SUPPLEMENTARY INFORMATION

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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Supplementary Note

Determination of T Cell Naïve vs. Memory vs. Effector Differentiation Status. T cell differentiation status was evaluated by flow cytometry, as previously described[1]. Thawed patient PBMCs that were collected post-HCT and donor T-Rapa cell products were stained with a cocktail of antibodies: CD8 FITC (BD), CCR7 PE (R&D), CD45RA ECD (Beckman Coulter), CD39 PerCP e710 (eBioscience), CD25 PE-Cy7 (eBioscience), CD127 APC (eBioscience), CD3 Alexa700 (BD), CD27 APC e780 (eBioscience), the viability marker NFL (OncoImmunin), and CD4 KromeOrange (Beckman Coulter). Stained samples were run on a Gallios flow cytometer (Beckman Coulter).

Post-HCT T Cell Phenotype by Cytokine Capture Flow Cytometry. Cytokine capture flow cytometry was performed at week 2 post-HCT (just prior to T-Rapa infusion) and after T-Rapa infusion (week 4 post-HCT). PBMC were isolated and stimulated for 24 hours using anti-CD3, anti-CD28 coated beads; the resultant T cells were then stained for surface markers (CD4, CD8; antibodies obtained from BD Biosciences) followed by cytokine capture flow cytometry according to the manufacturer's instructions (Miltenyi Biotec Inc., Auburn, CA). Data were acquired using a FACS Calibur flow cytometer (BD Biosciences) and data analysis was performed using FlowJo software (Tree Star, Inc, Ashland, OR).

Detection of CMV-specific T Cells by Dextramer Staining. Post-HCT PBMC from transplant recipients or PBMC from normal donors were thawed, washed in PBS/5% FCS, and stained with PE-conjugated HLA-A*0201 NLVPMVATV hCMV pp65 or HLA-A*0201 Negative Control Dextramers (generous gift from Immudex, Copenhagen Denmark) and FITC-conjugated anti-CD8 (BD Biosciences, San Diego, CA). Stained cells were washed twice in PBS containing 5% FCS, resuspended in PBS, and stained with 7-AAD (BD Biosciences) prior to data acquisition using a FACS Calibur flow cytometer (BD Biosciences). Analyses were performed using FlowJo software (Tree Star, Inc.).

Characterization of CMV-specific Immunity by CMV Peptide Stimulation Assay. Post-HCT PBMC from transplant recipients and donor T-Rapa cell products were thawed, washed in PBS, and plated in triplicate in 48-well flat-bottom plates at a final concentration of 10^7/ml in X-vivo 20 media supplemented with 20 IU/ml of rhuIL-2. Plated cells were either left unstimulated for 48 hours or stimulated for 48 hours with a pool of reconstituted CMV-pp65 peptides or the provided positive control T cell stimulation (Cytostim), according to the manufacturer's instructions (Miltenyi Biotec Inc.). Supernatants were harvested and frozen at -20C° overnight until processed for cytokine level measurements by multiplex immunoassays, according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA).

Determination of T Cell Receptor Repertoire by Spectratyping Method. Normal donor and patient (post-HCT) PBMCs were sorted for $CD4^+$ cells using MS magnetic columns (Miltenyi

Biotec Inc.). Sorted normal donor or post-HCT $CD4^+$ cells and donor $CD4^+$ T-Rapa cell products were pelleted, resuspended in Trizol, and stored at -80 C°. Samples were processed for RNA isolation, cDNA synthesis, and V-beta specific PCR was performed and analyzed, as previously described[2].

Supplemental Table I. Gene ontology (GO) families most significantly altered in T-Rapa cell products relative to culture input $CD4^+$ T cells

GO Families	Gene Symbol	Gene Name	Fold-Change (vs. input CD4)
Up-regulated			
Cell Cycle	DTL	denticleless E3 uniquitin protein ligase homolog	63-fold Increase
DNA Metabolism	ESCO2	establishment of cohesion 1 homolog 2	49-fold Increase
Stress Response	TOP2A	topoisomerase (DNA) II alpha 170kDa	23-fold Increase
Glucose Catabolism	OGDGL	oxoglutarate dehydrogenase-like	92-fold Increase
Oxidative Reduction	MAOA	monoamine oxidase A	35-fold Increase
Down-regulated			
Apoptosis	IL1B	interleukin 1, beta	134-fold Decrease
Transcription	EREG	epiregulin	45-fold Decrease
Inflammation	S100A8	S100 calcium binding protein A8	341-fold Decrease
Cytokine Production	NLRP3	NLR family, pyrin domain containing 3	173-fold Decrease
Immune Response	CXCL2	Chemokine (C-X-C motif) ligand 2	55-fold Decrease

