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Differential Gene Expression Profile of First- and Second-Generation T-Rapa Cell Clinical Products

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Abstract

We completed a phase II clinical trial evaluating rapamycin-resistant allogeneic T cells (T-Rapa) and now are evaluating a T-Rapa product manufactured in 6-days (T-Rapa₆) rather than 12-days (T-Rapa₁₂). Using gene expression microarrays, we addressed our hypothesis that the two products would express a similar phenotype. The products had similar phenotypes using conventional comparison methods of cytokine secretion and surface markers. Unsupervised analysis of 34,340 genes revealed that T-Rapa₆ and T-Rapa₁₂ products clustered together, distinct from culture input CD4⁺ T cells (CD4). Statistical analysis of T-Rapa₆ products revealed differential expression of 19.3% of genes (n=6641) compared to input CD4 cells; similarly, 17.8% of genes (n=6147) were differentially expressed between T-Rapa₁₂ products and input CD4 cells. Compared to input CD4 cells, T-Rapa₆ and T-Rapa₁₂ products were similar in terms of major gene families up-regulated (cell cycle, stress response, glucose catabolism, DNA metabolism) and down-regulated (inflammatory response, immune response, apoptosis, transcriptional regulation). However, when directly compared, T-Rapa₆ and T-Rapa₁₂ products showed differential expression of 5.8% of genes (n=1994; T-Rapa₆ vs. T-Rapa₁₂). Second-generation T-Rapa₆ cells therefore possess a similar yet distinct gene expression profile relative to first-generation T-Rapa₁₂ cells, and thus may mediate differential effects after adoptive transfer.

Keywords

Cell Therapy; Graft vs Host Disease; Sirolimus; T-Lymphocytes

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Disclosure of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Introduction

Although gene expression microarrays represents a feasible and unbiased method for the characterization of global cellular function[1], initial studies are only now underway to use this technology for the characterization of ex vivo manipulated clinical cellular products[2]. We reasoned that, in the setting of cellular therapy, expression profiling may have value in several respects, including: (1) as a quality control measure, with results providing a 'genetic fingerprint' that can be utilized to help ensure cell product efficacy; (2) the identification of putative functional pathways, thereby contributing to an increased understanding of cellular mechanisms of action; and (3) facilitation of an assessment of whether modifications of the ex vivo manufacturing process alter the global cellular phenotype.

In murine models, we have found that ex vivo manufacture of allogeneic CD4⁺ T cells in rapamycin, which inhibits mTOR and alters cellular function through a variety of mechanisms[3], generates a robust T cell population that can be adoptively transferred to beneficially modulate the balance of immune reactions that occur after hematopoietic cell transplantation. Such rapamycin-resistant T-helper (Th) cells could be manufactured ex vivo in either a Th1 or Th2 cytokine phenotype depending on whether antigen-presenting-cell (APC) free co-stimulation was performed in the presence of IL-12 or IL-4 polarizing cytokines, respectively[4]. Importantly, rapamycin-resistant T cells of both Th1 and Th2 phenotype had increased in vivo function after adoptive transfer into allogeneic hosts[4]. These results stood in contrast to previous results that found rapamycin to tolerize T cells[5] or induce an immune suppressive regulatory T (T_{REG}) phenotype[6] but are consistent with emerging data that indicate an immune augmentation effect of rapamycin on CD8⁺ T cell function[7].

Infusion of rapamycin-resistant donor Th2 cells represented a novel approach to balancing Th1/Th2 immunity after experimental murine allogeneic bone marrow transplantation for the mediation of graft-versus-tumor (GVT) effects with reduced graft-versus-host disease (GVHD)[8] and for the prevention of fully MHC-disparate graft rejection[9, 10]. The enhanced in vivo efficacy of rapamycin-resistant murine and human T cells in allogeneic[9] and xenogeneic[11] transplantation models was due in part to a multi-faceted anti-apoptotic phenotype that dictated increased in vivo T cell persistence after adoptive transfer. Finally, consistent with the known role of rapamycin-induced mTOR blockade in the promotion of autophagy[12], we have identified that the anti-apoptotic phenotype of rapamycin-resistant T cells emanated from a mitochondrial autophagy process[11, 13].

