental models, ex vivo induced T cell rapamycin-resistance occurred independent of T-helper 1 (Th1)/T-helper 2 (Th2) differentiation and yielded allogeneic CD4+ T cells of increased in vivo efficacy that facilitated engraftment and permitted graft-versus-tumor (GVT) effects while minimizing graft-versus-host disease (GVHD). To translate these findings, we performed a phase II multi-center clinical trial of rapamycin-resistant donor CD4+ Th2/Th1 (T-Rapa) cells after allogeneic matched-sibling donor hematopoietic cell transplantation (HCT) for therapy of refractory hematologic malignancy. T-Rapa cell products, which expressed a balanced Th2/Th1 phenotype, were administered as a pre-emptive donor lymphocyte infusion (DLI) at day 14 post-HCT. After T-Rapa cell infusion, mixed donor/host chimerism rapidly converted and there was preferential immune reconstitution with donor CD4+ Th2 and Th1 cells relative to regulatory T cells and CD8+ T cells. The cumulative incidence probability of acute GVHD was 20% and 40% at days 100 and 180 post-HCT, respectively. There was no transplant-related mortality. Eighteen of 40 patients (45%) remain in sustained complete remission (range of follow-up: 42 to 84 months). These phase II clinical trial results demonstrate the safety of this low-intensity transplant approach, and indicate the feasibility of subsequent randomized studies to compare T-Rapa cell-based therapy to standard transplantation regimens.
Study registered at: www.cancer.gov/clinicaltrials; NCT 00077480
INTRODUCTION

Allogeneic hematopoietic cell transplantation (HCT) using non-myeloablative host conditioning\textsuperscript{1,2} has reduced transplant-related mortality\textsuperscript{3} but is associated with increased tumor progression\textsuperscript{4} and graft rejection\textsuperscript{5}, and remains limited by graft-versus-host disease (GVHD)\textsuperscript{6}. Competing immune T cell reactions underlie these clinical events. Donor T cell-mediated GVHD and host T cell-mediated rejection are reciprocally related\textsuperscript{7}, whereas donor T cell-mediated graft-versus-tumor (GVT) effects and GVHD are intertwined\textsuperscript{8}. New approaches to modulate allogeneic T cell immunity are therefore required. Imbalance between T-helper 1 (Th1), Th2, and other CD4\textsuperscript{+} T cell subsets predisposes to human disease\textsuperscript{9}, including GVHD, which is primarily Th1-driven\textsuperscript{10}. As such, we hypothesized that allograft augmentation with T cells of mixed Th2 and Th1 phenotype may beneficially balance immunity after allogeneic HCT.

In murine models, we have evaluated the novel ex vivo application of rapamycin to control the Th2/Th1 balance post-transplant as an alternative to in vivo rapamycin drug therapy, which in various models has been found to: prevent graft rejection and GVHD but abrogate anti-tumor effects through inhibition of Th1-type cells and preservation of Th2-type cells\textsuperscript{11,12}, prevent GVHD through promotion of regulatory T (T\textsubscript{REG}) cells\textsuperscript{13} or modulation of host APC\textsuperscript{14}, and improve anti-viral immunity mediated by CD8\textsuperscript{+} T cells\textsuperscript{15}. The ex vivo approach that we developed allows one to dissect these seemingly disparate potential in vivo drug effects on a purified T cell subset under defined polarizing cytokine micro-environments. In our studies, we found that ex vivo rapamycin increased the capacity of IL-4 polarized donor Th2 cells to promote a balanced pattern of Th2/Th1 immune reconstitution for promotion of GVT effects and alloengraftment with reduced GVHD\textsuperscript{16-19}. Ex vivo rapamycin creates a state of T cell starvation
that induces autophagy\textsuperscript{20}, thereby resulting in an anti-apoptotic T cell phenotype that dictates persistent T cell engraftment in mouse-into-mouse\textsuperscript{18} or human-into-mouse\textsuperscript{21} transplantation models. Rapamycin-resistant Th2 cells inhibited GVHD by multiple mechanisms, including: IL-4 and IL-10 secretion, consumption of IL-2 required for propagation of pathogenic effector T cells, and modulation of host APC\textsuperscript{17}. Furthermore, delayed administration of rapamycin-resistant Th2 cells after an initial donor Th1-type response optimized the balance of GVT effects and GVHD\textsuperscript{16}, thereby indicating that a mixed pattern of Th2 and Th1 immune reconstitution was desirable in the setting of cancer therapy. And finally, rapamycin-resistant Th2 cells prevented graft rejection through host T cell conversion to a Th2-type profile\textsuperscript{19}, thus illustrating that this novel donor T cell population may have particular application in transplant settings associated with increased graft rejection, such as the use of low-intensity host conditioning.

Building on these data, we transitioned from a phase I clinical trial of IL-4 polarized donor CD4\textsuperscript{+} T cells not manufactured in rapamycin\textsuperscript{22} to the current trial that incorporated ex vivo rapamycin during IL-4 polarization to produce donor “T-Rapa” cells. To improve the safety of our transplantation method and to incorporate an engraftment endpoint into the clinical trial (conversion of mixed chimerism), we developed an outpatient treatment platform consisting of low-intensity host conditioning (75\% reduction in chemotherapy intensity relative to our previous studies of reduced-intensity transplantation)\textsuperscript{22}. And, in an attempt to tailor post-transplant immune suppression to favor the manufactured T-Rapa cells rather than the unmanipulated T cells contained in the T cell-replete hematopoietic cell allograft, we administered double-agent GVHD prophylaxis (cyclosporine plus sirolimus) in the early post-transplant period and subsequent single-agent cyclosporine prophylaxis after T-Rapa cell
adoptive transfer at day 14 post-transplant. This latter aspect of the protocol design was informed by our observation that ex vivo manufactured rapamycin-resistant allogeneic murine T cells, in particular the Th1 subset, were susceptible to the in vivo immune suppressive effects of rapamycin drug therapy.