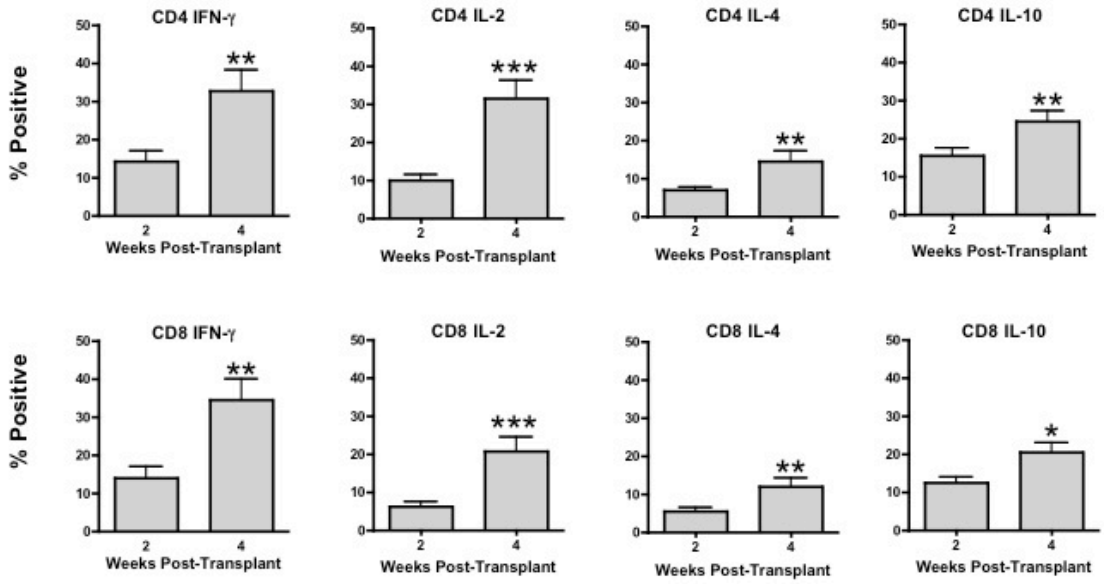
Supplemental Table II. Differentiation Status of T-Rapa Products and Post-HCT T Cells

Flow cytometry was used to characterize the differentiation status of T-Rapa cell products and post-HCT T cells (prior to T-Rapa infusion [day +14 post-HCT] and after T-Rapa infusion [days +60 and +180 post-HCT]). As previously detailed[1], differential expression of CD45RA and CCR7 was used to define naïve, central memory, effector memory, and effector subsets. Values are mean ± standard error of the mean. Abbreviations: N/A, not applicable; PBMC, peripheral blood mononuclear cells.