In a clinical translation of this research, we developed a method for the ex vivo manufacture of cytokine polarized allogeneic human CD4⁺ T cells in rapamycin and evaluated such cells on a phase II clinical trial (NCT #00074490). This method consisted of APC-free co-stimulation of purified CD4⁺ T cells in rapamycin, IL-4, and IL-2 over a 12-day culture interval; the resultant T-Rapa cell product was comprised of minimally differentiated effector T cells that secreted a balanced pattern of Th1 and Th2 cytokines[14]. In the setting of HLA-matched sibling allogeneic hematopoietic cell transplantation using low-intensity chemotherapy conditioning, we found that the delayed infusion of allogeneic T-Rapa cells at day 14 post-transplant induced a balanced pattern of immune reconstitution involving both

Th1 and Th2 cytokines, promoted alloengraftment as indicated by conversion of mixed chimerism towards full donor elements, associated with a low rate of classical acute GVHD (4 cases out of 40 patients), and resulted in sustained complete remissions in patients with chemotherapy refractory hematologic malignancy[15]. The main cause of post-transplant mortality was malignant disease progression, and as such, we are now evaluating whether alternative methods of T-Rapa cell manufacturing might augment GVT effects.

In experimental models of syngeneic anti-tumor T cell therapy, the state of T cell differentiation is an important determinant of T cell efficacy after adoptive transfer, with less differentiated T cells mediating more potent in vivo effects[16]. In our studies performed in the allogeneic transplantation setting involving rapamycin-resistant murine Th2 cells, which were manufactured by a truncated 6-day culture method, we found that: (1) such cells were minimally differentiated at the time of T cell transfer, as indicated by very low cytokine secretion potential and expression of cell surface molecules such as CD62L and CCR7[4]; and (2) in spite of this minimally differentiated state, adoptive transfer of such cells induced approximately one-log higher in vivo cytokine levels relative to control T cells not manufactured in rapamycin[8]. In sum, these data indicate that minimally differentiated T cells represent a suitable phenotype for adoptive T cell transfer.

Given this background and our desire to generate a more robust allogeneic T-Rapa cell product for more potent mediation of GVT effects, we truncated our manufacturing process from 12-days (T-Rapa₁₂ cells) to 6-days (T-Rapa₆ cells). Our current translational efforts are evaluating this second-generation T-Rapa₆ cell therapy on a phase II clinical trial using the exact same transplant conditions utilized in our evaluation of the first-generation T-Rapa₁₂ cells. In this project, we have utilized gene expression profiling to compare and contrast the global phenotype of the T-Rapa₁₂ and T-Rapa₆ clinical products.

Material and Methods

T Cell Isolation and Culture

Experiments were performed according to a Standard Operating Procedures (SOP) established to support the phase II clinical trial evaluating T-Rapa₁₂ cell therapy at the NIH Clinical Center (NCI Protocol 04-C-0055). Peripheral blood mononuclear cells (PBMCs) from 6 healthy donors were collected by apheresis (Amicus Separator, Fenwal; Lake Zurich, IL, USA) on an IRB-approved protocol in the Department of Transfusion Medicine, National Institutes of Health (Bethesda, Maryland, USA). CD4⁺ T cells were isolated by positive selection to > 99% purity using a CliniMACS system (Miltenyi Biotec; Bergish Gladbach, Germany). CD4 cells were cryopreserved using a controlled rate freezer (Kryosave, Integra, Planer plc; Sunbury-on-Thames, UK) and media containing 5% DMSO (Edwards Lifesciences; Inc, Ca), 6% pentastarch (Pharmaceutical Development Section, Pharmacy Dept, Clinical Center, NIH), and 4% human serum albumin (Baxter Health Care Corporation; Ca, USA). Thawed CD4⁺ T cells were cultured in PL732 bags in X-VIVO 20 media containing 5% heat inactivated human AB plasma, recombinant human IL-4 (1,000 I.U./mL; Schering; Whitehouse Station, NJ, USA), recombinant human IL-2 (20 I.U./mL; Chiron Corporation; Emeryville, CA, USA), and rapamycin (Rapamune, 1 uM; Wyeth Ayerst; West Greenwich, RI, USA). T cells were co-stimulated once at culture initiation