METHODS

CLINICAL TRIAL DESIGN, IMPLEMENTATION, AND ENDPOINTS

This phase II multi-institution protocol (Figure 1) was approved by the National Cancer Institute (NCI) and Hackensack University Medical Center (HUMC) institutional review boards and implemented according to an Investigational New Drug Application accepted by the Food and Drug Administration. Dates of transplant for this protocol ranged from 12/13/05 to 6/29/09; 35 patients were transplanted at the National Institutes of Health Clinical Center and 5 patients were transplanted at Hackensack (HUMC). Subjects provided written informed consent in accordance with the Declaration of Helsinki; enrollment was based on age (between 19 and 75), availability of 6/6 HLA-matched sibling donor, organ function, and hematologic malignancy diagnosis (acute and chronic myelogenous or lymphocytic leukemia, myelodysplastic syndrome, multiple myeloma, and Hodgkin’s and non-Hodgkin’s lymphoma). Patients were eligible independent of their response to prior chemotherapy regimens; acute leukemia patients were eligible if blast frequency was <10%. Patients who had previously received autologous transplantation were eligible. Prior to transplant, patients received one to three cycles of EPOCH-FR chemotherapy to achieve a pre-determined level of host immune depletion (CD4 count \( \leq 200/\mu l \)), to assess chemotherapy sensitivity, and attempt to reduce disease burden.
Fludarabine (30 mg/m²/d) and cyclophosphamide (300 mg/m²/d) were administered on days -6 to -3 prior to the G-CSF mobilized peripheral blood allograft; this dose of cyclophosphamide was 75% reduced relative to the regimen we previously evaluated. GVHD prophylaxis was cyclosporine (200-250 ng/ml; day -1 to +100, tapered at day +100 or earlier for progressive disease) and sirolimus (3-12 ng/ml; day -2 to +14). T-Rapa cells were infused at day +14 post-HCT (2.5 x 10⁷ cells/kg). The composite primary study objectives were to determine the safety and feasibility of pre-emptive T-Rapa cell DLI and to characterize alloengraftment, anti-tumor effects, GVHD effects, and the Th2/Th1 balance post-HCT. The sample size of n=40 was selected to give a reasonable estimation of the rate of acute GVHD: with this sample size, the maximum confidence interval width for the fraction of patients with grade II to IV acute GVHD will not exceed +/- 15%. Organ toxicity was evaluated by NCI Common Toxicity Criteria (version 2.0); GVHD was evaluated using acute and chronic grading. Disease responses were evaluated by computed tomographic measurements and marrow examinations, with lymphoma responses measured by standard criteria; progressive disease was treated with chemotherapy and/or unmanipulated donor lymphocyte infusion (DLI). Alloengraftment was monitored using variable N-terminal repeat PCR assays on total, CD3-, or CD15-enriched cells.

T-RAPA CELL MANUFACTURING

Donor lymphocytes were collected by a 10-liter steady-state apheresis performed prior to stem cell mobilization. CD4 cells were positively selected (Miltenyi; CliniMACS® device) and co-stimulated (tosylated magnetic beads [Dynal] conjugated with anti-CD3 [OKT3; Ortho] and GMP-grade anti-CD28 9.3 antibodies [3:1 bead:cell ratio]). Purified CD4+ T cells (900 x 10⁶ cells at culture initiation) were propagated in polyolefin bags (Baxter) using X-VIVO 20 media.
(Lonza), 5% donor plasma, recombinant human (rhu) IL-4 (1000 I.U./ml; Schering), rhu IL-2 (20 I.U./ml; Chiron), and Sirolimus® oral solution (Wyeth; 1 µM); cytokine- and rapamycin-replete media was added every 2-3 days to maintain cell concentrations at < 1 x 10^6 cells/ml. After 12 days, beads were removed; T cells were washed to remove cytokines and sirolimus, and then cryopreserved. All infused T-Rapa products met release criteria, which included: CD4 cell purity > 70% (median CD4 purity was 99%), CD8 cell content < 5% (median CD8 content was < 0.1%), viability > 70% (median viability was 95%), absence of bacterial and fungal growth, absence of endotoxin content by limulus assay, negative mycoplasma test, and < 100 magnetic beads per 3 x 10^6 cells. T-Rapa products were manufactured centrally (NIH Clinical Center Department of Transfusion Medicine).

GENE EXPRESSION PROFILING

Total RNA was extracted (mRNA Easy Kits; Qiagen), quantified (ND-1000 Spectrophotometer; NanoDrop), and quality was verified (2100 Bioanalyser; Agilent). RNA was amplified, Cy5-labeled, and hybridized (Agilent chip) alongside Cy3-labeled Human Reference RNA (Stratagene). Microarrays were scanned and images analyzed (Agilent; Software 9.5.1.1). Data were analyzed with BRB Array Tools (http://linus.nci.nih.gov/BRB-ArrayTools.html; 34,051 of 41,687 genes were evaluable; accession number, GSE34911).

T CELL PHENOTYPE

Culture supernatants were evaluated for Th1 cytokines (IFN-γ, IL-2, TNF-α), Th2 cytokines (IL-4, -5, -10, and -13), and IL-17 (Luminex; Bio-rad). For supernatant generation, T-Rapa products (culture day 12) were re-stimulated for 24 hours; to assess phenotype stability, T-Rapa products were co-stimulated, expanded without rapamycin or polarizing cytokines until culture
day 18, and re-stimulated for supernatant generation. To evaluate post-HCT cytokine phenotype, peripheral blood lymphocytes were isolated (weeks 1, 2, 4, and 7-8) and co-stimulated; supernatants were evaluated for cytokine content (Luminex). For transcription factor detection, T cells were cultured (4 h, no co-stimulation; with GolgiStop and GolgiPlug, BD), washed, surface stained (anti-CD4; -CD25), fixed and permeabilized (Fix/Perm buffer; eBioscience), and stained (anti-GATA-3; -T-bet; -FoxP3). Antibodies were purchased (BD, Biolegend, eBioscience); 6-color flow was performed (LSRII; BD). Post-HCT immune cell numbers were quantified.

