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Multiply restimulated human thymic Treg express distinct signature Treg transcription factors without evidence of exhaustion

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Abstract

Adoptive transfer of suppressive thymic CD4+CD25+ Tregulatory cells (tTreg) can control auto- and allo-immune responses, but typically require *in vitro* expansion to reach the target cell number for efficacy. Although the adoptive transfer of expanded tTreg purified from umbilical cord blood ameliorated GVHD in patients receiving hematopoietic stem cell transplantation for lympho-hematopoietic malignancy, individual Treg products of 100×10^6 cells/kg were manufactured over an extended 19 day time period using a process that yielded variable products and was both laborious and costly. These limitations could be overcome with the availability of 'off the shelf' Treg. Previously, we reported a repetitive restimulation expansion protocol that maintains

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Author Contributions

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Supplemental Information

Supplemental Information includes Supplemental figure legends along with five figures.

Treg phenotype (CD4+25++127-Foxp3+), potentially providing hundreds to thousands of patient infusions. However, repetitive stimulation of Teffectors induces a well-defined program of exhaustion that leads to reduced T cell survival and function. Unexpectedly, we find that multiply stimulated human tTreg do not develop an exhaustion signature and instead maintain their Treg gene expression pattern. We also find that tTreg expanded with 1 or 2 rounds of stimulation, and tTreg expanded with 3 or 5 round of stimulation, preferentially express distinct subsets of a group of five transcription factors that lock-in Treg Foxp3 expression, Treg stability and suppressor function. Multiply restimulated Treg also had increased transcripts characteristic of Tfollicular regulatory cells, a Treg subset. These data demonstrate that repetitively expanded human tTreg have a Treg locking transcription factor with stable FoxP3 and without classical T cell exhaustion gene expression profile, desirable properties that support the possibility of off-the-shelf Treg therapeutics.

Keywords

regulatory T cell; Treg; Cell therapy; cGMP production; graft versus host disease

Introduction

Regulatory T-cells (Treg) are CD4+ suppressor cells that co-express the high affinity IL-2Ralpha chain, CD25, and the master transcription factor, Foxp3. Treg are essential immune system controllers, limiting self-reactive T-cells responsible for autoimmunity, and alloreactive T-cells in transplant recipients that destroy foreign antigen-bearing tissues resulting in tissue inflammation and injury[1]. Treg generated in response to commensal bacteria or their products facilitate tissue and organism homeostasis[2–4]. Treg can be classified by their site of differentiation in the thymus (thymic Treg, or tTreg)[5–7] or induced in the periphery (pTreg) or *in vitro* (iTreg)[8,9]. Under specific inflammatory conditions, Treg can become unstable and convert to Teffectors (Teffs).

Allogeneic hematopoietic stem cell transplantation (HSCT) can be complicated by graft-versus-host disease (GVHD)[10–12] caused by donor T-cells that attack host tissues; GVHD occurs in up to 70% of transplant recipients, leading to high rates of morbidity and mortality[13,14]. The adoptive transfer of high Treg numbers can prevent murine GVHD[15–19]. Donor and third-party Tregs were similarly effective in suppressing murine GVHD[20]. Circulating human Treg are present at low frequency (1–2%), and as such may require *in vitro* expansion to achieve favorable Treg: Teff ratios needed to control adverse Teff responses. Because short-course expansion of Treg is often inadequate to obtain sufficient Treg yields[16], we developed an *in vitro* expansion protocol that preferentially supports Treg over Teffs. Robust expansion was achieved by repetitively stimulating Treg after return to resting size. Integral to this process is the immunosuppressant rapamycin, which selectively expands murine and human CD4+25+Foxp3+ Treg vs. Teffector/memory cells[21–23]. In the clinic, a cord blood tTreg expansion protocol that incorporated a re-stimulation step allowed high donor Treg:Teff ratios of ~7:1; the infusion of these *in vitro* expanded, third-party HLA 3–6/6 tTregs resulted in virtually no acute GVHD[24].

During the course of preclinical optimization of re-stimulation cultures, we tested an expansion platform consisting of flow cytometer-sorted peripheral blood (PB) human tTreg expanded on GMP-grade artificial antigen-presenting cells (aAPC) for a total of 5 stimulations with rapamycin present in the culture to suppresses Teff expansion and enhance Foxp3 and tTreg stability[22,25,26]. Treg expansion of ≥10,000,000-fold was attained, while retaining Foxp3 expression and *in vitro* and *in vivo* xenogeneic GVHD suppressor function[15,16,27]. Using a computational network inference approach, Fu identified 5 redundant transcription factors (TF) (Eos, Lef1, Satb1, IRF4 and GATA1) that individually synergize with Foxp3 to stabilize Foxp3 expression and "lock in" the Treg transcriptional signature[28]. Recent studies characterized additional TF that maintain Foxp3 expression and Treg stability[29–32]. Expression of two of these TF (TCF-1 and LEF1) defines three tTreg subsets as resting (TCF-1+LEF1+), activated (TCF-1+LEF1+/lo), and effector (TCF-1-LEF1-). TCF-1 and LEF1 were required for increased expression of specific Treg signature genes, *in vivo* Treg competitive fitness and Tfollicular regulatory (Tfr) cell development but were dispensable for *in vitro* suppressive function[31,32].