with tosylactivated paramagnetic M-450 beads (DynaL Invitrogen; Oslo, Norway) coated with anti-CD3 (OKT3; Ortho Biotech) and anti-CD28 (clone 9.3; kindly provided by Dr. Bruce Levine of the University of Pennsylvania Cancer Center) at a bead to cell ratio of 3:1. The number of CD4⁺ T cells loaded into each culture bag ranged from 62 to 154 × 10⁶ cells per culture; each culture was initiated at a T cell concentration of 1.5 × 10⁶ cells/ml. On culture days 2 and 4, 10× cytokines (in 10% total culture volumes) were added to each bag and as cultures were monitored for cell growth. In addition, 1× cytokine-containing media (in 10–25% volume amounts) was also added to each bag to ensure that cell concentrations did not exceed 0.5 to 1 × 10⁶ cells/ml. The cells were cultured at 37° C in incubators with 5% CO₂ for either 6 or 12 days to generate the T-Rapa₆ and T-Rapa₁₂ populations, respectively.

Assessment of T Cell Cytokine Secretion Profile

T cell culture supernatants of input CD4 cells, T-Rapa₆ cells, and T-Rapa₁₂ cells were generated by adjusting the T cells to a concentration of 1 × 10⁶ cells/ml and co-stimulating at a 3:1 bead to cell ratio for 24 hours; then, the cell-free supernatants were harvested and tested for cytokine content by Luminex multiplex assays following the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA, USA). To further assess cytokine secretion potential, T-Rapa₆ and T-Rapa₁₂ populations were co-stimulated and cultured for an additional 6 days in X-VIVO 20 media supplemented with heat inactive AB plasma and IL-2 (20 IU/ml) with or without polarizing cytokines (IL-4 or IFN-γ; then, the resultant expanded T cells were co-stimulated again and the 24 hour supernatants were tested for cytokine content by Luminex.

Total RNA Isolation

Total RNA was extracted from the input CD4⁺ T cells and the T-Rapa₆ and T-Rapa₁₂ populations using the mRNeasy mini kit (Qiagen; Valencia, CA, USA). Quality of the isolated RNA was evaluated by the Agilent Bioanalyzer 2100 using an RNA 6000 Nano-LabChip (Agilent Technologies; Santa Clara, CA, USA).

Microarray Expression Analysis

Microarray expression experiments were performed on 4×44 K Whole Human Genome Microarray (Agilent Technologies), according to the manufacturer's instructions. Universal Human Reference RNA (Stratagene, Santa Clara, CA, USA) was used as a common reference. Test samples (6 samples for each cell product) and reference RNA were amplified and labeled using Agilent kit according to the manufacturer's instructions. Images of the arrays were acquired using a microarray scanner G2505B (Agilent Technologies) and image analysis was performed using feature extraction software version 9.5 (Agilent Technologies). Resulting data files were uploaded to the mAdb database (<http://nciarray.nci.nih.gov/>) and further analyzed using BRBArrayTools developed by the Biometric Research Branch, National Cancer Institute (<http://linus.nci.nih.gov/BRB-ArrayTools.html>).

Statistical and Microarray Data Analysis

The raw data set was filtered according to a standard procedure to exclude spots below a minimum intensity of 10 in both fluorescence channels and not represented in more than 80% of samples. Class comparison was conducted with BRB Array Tools using a random variance model. Significant genes were defined as $p\text{-value} < 0.001$ and an $\text{FDR} < 0.10$. Partek Genomic Suite 6.4 (Partek Inc., St. Louis, MO, USA) was used for data visualization, hierarchical cluster analysis, and Principal Component Analysis (PCA)[17]. 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[18]; specific gene annotations were retrieved on Gene Cards (<http://www.genecards.org/index.shtml>). The microarray data used in this study were deposited on the NCBI GEO database (GSE34911).