STATISTICAL ANALYSES

Gene expression was analyzed (Genomic Suite 6.4; Partek); induced genes were selected by t-test (filters of p < 0.001, FDR < 0.01). For hierarchical clustering, genes and samples were organized with Pearson correlation metric dissimilarity to measure distance on gene-averaged values. Enrichment of biological pathways for differentially expressed genes and Gene Ontology designations were determined using the Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics resource; specific gene annotations were retrieved on Gene Cards (http://www.genecards.org/index.shtml). Changes in laboratory parameters between stated time points were analyzed using absolute or relative differences, depending on change distribution. Differences were tested to determine if they differed from zero. The GATA-3:T-bet ratio was tested to determine if it was different from one using Wilcoxon signed-rank analyses. P-values are presented without multiple comparison adjustment, as this is an exploratory analysis. The cumulative incidence of acute GHVD (combining both classical acute and late acute forms, including liver transaminase elevation) and the cumulative incidence of chronic GVHD were determined using the method of Gooley, adjusting for the competing risk of death;
the cumulative incidence of relapse was adjusted for the competing risk of death due to transplant-related mortality.

RESULTS

TH2/TH1 PHENOTYPE OF T-RAPA CELLS

During the 12-day culture interval, median CD4⁺ T cell number was 6.4-fold increased relative to day 0 culture input values (range, 4.5- to 19.1-fold increased). Relative to culture input CD4⁺ T cells, T-Rapa cells differentially expressed 18.1% (6147/34,051) of mRNA species, with similar numbers of genes up-regulated (3185) or down-regulated (2962) (illustrated in heat map, Figure 2). By gene ontology analysis, the five gene families most significantly up-regulated in T-Rapa cells relative to day 0 culture input CD4⁺ T cells were: cell cycle, DNA metabolism, stress response, glucose catabolism, and oxidative reduction; representative gene members in these families were up-regulated 23- to 92-fold above values in the day 0 culture input cells (Supplemental Table I). On the other hand, the five gene families most significantly down-regulated in T-Rapa cells relative to the day 0 culture input CD4⁺ T cells were: apoptosis, transcription, inflammation, cytokine production, and immune response; representative gene members in these families were down-regulated 45- to 341-fold below values in the day 0 culture input cells (Supplemental Table I). In spite of this evidence that cytokine and immune response genes were dramatically down-regulated in T-Rapa cell products, a limited number of Th2 and Th1 genes were significantly up-regulated in T-Rapa cells: most notably, the Th2 cytokine IL-13 was 21.5-fold and the Th1 cytokine IL-12Rβ2 was 18.4-fold, respectively. The gene expression pattern of T-Rapa cells was remarkably reproducible: in an evaluation of n=21
T-Rapa cell clinical products, the inter-product gene expression variability by intra-class correlation coefficient analysis was 0.93.

T-Rapa products had minimal contamination with CD4⁺Foxp3⁺ cells (<1%) and preferentially expressed GATA-3 (median CD4⁺GATA-3⁺, 11.8%) relative to T-bet (median CD4⁺T-bet⁺, 5.1%); the median intra-product GATA-3/T-bet ratio was 2.1 (Figure 3A). T-rapa products secreted low levels of Th2 cytokines, which increased after extended culture without polarizing cytokines and rapamycin (Figure 3B; median values; d12 to d18 of culture: IL-4 [1 to 11 pg/ml], IL-5 [21 to 363 pg/ml], IL-10 [10 to 159 pg/ml], and IL-13 [24 to 725 pg/ml]). T-Rapa products secreted low levels of IFN-γ and TNF-α, which increased after extended culture (IFN-γ [10 to 418 pg/ml]; TNF-α [1 to 41 pg/ml]). T-Rapa cell IL-2 secretion actually decreased after extended culture, whereas IL-17 secretion increased from undetectable levels to <10 pg/ml after extended culture.

CONVERSION OF MIXED CHIMERISM AFTER T-RAPA INFUSION

Although each patient engrafted with donor cells, the low-intensity conditioning yielded mixed donor/host T lymphoid chimerism at day 14 post-HCT (Figure 4A; median donor chimerism, 61%). After T-Rapa cell infusion at day 14 post-HCT, median values increased to 89% and 94% at days 28 and 100 post-HCT, respectively. Median estimated absolute number of donor CD4⁺ T cells increased from 89/µl (day +14) to 198/µl and 250/µl (days 28 and 100 post-HCT). Reciprocally, median estimated numbers of host CD4⁺ T cells decreased from 67/µl (day +14) to 24/µl and 8/µl (days 28 and 100 post-HCT). Median estimated numbers of donor CD8⁺ T cells did not increase significantly after T-Rapa cell infusion (values at days 14, 28, and 100 post-HCT: 89, 92, and 158 cells/µl, respectively); in contrast, median estimated numbers of host
CD8⁺ T cells decreased from 51/µl (day +14) to 13/µl and 6/µl (days 28 and 100 post-HCT). As detailed in Supplemental Table II, the T-Rapa cell products were comprised primarily of central memory CD4⁺ T cells (mean value, 66.4%). Immune reconstitution post-SCT was characterized by relatively balanced numbers of naïve, central memory, and effector memory cells in the CD4 compartment through day 180 post-SCT; by comparison, CD8 cell immune reconstitution was biased towards the effector memory subsets, including both CD45RA⁺ and CD45RA⁻ populations (Supplemental Table II). At one year post-HCT, median values for CD4, CD8, and B cell counts were 423, 297, and 151 cells/µl, respectively (n=22 evaluated); at one year post-HCT, median values for serum IgG, IgM, and IgA were 502, 46, and 40 mg/dl, respectively (n=14 evaluated). Donor myeloid chimerism was mixed at day 14 post-HCT (Figure 4B; median, 37%); after T-Rapa cell infusion, median values increased to 81% and 99% at days 28 and 100 post-HCT.