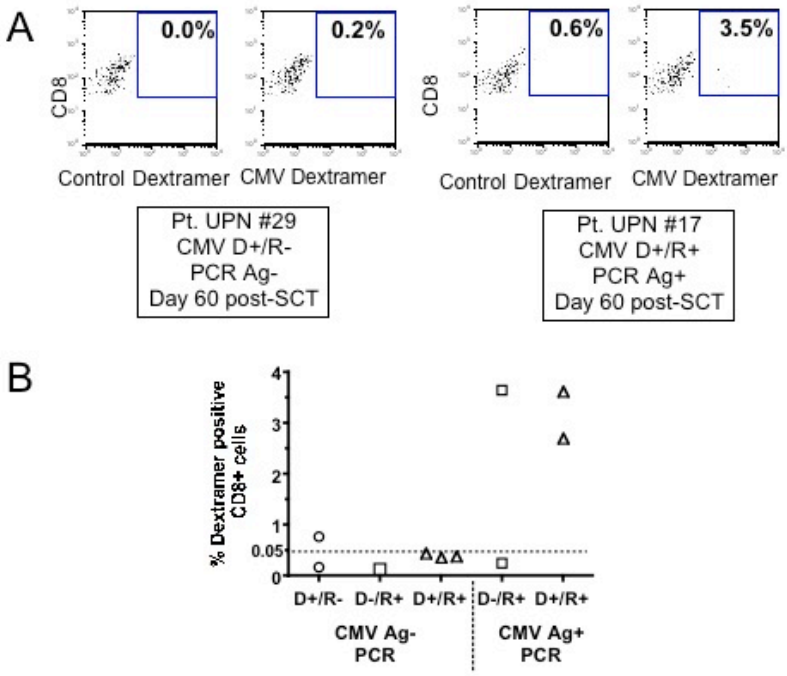
Sample Type	Time Post-HCT	n=	% Naive[1]	% CM[2]	% EM[3]	% TEMRA[4]
$CD4^+$ T-Rapa Products	N/A	5	1.3 ± 0.6	66.4 ± 2.8	31.7 ± 3.2	0.6 ± 0.2
$CD4^+$ PBMC	Day +14	5	24.7 ± 2.5	38.1 ± 6.2	32.3 ± 4.9	4.9 ± 2.5
	Day +60	5	38.5 ± 3.5	26.9 ± 3.9	27.8 ± 1.0	6.7 ± 1.6
	Day +180	5	38.1 ± 6.2	35.7 ± 3.9	24.3 ± 3.2	1.9 ± 0.5
$CD8^+$ PBMC	Day +14	5	26.9 ± 10.2	18.7 ± 5.3	36.6 ± 7.3	17.8 ± 5.8
	Day +60	5	16.7 ± 4.5	4.0 ± 1.4	40.9 ± 9.3	38.4 ± 9.1
	Day +180	5	23.5 ± 3.2	5.7 ± 1.2	30.5 ± 3.2	40.4 ± 5.2

[1] Naïve subset definition: $CD45RA^+$ $CCR7^+$
[2] Central Memory (CM) subset definition: $CD45RA^-$ $CCR7^+$
[3] Effector Memory (EM) subset definition: $CD45RA^-$ $CCR7^-$
[4] $CD45RA^+$ effector memory (TEMRA) subset definition: $CD45RA^+$ $CCR7^-$

Supplemental Figure 1. T-Rapa cell recipients have mixed type I and type II cytokine secretion in both post-HCT $CD4^+$ and $CD8^+$ T cells. At week 2 and week 4 post-HCT, peripheral blood mononuclear cells were co-stimulated for 24 hours and evaluated for cytokine secretion by cytokine capture flow cytometry for determination of the percentage of $CD4^+$ and $CD8^+$ cells secreting type 2 (IL-4 and IL-10) or type 1 (IFN-γ and IL-2) cytokines. Results shown are mean ± SEM, with between n=26 to n=28 evaluated for each paired analysis (comparisons are week 4 vs. week 2; ***, $p<0.001$; **, $p<0.01$; *$p<0.05$).



Supplemental Figure 2. Detection of CMV-specific CD8$^+$ T cells in T-Rapa cell recipients. For transplant recipients who were HLA-A2$^+$, cryopreserved post-HCT PBMC (from day 60 post-HCT) were thawed and evaluated by flow cytometry for the frequency of CD8$^+$ T cells that were CMV-specific by dextramer analysis. (A) The left two panels show results from patient UPN #29, who did not develop CMV viremia by DNA-PCR analysis; in this case, where the pre-HCT CMV status by antibody testing was negative for the recipient and positive for the donor ("R$^-$/D$^+$"), the day 60 post-HCT flow result showed a relatively low frequency of CMV-specific CD8$^+$ T cells (0.2%). By comparison, the right two panels show results from patient UPN #17, who developed CMV viremia by DNA-PCR analysis; in this case, where the pre-HCT CMV status by antibody testing was positive for both recipient and donor ("R$^+$/D$^+$"), the day 60 post-HCT flow result showed a relatively high frequency of CMV-specific CD8$^+$ T cells (3.5%). (B) The summation of CMV dextramer results from n=10 T-Rapa cell recipients who were HLA-A2$^+$ are shown; all results were from day 60 post-HCT. In three out of the four cases where CMV viremia developed post-HCT, an increased frequency of CMV-specific CD8$^+$ T cells was detected (right side of Supplemental Figure 2B). By comparison, in the six recipients who did not develop CMV viremia post-HCT, the frequency of CMV-specific CD8$^+$ T cells was similar to background levels (dotted line indicates background level of assay).