Results

T-Rapa₆ vs. T-Rapa₁₂ Cells: Differential Yield But Similar Cytokine Phenotype

Relative to the absolute number of input CD4^+ T cells at culture initiation, there was reduced T cell expansion in the T-Rapa₆ culture condition relative to the T-Rapa₁₂ culture condition (mean fold-expansion $\pm$ SEM, $n=6$: 2.2 ± 0.3 vs. 17.8 ± 3.0 , $p < 0.05$). However, there were no consistent differences in T-Rapa₆ and T-Rapa₁₂ capacity to secrete IFN- γ , IL-2, IL-4, and IL-10; of the 16 comparisons in totality, we only found statistically significant differences in two comparisons (Figures 1A through 1D). At the end of T-Rapa₆ and T-Rapa₁₂ cell manufacturing, T cell capacity for cytokine secretion was minimal, thereby consistent with a minimally differentiated state; specifically, median cytokine secretion values were: IFN- $\gamma < 100$ pg/ml, IL-2 < 1 pg/ml, IL-4 < 1 pg/ml, and IL-10 < 100 pg/ml (all comparisons of T-Rapa₆ vs. T-Rapa₁₂: $p = \text{NS}$). Extended culture of T-Rapa₆ and T-Rapa₁₂ cell products resulted in T cells with an enhanced capacity for cytokine secretion, in particular IFN- γ and IL-4. Extended culture in the presence of a type I polarizing cytokine (IFN- γ) similarly increased both T-Rapa₆ and T-Rapa₁₂ cell capacity for IL-2 secretion, whereas extended culture in the presence of the type II polarizing cytokine IL-4 similarly increased both T-Rapa₆ and T-Rapa₁₂ cell capacity for IL-4 secretion. Because both T-Rapa₆ and T-Rapa₁₂ cell products maintained a balanced pattern of secretion of IFN- γ , IL-2, IL-4, and IL-10, each population was comprised of a mix of Th1 and Th2 effectors that manifested minimal T-helper subset differentiation plasticity.

Gene Expression Unsupervised Analysis Differentiates T-Rapa₆ and T-Rapa₁₂ Cell Products

Principal component analysis (PCA) of the entire gene expression dataset (34,340 genes) separated the three T cell populations into three distinct groups: (1) input CD4^+ T cells; (2) T-Rapa₁₂ cells; and (3) T-Rapa₆ cells (Fig. 2A). The first principal component (PC #1) clearly separated input CD4^+ T cells from each of the T-Rapa cell populations, whereas the third principal component (PC #3) separated the T-Rapa₆ and T-Rapa₁₂ cell populations. Given the high signal to noise ratio in microarray data[17, 19], we performed an unsupervised hierarchical clustering on the most informative genes by removing genes that had low variation across the dataset (as defined by a coefficient of variation < 0.05). The

unsupervised hierarchical clustering of the resulting 14,780 genes revealed two distinct main clusters: (1) one with all of the input CD4⁺ T cell samples (Fig. 2B); and (2) one with all of the T-Rapa₆ cell samples separated from all of the T-Rapa₁₂ cell samples (Fig. 2C).

The Majority of Differentially Expressed Genes are of Limited Magnitude

To better delineate differential gene expression across the three T cell populations, we compared the number of differentially expressed genes using an additional filter that incorporated relative fold-change of gene expression (Fig. 3). The total number of differentially expressed genes between the input CD4⁺ T cells and the T-Rapa₆ cells was 6641 (p-value < 0.001 and FDR < 0.10; 19.3% of all evaluable genes), with a relatively equal number of genes up-regulated (n=3614) or down-regulated (n=3027) by the T-Rapa₆ culture condition. Similarly, the total number of differentially expressed genes between the input CD4⁺ T cells and the T-Rapa₁₂ cells was 6147 (p-value < 0.001 and FDR < 0.10; 17.8% of all evaluable genes), again with a relatively equal number of genes up-regulated (n=3185) or down-regulated (n=2962) by the T-Rapa₁₂ culture condition. Instead, the total number of differentially expressed genes between the T-Rapa₆ and T-Rapa₁₂ cells was 1994 (p-value < 0.001 and FDR < 0.10; 5.8% of all evaluable genes; 1144 genes up-regulated in T-Rapa₆ cells, 800 genes down-regulated in T-Rapa₆ cells); furthermore, whereas both T-Rapa₆ and T-Rapa₁₂ cells had more than 2000 genes that differed from the input CD4⁺ T cells by relatively large fold-changes of 5-fold, less than 200 genes were of that magnitude difference when comparing the T-Rapa₆ and T-Rapa₁₂ cell populations. As such, the T-Rapa₆ and T-Rapa₁₂ cell products showed fewer differences from each other than from the input CD4⁺ T cells.