T-RAPA RECIPIENTS EXPRESS A BALANCED TH2/TH1 PROFILE

Low frequencies of Th2 and Th1 cells were detected at day 14 post-HCT just prior to T-Rapa cell infusion (Figure 5A, median values; CD4⁺GATA-3⁺, 0.6%; CD4⁺T-bet⁺, 1.1%). After T-Rapa cell infusion, median frequencies of CD4⁺GATA-3⁺ cells increased to 20.2% and 13.9% at one and three months post-HCT, respectively; median frequencies of CD4⁺T-bet⁺ cells modestly increased to 4.0% and 1.8% (one and three months post-HCT). The median intra-patient GATA-3/T-bet ratio was 0.6 at day 14 post-HCT, and increased to 5.4 and 8.1 at one and three months post-HCT, respectively. Median frequencies of CD4⁺FoxP3⁺ cells were low at day 14 post-HCT (0.3%) and increased at one and three months post-HCT (2.5% and 2.4%).
Post-HCT T cells secreted low levels of Th2 cytokines at days 7 and 14 post-HCT (Figure 5B; IL-4, IL-5, IL-10, and IL-13 values were typically < 10 pg/ml); after T-Rapa cell infusion, these values were generally increased at one and three months post-HCT. By comparison, post-HCT T cells secreted higher levels of Th1 cytokines at days 7 and 14 post-HCT (IL-2, INF-\( \gamma \), and TNF-\( \alpha \) levels ranging from 100 to 10,000 pg/ml); after T-Rapa cell infusion, these values were either stable or increased at one and three months post-HCT. Post-HCT T cell secretion of IL-17 was not detected prior to T-Rapa infusion and modestly increased at one and three months post-HCT. Just prior to T-Rapa infusion (at day 14 post-HCT), low frequencies of post-HCT CD4\(^+\) and CD8\(^+\) T cells secreted cytokines by cytokine capture flow cytometry analysis; by comparison, at day 28 post-HCT, increased frequencies of both CD4\(^+\) and CD8\(^+\) T cells secreted the type I cytokines IL-2 and IFN-\( \gamma \) and the type II cytokines IL-4 and IL-10 (Supplemental Figure 1).

To evaluate antigen-specific T cell responses post-HCT, transplant recipients were evaluated for immune responses against CMV. In three of the four HLA-A02\(^+\) recipients who developed CMV viremia post-HCT, there was an increased frequency of CMV-specific T cells by flow cytometry analysis (Supplemental Figure 2); by comparison, each of the six HLA-A02\(^+\) recipients who did not develop CMV viremia post-HCT had frequencies of CMV-specific T cells near background levels. In addition, in 13 transplant cases where the donor or host (or both) was CMV seropositive, we observed increased secretion of IFN-\( \gamma \) and IL-4 in response to overlapping CMV peptides at day 60 post-HCT (Supplemental Table III). Consistent with their minimally differentiated effector state, the T-Rapa cell products secreted minimal cytokines in response to either overlapping CMV peptides or a superantigen-like positive control stimulation.
Finally, we evaluated the T cell receptor V-β repertoire of both the T-Rapa cell products and day 60 post-HCT CD4+ T cells: the T-Rapa products had a diverse T cell repertoire similar to normal donor CD4 cells whereas the post-HCT CD4+ T cells tended to have a more skewed T cell receptor repertoire (Supplemental Figure 3).

PATIENT CHARACTERISTICS AND POST-HCT OUTCOME

Median patient age was 51 years (range, 23-69; 17 females, 23 males; Tables I and II). To assess risk of disease progression after reduced-intensity HCT\(^4\), patients were classified as having low-risk (n=9, 22.5%), standard-risk (n=7, 17.5%), or high-risk (n=24, 60%) diagnoses. Median number of prior regimens was 3 (range, 1 to 5). Twenty of 40 patients (50%) were refractory to their most recent prior chemotherapy, with eight being primary refractory. Twenty-three of 40 patients (57.5%) were refractory to an outpatient chemotherapy regimen consisting of EPOCH-FR\(^2\). Thirty-two of 40 patients (80%) proceeded to low-intensity transplant with measurable disease.

There were no deaths directly related to transplantation. There were no infusional toxicities or serious adverse events attributable to T-Rapa cell therapy. There were no cases of veno-occlusive disease, engraftment syndrome, or transplant-associated microangiopathy. CMV viremia by DNA-PCR test was detected in 3/21 (14.3%) of CMV-negative recipients and 6/19 (31.6%) of CMV-positive recipients. One of 40 recipients (5%) developed CMV infection (gastritis); one patient developed disseminated adenoviral infection after intensive therapy of progressive AML. Sixteen of 40 patients (40%) had bacteremia (n=18 episodes) or candidemia (n=1) attributable to neutropenia and/or line infection; there were no cases of bacterial or fungal pneumonia, one case of mycobacterium avium complex pneumonia, and one case of fungal infection (Paecilomyces...
variotti). Late, non-relapse mortality was due to post-surgical pulmonary embolus (n=1) and myocardial infarction (n=1).

Classical acute GVHD (grade II-IV through day 100 post-HCT) was observed in 4/40 patients (10%; each case steroid-responsive); in three cases, acute GVHD was preceded by early immune suppression taper and/or unmanipulated donor lymphocyte infusion for therapy of early malignant disease progression. Late acute GVHD, which occurred in 14/37 patients (37.8%), consisted primarily of liver transaminitis without other organ involvement (9/14 patients); four cases of late acute gut GVHD occurred (each case steroid-responsive). After combining all cases of classically-defined acute GVHD and all cases of late acute GVHD (including liver transaminase elevation, which occurred prior to day 100 post-HCT in some patients), there was a cumulative incidence probability of acute GVHD of 20% at day 100 post-HCT and 40% at day 180 post-HCT. Seventeen of 37 evaluable patients (45.9%) had classical chronic GVHD, with global severity scores of mild (n=9), moderate (n=6), or severe (n=2); median number of organs involved was 2 (range, 1 to 4), with tissue distribution of skin (n=15), oral (n=10), ocular (n=7), vulvo-vaginal (n=3), and lung (n=2); the cumulative incidence probability of chronic GVHD at 2 years post-HCT was 42.5%. Ten of 40 patients (25%) developed overlap GVHD (classical chronic in combination with classical or late acute); sixteen of 40 patients (40%) did not develop any form of GVHD.