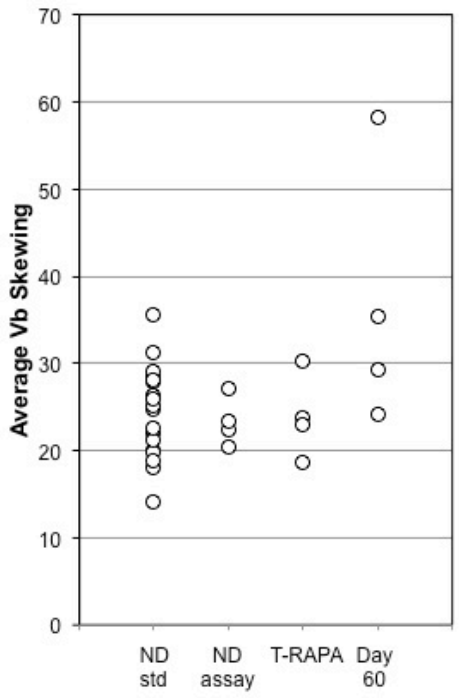
Supplemental Table III. Post-HCT T Cells Secrete IFN-γ and IL-4 in a CMV-specific Manner (CMV peptide stimulation assay)

Sample Type	Donor/Recipient Seropositive Status	n=	Stimulation Type	IFN-γ (pg/ml)	IL-4 (pg/ml)
T-Rapa Products	D^{NEG}	2	None	93 ± 43	2 ± 2
			CMV P.A.[2]	89 ± 38	4 ± 1
			TCR[3]	84 ± 35	4 ± 1
	D^{POS}	3	None	104 ± 35	13 ± 10
			CMV P.A.	85 ± 36	10 ± 4
			TCR	82 ± 34	7 ± 4
PBMC[1]	D^{NEG}/R^{NEG}	3	None	1 ± 1	11 ± 9
			CMV P.A.	1 ± 1	6 ± 6
			TCR	1574 ± 947	3343 ± 1452
	D^{POS}/R^{NEG}	3	None	46 ± 1	1 ± 1
			CMV P.A.	430 ± 278	53 ± 33
			TCR	1301 ± 441	2022 ± 904
	D^{NEG}/R^{POS}	3	None	16 ± 7	4 ± 3
			CMV P.A.	70 ± 29	24 ± 13
			TCR	2818 ± 196	5414 ± 4371
	D^{POS}/R^{POS}	7	None	59 ± 27	8 ± 5
			CMV P.A.	446 ± 151 *	89 ± 27 **
			TCR	3706 ± 2330	2274 ± 1090

[1] PBMC obtained from patients at day +60 post-HCT were evaluated.
[2] PBMC were stimulated for 48 hr with a pool of CMV peptides (P.A., PepTivator).
[3] PBMC were stimulated for 48 hr with Cytostim (superantigen-like TCR stimulation)
* $P<0.04$, increased IFN-γ secretion (CMV P.A. stimulation vs. No stimulation)
** $P<0.03$, increased IL-4 secretion (CMV P.A. stimulation vs. No stimulation)

Supplemental Figure 3: T-Rapa Cell Products and Post-HCT T Cells from T-Rapa Cell Recipients Have a Diverse TCR Repertoire by V-β Spectratype Analysis

The following $CD4^+$ T cell populations were subjected to V-β spectratype analysis, as indicated in the figure below: "ND std", normal donor $CD4^+$ T cell standard values obtained from previous experiments in our lab; "ND assay", normal donor $CD4^+$ T cells from the current experiment; "T-RAPA", manufactured T-Rapa cell clinical products; and "Day 60", purified $CD4^+$ T cells from T-Rapa cell recipients at day 60 post-HCT. A total of 17 V-β families were evaluable for analysis. These results indicate that the T-Rapa cell products had a TCR repertoire diversity that was similar to normal donor CD4 cells; at day 60 post-HCT, there tended to be some skewing of the CD4 cell TCR repertoire.



References for Supplementary Information

1. Sportes C, Hakim FT, Memon SA, et al. Administration of rhIL-7 in humans increases in vivo TCR repertoire diversity by preferential expansion of naïve T cell subsets. J Exp Med. 2008. Jul 7;205(7):1701-14.

2. Memon SA, Sportes C, Flomerfelt FA, et al. Quantitative analysis of T cell receptor diversity in clinical samples of human peripheral blood. J Immunol Methods. 2012. Jan 31; 375(1-2):84-92.