T-Rapa₆ and T-Rapa₁₂ Cell Products Each Up-regulate Cell Cycle and Metabolic Genes and Each Down-regulate Apoptosis and Immune Response Genes

Next, we performed Gene Ontology (GO) analysis using DAVID software to determine which gene families were most significantly altered in their expression when comparing either T-Rapa₆ or T-Rapa₁₂ cell populations to the input CD4⁺ T cells. Relative to input CD4⁺ T cells, gene families that were the most significantly over-represented in the genes up-regulated in T-Rapa₁₂ cell clinical products included cell cycle, modulation of DNA metabolism, cellular response to stress, glucose catabolic process, nitrogen compound biosynthetic process, and oxidative reduction (Fig. 4). On the other hand, gene families that were the most significantly over-represented in the genes down-regulated in the T-Rapa₁₂ cell clinical products included the inflammatory response, regulation of apoptosis, positive regulation of cytokine production, leukocyte activation, regulation of transcription, and the immune response. Remarkably, 10 out of 12 of these gene families were also the most significant families that had differential gene expression when comparing T-Rapa₆ cell clinical products to input CD4⁺ T cells (Fig. 5). Taken together, these gene family ontology results indicate that the majority of the gene expression differences induced by the culture system were apparent by day 6 of culture (T-Rapa₆ cell product) and maintained throughout day 12 of culture (T-Rapa₁₂ cell product). Table I details specific genes from these families that were commonly differentially expressed in the T-Rapa₁₂ and T-Rapa₆ cells relative to the day 0 input CD4⁺ T cells.

T-Rapa₆ and T-Rapa₁₂ Cell Products Differentially Express Genes Related to Hypoxia, Cell Death, and Immune Function

We next utilized GO analysis to evaluate families of genes that were up- or down-regulated in the second-generation T-Rapa₆ cell product relative to the first-generation T-Rapa₁₂ cells. Moreover, it is important to note that the T-Rapa₆ cell products were similar to the T-Rapa₁₂ products in terms of expression of genes in the Th2 family, the Th1 family, and the T_{REG} family (Table II); consistent with the cytokine secretion results (Figure 1), these results are consistent with the conclusion that both the T-Rapa₁₂ and T-Rapa₆ cell products can be characterized as having a mixed Th1/Th2 phenotype.

The gene families that were differentially expressed between the T-Rapa₆ and T-Rapa₁₂ cell products are shown in Figure 6. Relative to the T-Rapa₁₂ cell product, the T-Rapa₆ cell product had up-regulation of hypoxia response genes, anti-apoptosis genes, cell motility genes, sterol hormone synthesis genes, and regulation of the immune system genes; and conversely, relative to the T-Rapa₁₂ cell product, the T-Rapa₆ cell product had down-regulation of families of genes relating to antigen processing and presentation, negative regulation of protein ubiquitination, cell death, positive regulation of macromolecule metabolic process, and the JAK-STAT cascade. It should be noted that the levels of significance of the changes in these gene families were markedly lower than the levels of significance observed when comparing either T-Rapa cell product to the input CD4⁺ T cells (that is, the most significant family difference p value was in the 10⁻⁵ range rather than in the 10⁻⁵⁰ range). Table III details specific genes from these families that were differentially expressed when comparing the T-Rapa₁₂ and T-Rapa₆ cell products. In general, the magnitude of these fold-changes were less than the fold-changes observed when comparing the T-Rapa cell products to the day 0 CD4⁺ T cells.

Discussion

Gene expression profiling represents an important tool to more fully characterize ex vivo manufactured T cell products in an unbiased manner, can be utilized to provide a T cell 'fingerprint' as a component of a quality control program, and may offer new insights into T cell biology and the modulation of transplantation responses. These attributes of microarray analysis are particularly relevant to our clinical translation efforts using ex vivo manufactured allogeneic donor T-Rapa cells because our murine models indicated that such T cells had diverse functional characteristics above and beyond classical definitions of T cell function such as the Th1/Th2 cytokine profile, including a post-autophagy state, an anti-apoptotic phenotype, and altered mitochondrial biology. A more detailed investigation of T cell product characteristics is particularly important as we have now transitioned from an initial 12-day interval of T-Rapa cell manufacturing to the current culture interval of 6 days. In this report, we have found that the T-Rapa₁₂ and T-Rapa₆ populations, which are relatively indistinguishable from one another in terms of conventional definitions of Th1/Th2 cytokine secretion, were also indistinguishable from one another in terms of the majority of genes that were up- or down-regulated during T cell culture. However, we did identify that the T-Rapa₁₂ and T-Rapa₆ populations differentially expressed a significant

number of genes, thereby indicating that these T cell populations would best be viewed as distinct cellular products.