Each patient with low-risk diagnoses achieved complete remission, although one patient died from isolated CNS disease; six of 9 low-risk diagnosis recipients (66.6%) are in sustained complete remission (median follow-up, 1647 days post-HCT; range, 1312-2566). Three of 7 patients (42.9%) with standard-risk diagnoses are in sustained complete remission at days 1326,
1356, and 2468 post-transplant. Nine of 24 recipients (37.5%) with high-risk diagnoses are in sustained complete remission (median follow-up, 1402 days post-HCT; range, 1285-1669). Post-transplant chemotherapy and/or unmanipulated donor lymphocyte infusion contributed to sustained complete remission in 5/18 (27.8%) of patients; a total of 22 patients received chemotherapy and/or DLI for management of progressive disease. The cumulative probability of disease progression was 32.5% at 6-months post-HCT, 50% at 12-months post-HCT, 57.5% at 24-months post-HCT, and 57.5% at 36 months post-HCT. In total, eighteen of 40 patients (45%) remain in sustained complete remission (range of follow-up: 42 to 84 months).

**DISCUSSION**

Reduced-intensity allogeneic HCT yields mixed donor/host chimerism, reduces morbidity and mortality after transplant, and places the therapeutic emphasis on donor immunity rather than host conditioning. However, progress in the field has been generally restricted by an inability of current approaches to: (1) preferentially favor donor immunity rather than residual host immunity; and (2) balance donor T cell effects once they predominate. As a result, transplant outcome can be variably limited by graft rejection, persistent mixed chimerism with associated reduced GVT effects, and GVHD. These obstacles would be anticipated to be particularly relevant with the low-intensity of chemotherapy that we used in this study, which is 75% lower in alkylator dose than the reduced-intensity regimen that we previously evaluated. Here, we describe a new transplant platform that begins to overcome these obstacles through a unique allograft augmentation strategy that uses ex vivo rapamycin to manufacture a novel donor T cell product that promotes a balanced Th2/Th1 immune reconstitution.
Ex vivo application of rapamycin to the T cell manufacturing process yielded CD4$^+$ effector T cells that expressed a mixed Th2/Th1 phenotype. Micro-array analysis of such T-Rapa cells revealed a broad-based gene expression identity that was remarkably reproducible during clinical trial implementation; such reproducibility was achieved with a manufacturing method that incorporated clinical product cryopreservation and shipment to a multi-center site, thereby demonstrating that subsequent, definitive trials using T-Rapa cells will be feasible. Further studies will be required to evaluate if this gene expression identity dictates functional characteristics of the T-Rapa cell product; in subsequent clinical trials, it may be advantageous to use the gene expression identity as a release criterion for the T-Rapa cell product. Consistent with our results in experimental models$^{16,18,21}$, the clinical T-Rapa cell products had down-regulation of apoptosis genes and were minimally differentiated on the basis of global down-regulation of inflammation, cytokine production, and immune response genes. T-Rapa products were minimally contaminated with FoxP3$^+$ cells and thus distinct from T$_{REG}$ cell products evaluated for GVHD prevention in the cord blood transplantation setting$^{32}$. At the time of infusion, T-Rapa cell products secreted low levels of cytokines and had a diverse T cell receptor repertoire; this observation further supports our conclusion that T-Rapa cells represent minimally differentiated effectors, which in experimental models$^{33}$, mediate increased therapeutic effects. T-Rapa cell products secreted high levels of cytokines ex vivo after removal of rapamycin (thus demonstrating their effector potential) and maintained a mixed Th2/Th1 profile ex vivo without exogenous cytokine addition and in the absence of rapamycin (thus illustrating their limited polarization plasticity$^{34}$). The T-Rapa cell products were therefore comprised of minimally-differentiated Th2/Th1 effector CD4$^+$ T cells rather than an anergic T cell population$^{35}$ or a T$_{REG}$-
enriched population observed in other experimental systems that also evaluated ex vivo rapamycin.

The clinical treatment platform we evaluated can be considered a low-intensity regimen as it can be administered in the outpatient setting and universally resulted in mixed donor/host T cell chimerism, with a median of only 61% donor chimerism at day 14 post-HCT. This result, which stands in contrast to results using a reduced-intensity regimen that we previously evaluated, is likely due to two aspects of the current platform: (1) a 75% reduction in cyclophosphamide intensity during host conditioning; and (2) a requirement that the host CD4 count be <200 cells/µl prior to transplantation rather than the more stringent previous value that we previously utilized (<50 cells/µl). Pre-emptive DLI with T-Rapa cells in this setting was associated with the conversion of mixed chimerism towards predominant donor chimerism; no graft rejection occurred in T-Rapa recipients, which included many patients with <50% donor T cell chimerism at day 14 post-HCT (n=14). Previously, this level of mixed chimerism at the day 14 post-HCT time-point was associated with increased graft rejection. It is difficult to compare engraftment results across clinical trials because of differences in patient selection and pre-transplant treatment history; nonetheless, the current results indicate that the T-Rapa cell DLI did not promote donor-host tolerance or impair donor T cell responses post-transplant. Although the T-Rapa cell DLI likely contributed to the rather rapid increase in donor chimerism, definitive clinical trials evaluating this transplant regimen without a DLI or with a control DLI consisting of unmanipulated donor T cells would be required to better address this question. Of note, previous investigations have evaluated CD4-enriched DLI, including in the following settings:
for decreasing chimerism after T cell-depleted transplantation\textsuperscript{38}; for treatment of post-transplant relapse\textsuperscript{39}; or, when used in combination with IL-2 therapy, for the treatment of chronic GVHD\textsuperscript{40}.