In comparing the T-Rapa₁₂ and T-Rapa₆ cell products, conventional methods of T cell phenotyping did not identify differences that might indicate that the T cell products were functionally distinct. That is, although the T-Rapa₁₂ cell product expanded to a greater degree in culture relative to the T-Rapa₆ cell product, the two products were nearly identical in terms of their ability to secrete the Th1 cytokines IL-2 and IFN- γ or the Th2 cytokines IL-4 and IL-10 in response to anti-CD3, anti-CD28 co-stimulation. In addition, we assessed the important functional characteristics of T cell differentiation plasticity by performing an extended ex vivo culture without rapamycin and in the presence of variable cytokine polarizing conditions, and again found the T-Rapa₁₂ and T-Rapa₆ cell products to be nearly identical. It is possible that protein-based methods of cell product phenotyping may have limited applicability in the setting of rapamycin-based manufacturing due to the ability of mTOR blockade to inhibit protein translation. The above-mentioned functional similarities that we observed between the two T-Rapa clinical products necessitated that we utilize additional methods such as gene expression microarray to identify differentially expressed genes and identify differences in less expected functions.

Given these functional similarities using conventional assays, it was somewhat surprising to observe that the T-Rapa₁₂ and T-Rapa₆ populations could be easily segregated from one another using global microarray analytical tools such as the principal component analysis and unsupervised hierarchical clustering. Remarkably, the T-Rapa₁₂ and T-Rapa₆ populations had similar numbers of differentially expressed genes relative to the input CD4⁺ T cells (17.8% and 19.3 % of all genes, respectively). Although these results indicate that T cell culture using co-stimulation, polarizing cytokines, and rapamycin altered expression of a multitude of genes, the great majority of these genes had small magnitudes of differential expression relative to the day 0 input CD4⁺ T cells (< 3-fold). Nonetheless, several hundred genes were massively up- or down-regulated in these culture conditions (>10-fold difference relative to input CD4⁺ T cells), thereby indicating the dramatic effect that culture in rapamycin and polarizing media had on T cell biology; it should be emphasized that further studies will be required to delineate which of the gene expression changes were due to individual culture components (that is, co-stimulation, cytokines, or rapamycin). Using this fold-change approach to the microarray analysis, one can clearly conclude that the T-Rapa₁₂ and T-Rapa₆ cell products were more similar than different: specifically, only a few genes were differentially expressed at a level of greater than 10-fold when comparing these two clinical T cell products.

Using GO to better understand patterns of differential gene expression, we found that the first-generation T-Rapa₁₂ and second-generation T-Rapa₆ cell populations were virtually identical in terms of the major families that were altered. With both T cell products, there was a great up-regulation of genes that promote the cell cycle; potentially, this attribute of the T-Rapa cell products may help ensure cell product expansion in vivo after adoptive transfer and thus may be a critical factor in any observed clinical effects. Several families related to cellular metabolism and stress were commonly up-regulated in both T-Rapa cell products, including the family of genes related to DNA metabolic process, glucose catabolic

process, and oxidative reduction. The T-Rapa₁₂ cells had a further signature of up-regulated nitrogen compound biosynthetic process whereas the T-Rapa₆ cells had a further signature of up-regulated sterol biosynthetic process. In sum, these data indicate that both the T-Rapa₁₂ and T-Rapa₆ populations had up-regulation of multiple pathways related to cellular energetics.

In a similar way, the T-Rapa₁₂ and T-Rapa₆ populations were virtually identical in terms of the gene ontology families that were consistently down-regulated. That is, apoptosis, transcription, inflammation, cytokine production, and overall immune response genes were commonly down-regulated, with T-Rapa₁₂ cells also having down-regulation of the leukocyte activation family of genes and T-Rapa₆ cells also having downregulation of antigen processing and presentation genes. Reduction in apoptosis genes is consistent with our previous results, which found that murine and human rapamycin-resistant T cells expressed an anti-apoptotic phenotype that conferred increased T cell survival in vivo [9, 11]. Furthermore, the observed reduction of inflammatory and immune response genes is consistent with our previous findings that rapamycin-resistant murine [8] and human [14] T cells existed in a minimally-differentiated effector status. Although such a relatively primitive T cell effector state may seem counter-productive, we [8] and others [16] have shown that T cells possessing nominal effector function at the time of T cell transfer associate with an increased in vivo efficacy.

Although these results in sum indicate that the T-Rapa₁₂ and T-Rapa₆ cell products are remarkably similar in terms of the number and types of genes differentially regulated relative to the day 0 input CD4⁺ T cells, it is also recognized that these populations are distinct, and to the extent, it is important to consider in greater detail the differences that exist. There were several gene ontology families that were not identified in the initial comparisons to the day 0 input CD4⁺ T cells; specifically, relative to the T-Rapa₁₂ cells, the T-Rapa₆ cells had increased expression of hypoxia response genes and cell motility genes and reduced expression of genes in the JAK-STAT cascade and protein ubiquitination families. Further studies are required to better characterize the differential gene expression that exists between these two T-Rapa cell products and to evaluate whether such differences are associated with differences in clinical outcomes.

In conclusion, using gene expression microarray, we have characterized two ex vivo manufactured T cell populations currently being evaluated in the setting of allogeneic hematopoietic cell transplantation, the first-generation T-Rapa₁₂ and the second-generation T-Rapa₆ cell products. Using this unbiased approach to T cell analysis, we have determined that these T cell products were remarkably similar in their differential expression of genes relative to the CD4⁺ T cells used for culture initiation. The families of genes that were commonly differentially expressed would predictably associate with an increased T cell function after adoptive cell transfer: that is, in general, both the T-Rapa₁₂ and T-Rapa₆ cell populations had a favorable genetic profile for increased cell cycle machinery, an anti-apoptotic phenotype, and a minimally-differentiated effector state. Nonetheless, the T-Rapa₁₂ and T-Rapa₆ populations were easily segregated on a gene expression basis, and as such, future research will more closely evaluate the functional differences that appear to exist between these two T cell products. Specifically, it will be important to validate these

research findings in an expanded dataset during clinical trial implementation, to confirm differences at the protein level and through functional assays, and to correlate T cell product phenotype and transcription profile with clinical outcomes after allogeneic transplantation, including engraftment, graft-versus-host disease, and graft-versus-tumor effects.

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Abbreviations

APC	antigen-presenting-cell
GO	Gene Ontology
GVT	graft-versus-tumor
GVHD	graft-versus-host disease
PCA	Principal component analysis
Th	T-helper cells
T_{REG}	regulatory T cells

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Figure 1. Cytokine expression analysis of T-Rapa₆ and T-Rapa₁₂ cells generated from CD4⁺ cells (n=6)

Cell supernatants were analyzed for cytokine content by Luminex assay. Cytokine levels were measured in cell supernatants generated over a 24 hr restimulation period. In contrast, cytokine levels in supernatants of cultures generated over a 24 hr restimulation period after extending the cultures for an additional 6 days in absence of Rapamycin were setup under costimulation conditions with IL-2 alone, IL-2 and IFN- α , or IL-2 and IL-4. The production of IFN- γ (a), IL-2 (b), IL-4 (c) and IL-10 (d) are shown.

Figure 2. Gene expression analysis of T-Rapa₆ and T-Rapa₁₂ cells

a) Principal component analysis of the whole dataset (34340 genes); b) Unsupervised hierarchical clustering analysis of 14780 genes that remained after filtering out genes with a coefficient of variation < 0.05. The heat map is average corrected. c) Magnification of the dendrogram in b showing sample identifiers. The T-Rapa₆ cells, T-Rapa₁₂ cells and input CD4⁺ cells are indicated in green, blue, and red, respectively.

Figure 3. The number of T-Rapa₆ and T-Rapa₁₂ cells that differ from input CD4⁺ cells

The number of genes up- and down-regulated among the three cell populations for which differences in expression exceeded specified levels of fold change (2-, 3-, 5- and 10-fold change as indicated by p, p2, p3, p5, p10 respectively) and whose difference in expression was highly significant (p-value < 0.001). The y-axis indicates the number of genes selected by filtering only using the p-value < 0.001 or the p-value plus 2-, 3-, 5- and 10-fold difference in expression.