In contrast to autologous adoptive T cell therapy approaches that maximize host immune depletion through high-dose conditioning and immediate T cell transfer\textsuperscript{41}, T-Rapa cells were administered remote from conditioning, in a state of host immunity that was relatively T cell-replete, and during calcineurin inhibitor therapy. Nonetheless, the CD4-purified donor T-Rapa cells appeared to break immune tolerance and predominate in vivo, as indicated by: (1) a rapid post-HCT increase in donor CD4\(^+\) T cells; (2) restricted expansion of donor CD8\(^+\) T cells; (3) concomitant reductions in host CD4\(^+\) and CD8\(^+\) T cells; (4) balanced CD4\(^+\) Th2/Th1 immune reconstitution with modest reconstitution of T\textsubscript{REG} cells; and (5) conversion of early mixed myeloid chimerism through an apparent graft-versus-myeloid lineage effect. The donor CD4-predominance observed after T-Rapa cell DLI suggests that the infused product expanded in vivo; however, the T-Rapa cells were not labeled for cell tracking, and as such, it is possible that CD4 cells contained in the mobilized allograft may have also contributed to the observed pattern of immune reconstitution. Indeed, our finding that both CD4\(^+\) and CD8\(^+\) T cells had balanced secretion of Th1 and Th2 cytokines post-HCT suggests that the CD4-purified T-Rapa cell DLI may have resulted in the in vivo modulation of T cells emanating from the mobilized allograft. Nonetheless, the preferential expansion of donor CD4 cells rather than donor CD8 cells after T-Rapa cell DLI stands in marked contrast to other clinical results in myeloablative or non-myeloablative transplantation, where a predominant CD8 cell reconstitution has been observed\textsuperscript{42}. Taken together, these results suggest that T-Rapa cells may represent a particularly potent
effector T cell population, as an array of biologic effects were observed after adoptive transfer in the face of systemic immune suppression.

Only 4/40 patients (10%) developed classical acute grade II-IV GVHD through day 100 post-HCT, which compares favorably to our first-generation clinical trial of ex vivo expanded CD4 cells where we observed a rate of 64% (18/28 cases)\textsuperscript{22}. The low rate of classical acute GVHD we observed is similar to results obtained using host conditioning with total lymphoid irradiation\textsuperscript{43}, which also promoted Th2 cytokines. We did observe a significant incidence of late acute GVHD, in particular liver transaminase elevation (which occurred in several patients before day 100 post-HCT), which resulted in a cumulative overall incidence probability of all forms of acute GVHD of 20% and 40% at days 100 and 180 post-HCT. Several factors may have influenced the incidence, type, and severity of acute GVHD in our study, including: balanced CD4\textsuperscript{+} Th2/Th1 immune reconstitution post-transplant with restricted donor CD8\textsuperscript{+} T cell expansion post-transplant, low-level persistent mixed T cell chimerism\textsuperscript{44}, low-intensity conditioning\textsuperscript{45}, and sirolimus prophylaxis\textsuperscript{46}. With respect to this latter point, in a recent study performed by our collaborators\textsuperscript{47}, the rate of acute grade II-IV GVHD in recipients of a matched related donor transplant after reduced-intensity conditioning and GVHD prophylaxis consisting of cyclosporine plus a short course of peri-transplant sirolimus without T-Rapa cell DLI was 27% (6/22 cases). Classical chronic GVHD was primarily of mild-to-moderate global severity. A substantial proportion of patients did not develop acute or chronic GVHD. As such, the overall GVHD profile of the current platform was not excessive, particularly considering that ex vivo activated donor T cells were administered in the context of a T cell replete peripheral blood allograft.
This study provided a rigorous clinical test for immune GVT effects because of the low-intensity of host conditioning and because of the patient population, which had relatively low percentages of patients having a low-risk malignancy diagnosis (22.5%), receiving transplantation in complete remission (18%), and having chemotherapy-sensitive disease (50%). By comparison, previous studies using other low-intensity conditioning methods had higher frequencies of patients transplanted in remission and having low-risk diagnoses. In our trial, sustained complete remissions were achieved in some patients with primary refractory disease and diagnoses such as refractory CML that are difficult to eradicate with reduced-intensity transplantation. As such, the current transplant platform appears to be suitable in terms of anti-tumor potency for the majority of patients typically considered for reduced-intensity transplantation. However, T-Rapa cell infusion did not promote sufficient GVT effects in patients with refractory diffuse large cell NHL or multiply relapsed AML; ongoing efforts seek to overcome this GVT limitation through infusion of T-Rapa cells with an increased Th1 phenotype or enhanced tumor specificity through incorporation of chimeric antigen receptors.

In sum, in this first-in-human clinical trial of ex vivo manufactured allogeneic T-Rapa cells, we have demonstrated that the combined use of ex vivo and in vivo sirolimus can be combined to provide a new platform for the safe implementation of low-intensity allogeneic hematopoietic cell transplantation. These phase II clinical trial results suggest that the CD4-purified T-Rapa cells mediated distinct effects in vivo when administered as a pre-emptive DLI, in particular the rapid conversion of mixed chimerism towards full donor chimerism that was predominated by CD4+ T cells of a balanced Th2/Th1 cytokine phenotype. Pre-emptive T-Rapa cell infusion after low-intensity allogeneic HCT therefore represents a suitable platform for further studies. Our
demonstration that the T-Rapa cell product can be safely administered in a multi-center manner indicates that it will be feasible to perform subsequent randomized studies comparing T-Rapa cell-based therapy to other types of DLI or other transplant regimens.
ACKNOWLEDGMENTS

This work is dedicated to the memory of our good friend and collaborator in this research, Dr. Thea Friedman, who recently tragically passed away much too young. Special recognition goes to numerous individuals who made outstanding contributions to this work, including: Bazetta Blacklock-Schuver, R.N. and Sheila Phang, R.N. for their creative and efficient efforts in patient recruitment and care coordination; Roger Kurlander, M.D. NIH Department of Laboratory Medicine, for his collaboration and professionalism in chimerism analysis; Maryalice Stetler-Stevenson, M.D. and Constance Yuan, M.D. for their care in providing clinical flow cytometry analyses; Jeanne Odom, R.N., Paula Layton, R.N., and Brenna Hansen, R.N. for excellence in research nursing; Vicki Fellowes, for her irreplaceable efforts towards the development and implementation of T-Rapa cell manufacturing; Xiao-Yi Yan, Ph.D., Sarfraz Memon, M.D., Shoba Amarnath, Ph.D., Tania Felizardo, Ph.D., Jason Foley, M.S., M.B.A., and Yelena Kogan, M.D. for their efforts with respect to laboratory studies and immunology endpoints; Elizabeth J. Read, M.D., for her involvement in cell manufacturing at the NIH during initial aspects of study implementation; Daniele Avila, Amanda Urban, Jennifer Mann, and Tiffani Taylor for their excellence and dedication in the care of protocol patients; Suzanne Murphy for expert assistance with the protocol and Investigation New Drug Application; the NIH Clinical Center and Department of Nursing; the Medical Oncology Fellows at the NCI; and numerous individuals at HUMC, including Tatyana Feldman, M.D, Anthony Mato, M.D., Carol Carini, R.N., and Andrea Ortega, R.N.
AUTHOR CONTRIBUTIONS

Daniel H. Fowler, M.D. (designed trial, patient care, data interpretation, wrote manuscript), Miriam E. Mossoba, Ph.D. (performed laboratory endpoint experiments, data interpretation, assisted in writing manuscript), Seth M. Steinberg, Ph.D. (statistician; data analysis and interpretation), David C. Halverson, M.D. (patient care, data interpretation), David Stroncek, M.D. (clinical product manufacturing, data interpretation), Hahn M. Khuu, M.D. (clinical product manufacturing, data interpretation), Frances T. Hakim, Ph.D. (performed laboratory endpoint experiments, data analysis, assisting in writing manuscript), Luciano Castiello, Ph.D. (performed laboratory endpoint experiments), Marianna Sabatino, M.D. (performed laboratory endpoint experiments), Susan F. Leitman, M.D. (protocol design, transfusion medicine support), Jacopo Mariotti, M.D. (patient care, protocol design), Juan C. Gea-Banacloche, M.D. (patient care, data analysis), Claude Sportes, M.D. (patient care, data analysis), Nancy M. Hardy, M.D. (patient care), Dennis D. Hickstein (patient care), Steven Z. Pavletic, M.D. (patient care, data analysis), Scott Rowley, M.D. (protocol design, patient care), Andre Goy, M.D. (protocol design, patient care), Michele Donato, M.D. (protocol design, patient care), Robert Korngold, Ph.D. (protocol design), Andrew Pecora, M.D. (patient care, research support), Bruce L. Levine, Ph.D. (protocol design, manufacturing of clinical cellular product), Carl H. June, M.D. (protocol design, manufacturing of clinical cellular product), Ronald E. Gress, M.D. (protocol design, patient care, assisted in writing manuscript), and Michael R. Bishop, M.D. (protocol design, patient care, data analysis, assisted in writing manuscript).
CONFLICT OF INTEREST STATEMENT

Daniel H. Fowler, Ronald E. Gress, Bruce L. Levine, and Carl H. June are listed as co-inventors on a U.S. Patent relating to Rapamycin Resistant T Cells and Therapeutic Uses Thereof (US-7718196-2010).
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Clinical Trial of Allogeneic T-Rapa Cells


30. Kozuka T, Sugita M, Shetzline S, Gewirtz AM, Nakata Y. c-Myb and GATA-3 Cooperatively Regulate IL-13 Expression via Conserved GATA-3 Response Element and


Table I: Transplantation Outcome According to Malignancy Risk Factors

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<sup>a</sup>Risk of progressive disease post-HCT: Level I: low risk; Level II: standard risk. UPN, unique patient number; M, male; F, female.

<sup>b</sup>Diagnoses (Dx): mantle cell lymphoma (MCL); myelodysplastic syndrome (MDS); follicular non-Hodgkins lymphoma (FCL); myelofibrosis (MF); chronic myelogenous leukemia (CML); chronic lymphocytic leukemia (CLL); multiple myeloma (MM). #, number of prior regimens. Res, disease response: 1º R, primary refractory; S, sensitive to last regimen; and R, refractory to last regimen. Pre-HCT, indicates disease status at time of low-intensity transplant: Res, response to EPOCH-F(R): PR, partial response; CR, complete response; SD, stable disease; hCR, hematologic CR. NED, no evidence of disease status (Yes/No).

<sup>c</sup>Acute GVHD score of skin (s), gut (g), and liver (l); Gr, overall grade (0-IV). Late acute GVHD, manifested as elevated liver transaminase levels (tr) or gut involvement (g). Chronic GVHD sites of involvement: oral (o), skin (s), eye (e), vaginal (v), lung (l).

<sup>d</sup>Overall outcome for last tumor staging: PD, progressive disease; CR, complete remission; mCR, molecular CR; SD, stable disease. Ongoing survival, in days post-HCT (+). Death, day post-HCT; cause due to progressive disease (PD), presumed pulmonary embolus (PE), or myocardial infarction (MI).
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<sup>a</sup>Risk of progressive disease post-HCT: Level III: high risk. UPN, unique patient number; M, male; F, female.

<sup>b</sup>Diagnoses (Dx): acute myelogenous leukemia (AML); non-Hodgkins lymphoma (NHL), diffuse large cell (DLC); Hodgkins disease (HD); anaplastic T cell NHL (A-TCL); EBV-driven DLC (DLC-EBV); transformed follicular NHL to DLC [DLC(trFL)]; gray-zone NHL (NHL-GZ); chronic myelogenous leukemia (CML); chronic lymphocytic leukemia (CLL) transformed to DLC [DLC(trCLL)]; plasmablastic dendritic cell NHL (NHL-pDC). #, number of prior regimens. Res, disease response to last prior regimen: S, sensitive; R, refractory; 1º R, primary refractory. Pre-HCT, indicates disease status at time of low-intensity transplant; Res, response to EPOCH-F(R): PR, partial response; CR, complete response; SD, stable disease; hCR, hematologic CR; PD, progressive disease. NED, no evidence of disease status (Yes/NO).

<sup>c</sup>Acute GVHD score of skin (s), gut (g), and liver (l); Gr, overall grade (0-IV). Late acute GVHD, manifested as elevated liver transaminase levels (tr) or gut involvement (g). Chronic GVHD sites of involvement: oral (o), skin (s), eye (e), vaginal (v), lung (l).