Figure 4. Gene ontology analysis of genes differentially expressed between T-Rapa₁₂ cells and input CD4⁺ cells

The most overrepresented GO families among T-Rapa₁₂ cells are represented as $-\text{Log}_{10}(\text{p-value})$ for up-regulated genes and $\text{Log}_{10}(\text{p-value})$ for down-regulated ones.

Figure 5. Gene ontology analysis of genes differentially expressed between T-Rapa₆ cells and input CD4⁺ cells

Most overrepresented GO families among T-Rapa₆ are represented as $-\text{Log}_{10}(\text{p-value})$ for up-regulated genes and $\text{Log}_{10}(\text{p-value})$ for down-regulated genes.

Figure 6. Gene ontology analysis of genes differentially expressed between T-Rapa₆ and T-Rapa₁₂ cells

Most overrepresented GO families are represented as $-\text{Log}_{10}(\text{p-value})$ for genes up-regulated in T-Rapa₆ and $\text{Log}_{10}(\text{p-value})$ for those down-regulated.

Table I

Genes differentially regulated in both T-Rapa₁₂ and T-Rapa₆ cells (vs. day 0 input cells).

Gene Family	Gene Symbol (Reference)	T-Rapa ₁₂	T-Rapa ₆
Up-Regulated Families			
Cell Cycle	DTL ([20])	63-fold up	57-fold up
DNA Metabolism	ESCO2 ([21])	49-fold up	38-fold up
Stress Response	TOP2A ([22])	23-fold up	31-fold up
Glucose Catabolism	OGDHL ([23])	92-fold up	23-fold up
Oxidative Reduction	MAOA ([24])	35-fold up	41-fold up
Down-Regulated Families			
Apoptosis	IL-1B ([25])	134-fold down	90-fold down
Transcription	EREG ([26])	45-fold down	100-fold down
Inflammation	S100A8 ([27])	341-fold down	451-fold down
Cytokine Production	NLRP3 ([28])	173-fold down	11-fold down
Immune Response	CXCL2 ([29])	35-fold down	98-fold down

Table II

T-Rapa₁₂ and T-Rapa₆ cell products: similar expression of Th2, Th1, and T-Reg genes.

Gene Family	Gene Symbol (Ref.)	T-Rapa ₁₂ (Ref.)	T-Rapa ₆ (vs. Day 0)	T-Rapa ₆ (vs. T-Rapa ₁₂)
Th2 Genes				
	IL4 ([30])	4-fold up	6-fold up	NS
	IL13 ([31])	17-fold up	27-fold up	NS
	NFIL3 ([32])	4-fold up	4-fold up	NS
Th1 Genes				
	IFNG ([33])	NS	NS	NS
	IL12Rβ2 ([34])	21-fold up	24-fold up	NS
	HAVCR2 ([35])	6-fold up	6-fold up	NS
T-Reg Genes				
	FOXP3 ([36])	NS	NS	NS
	NRP1 ([37])	NS	NS	NS
	IKZF2 ([38])	NS	NS	NS

NS: not statistically significant

Table IIIGenes differentially regulated T-Rapa₆ cells relative to T-Rapa₁₂ cells.

Gene Ontology Family Up	Gene Symbol (Reference)	T-Rapa ₆ vs. T-Rapa ₁₂
Up-Regulated Families		
Response to Hypoxia	FLT1 ([39])	14-fold up
Regulation of Apoptosis	IER3 ([40])	7-fold up
Regulation of Cell Motion	PDGFA ([41])	4-fold up
Sterol Hormone Synthesis	ADM ([42])	5-fold up
Immune System Regulation	CD86 ([43])	18-fold up
Down-Regulated Families		
Antigen Processing and Presentation	HLA-DRA([44])	4-fold down
Protein Ubiquitination	CDKN2A ([45])	3-fold down
Cell Death	XAF1 ([46])	9-fold down
Macromolecule Metabolic Process	KLF2 ([47])	78-fold down
JAK-STAT Cascade	JAK2 ([48])	4-fold down