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Figure Legends

Figure 1: Phase II Clinical Trial Design of Ex Vivo and In Vivo Sirolimus for Low-Intensity Allogeneic Hematopoietic Cell Transplantation. Donors underwent steady-state apheresis #1, whereby CD4+ T cells were purified by positive selection, co-stimulated with anti-CD3 and anti-CD28, and exposed to IL-4, IL-2, and rapamycin in culture for 12-days; the resultant “T-Rapa” cell product was cryopreserved and administered as a pre-emptive DLI at day 14 post-HCT. Donors underwent apheresis #2 after G-CSF mobilization; the peripheral blood stem cell (PBSC) product was unmanipulated (T cell replete). The recipient was treated with four consecutive days (days -6 through -3) with concomitant fludarabine (Flu, 30 mg/m²/d) and cyclophosphamide (Cy, 300 mg/m²/d). GVHD prophylaxis consisted of short-course sirolimus (from day -2 until day +14 post-HCT) and cyclosporine A (from day -1 until day +100 post-HCT).

Figure 2: T-Rapa cell products have relatively equal numbers of genes that are up- or down-regulated in expression relative to culture input CD4+ T cells. T-Rapa cell products were manufactured ex vivo (n=6) and compared to the purified CD4+ T cells used to initiate the cultures (n=6). RNA was purified from each paired sample, with further analysis by gene expression microarray. The heat map illustrates that genes were consistently differentially expressed, with 18.1% of genes (6147/34,051) being differentially expressed in T-Rapa products relative to input CD4 cells (p<0.001); the number of up-regulated genes in T-Rapa cells was relatively equal to the number of down-regulated genes.

Figure 3. T-Rapa cells express a balanced Th2/Th1 cytokine phenotype. (A) T-Rapa cell clinical products were analyzed by intra-cellular flow cytometry for expression of GATA-3 (Th2 cell transcription factor), T-bet (Th1 cell transcription factor), and FoxP3 (TREG cell transcription
factor) (left panel). The intra-product ratio of GATA-3:T-bet in T-Rapa cells was greater than 1:1 (*, p<0.05). (B) T-Rapa cell clinical products were evaluated at the time of DLI (day 12 of culture) and after an additional culture interval intended to evaluate effector function and differentiation plasticity (day 18 of culture). At each time point, the T-Rapa cells were co-stimulated, and the resultant supernatants were tested for cytokine content by Luminex (mean ± SEM; n=40; *, increase from day 12 to day 18, p<0.05). Values are expressed as pg/ml (1 x 10^6 cells per ml per 24 hours).

Figure 4: T-Rapa cell infusion results in predominate donor CD4^+ T cell reconstitution. (A) Percent donor T lymphoid chimerism for each patient at days 14, 28, and 100 post-HCT (top panel; ***, d28 > d14 and d100 > d28; p<0.0001). Post-HCT numbers of donor vs. host CD4^+ or CD8^+ T cells were estimated by multiplying CD4 and CD8 cell absolute numbers by percent CD3 chimerism values. The figure shows median estimated values for absolute numbers of donor and host CD4^+ T cells (left-panel) and CD8^+ T cells (right panel) at days 14, 28, and 100 post-HCT (comparisons are d28 vs. d14 and d100 vs d28; ***, p<0.001; **, p<0.01; *, p<0.05; NS, not significant; between n=23 to n=33 evaluated for each paired analysis). (B) Percent donor myeloid chimerism for each patient at days 14, 28, and 100 post-HCT (***, d28 > d14 and d100 > d28; p<0.0001).

Figure 5: T-Rapa cell recipients have immune reconstitution of a mixed Th2 and Th1 cytokine phenotype. (A) Percentage of CD4^+ T cells expressing GATA-3, T-bet, and FoxP3 as measured by intra-cellular flow cytometry at day +14 post-transplant (just before T-Rapa cell DLI), at one month, and at three months post-HCT (comparisons are one month vs. day 14 and 3 months vs. day 14; **, p<0.001; *, p<0.05). Intra-patient ratio of GATA-3 to T-bet expressing CD4^+ T cells
is shown for each time point. (B) At one, two, four, and eight weeks post-HCT, peripheral blood mononuclear cells were co-stimulated, and the 24 hour supernatant was tested for cytokine content by Luminex (mean ± SEM; between n=32 to n=34 evaluated for each paired analysis; comparisons are week 4 vs. week 2 and week 8 vs. week 2; **, p<0.001; *, p<0.01).
Figure 1

Donor

Apheresis #1 CD4⁺ anti-CD3, anti-CD28 IL-4, IL-2, rapamycin

Apheresis #2 G-CSF PBSC Product

Recipient

-6 -5 -4 -3 -2 -1 0 14 100

Flu, 30 mg/m²/d x 4 Sirolimus, 3-12 ng/ml

Cy, 300 mg/m²/d x 4 Cyclosporine A, 200-250 μg/ml

Clinical Trial of Allogeneic T-Rapa Cells
Figure 2
Figure 3

A

Transcription Factor Levels

GATA-3: T-bet Ratio

B

IL-4

IL-5

IL-10

IL-13

IL-17

[cytokines] (pg/ml)

Day 12
Day 18

Day 12
Day 18

Day 12
Day 18

Day 12
Day 18

Day 12
Day 18

Day 12
Day 18
Figure 5

A

GATA-3

% Positive

2 WKs 1 MONTH 3 MONTHS

T-bet

% Positive

2 WKs 1 MONTH 3 MONTHS

GATA-3:T-bet Ratio

Ratio Value

2 WKs 1 MONTH 3 MONTHS

FoxP3

% Positive

2 WKs 1 MONTH 3 MONTHS

B

IL-4

[Cytokine] (pg/ml)

Weeks Post-transplant

IL-5

[Cytokine] (pg/ml)

Weeks Post-transplant

IL-10

[Cytokine] (pg/ml)

Weeks Post-transplant

IL-13

[Cytokine] (pg/ml)

Weeks Post-transplant

IL-17

[Cytokine] (pg/ml)

Weeks Post-transplant

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Phase II clinical trial of rapamycin-resistant donor CD4+ Th2/Th1 (T-Rapa) cells after low-intensity allogeneic hematopoietic cell transplantation


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