Chronic Teff stimulation can drive cells into a state of exhaustion resulting in Teff dysfunction[33–35]. The TF and gene sets enforcing exhaustion have been documented and include TOX, associated with exhausted murine and human CD8 T-cells, and TCF-1 associated with exhausted T-cell precursors[36–39]. One of the dominant T-cell exhaustion characteristics is the upregulation of multiple inhibitory receptors, including PD-1, Lag-3, Tim-3, CD160 and CTLA4[40].

Herein, we show that naïve PB Treg either stimulated 2 times without rapamycin (resulting in ~10,000-fold expansion) or Treg stimulated 5 times in rapamycin (resulting in ~10,000,000-fold expansion) preferentially express different sets of previously defined Treg locking factors (Eos, Lef1, Satb1, IRF4, GATA1), TCF1 and Bach2 that promotes the differentiation of Treg and homeostasis. Importantly, Treg cultured in rapamycin did not increase exhaustion gene expression even after 5 stimulations, in contrast to Treg stimulated 2 times without rapamycin that upregulated genes found in previously annotated exhaustion gene sets. The lack of observed signs of exhaustion of Tregs stimulated at resting size in rapamycin suggests that highly expanded Treg could become a clinically applicable off-the-shelf cellular therapeutic.

Methods

Thymic Treg and CD4 T-cell purification and validation

Non-mobilized peripheral blood (PB) leukapheresis products were purchased from Memorial Blood Center (St. Paul, MN). Naïve human PB tTreg ($CD4^+25^+127^-45RA^+$) were sort-purified from PB mononuclear cells (PBMNCs) (Ficoll-Hypaque, Amersham Biosciences, city, state) in a two-step procedure in which CD25+ cells were initially enriched from PBMNCs by AutoMACS (PosselD2) with GMP grade anti-CD25 microbeads (Miltenyi Biotec, Auburn, CA). CD25high cells were stained with CD4, CD25, CD127 and CD45RA and sorted via FACSAria as CD4+, CD25high, and CD127− CD45RA using a fluorochome-conjugated anti-CD25 antibody that recognizes a different epitope from bead-bound anti-CD25. Naïve human CD4 T-cells were sort-purified from the CD25- fraction as

CD4+25–127+45RA+ (Figure S1). To determine the methylation status of the TSDR in the Foxp3 gene, DNA was purified from sort-purified tTregs expanded. Samples were submitted to EpigenDx Inc. (Worcester, MA 01606) for bisulfite modification and sequencing of 11 CpG motifs in the TSDR.

In vitro tTreg and CD4 T-cell expansion

Purified naïve tTreg and CD4 T-cells were stimulated with a human myelogenous leukemia cell line, K562, engineered to express CD86 and CD64, the high-affinity Fc receptor (KT64/86) (2:1 tTreg:KT64/86), which had been irradiated with 10,000 cGy and loaded with anti-CD3 mAb (Miltenyi Biotec, Auburn, CA)[16,24]. tTreg and CD4+ Teff were re-stimulated up to 4 times as shown in Figure S2. tTreg and CD4+ Teff cell size was monitored 3x weekly, and re-stimulation was performed after they had returned to a resting size (8.5μm and 9.2μm, respectively), which we have shown maximizes expansion[16]. In some experiments, tTregs that had been stimulated with KT64/86 cells that were preloaded, irradiated, and frozen were used at a 1:1 tTreg:KT64/86. Naive tTreg and CD4 T-cells were cultured in X-*Vivo*-15 (BioWhittaker, Walkersville, MD) media supplemented with 10% human AB serum (Valley Biomedical, Winchester, VA). Recombinant IL-2 (300 IU/mL for Treg, 50 IU/mL for CD4+ T-cells; Chiron, Emeryville, CA) was added on day 2 and maintained for culture duration. Cultures were refed at least every Monday, Wednesday and Friday, and plated at 0.25×10^6 to 0.5×10^6 viable nucleated cells/ml. Where indicated, rapamycin (Rapamune, Wyeth-Ayerst, Princeton, NJ) at 109 nM was added on day 0 and with subsequent media supplementations. At the termination of each culture (7–10 days following the last stimulation), cells were aliquoted and frozen. To avoid experiment variability, analyses were conducted concurrently with all samples after thawing.

Suppression assays

The in *vitro*–suppressive capacity of expanded tTreg was assessed with a carboxyfluorescein succinimidyl ester inhibition assay as published[41]. Briefly, PBMNCs were purified, labeled with the intracellular dye CFSE (Invitrogen) that dilutes with each division, and stimulated with anti-CD3 mAb-coated beads (Dynal, InVitrogen) ± cultured tTreg (1:2–1:32 tTreg/PBMNCs). On day 4, cells were stained with antibodies to CD4 and CD8 and suppression was determined from Division Index (FlowJo). tTreg comparably suppressed CD4 and CD8 T cell responses; therefore, only CD8 data are presented.

Flow cytometry and antibodies

Human-specific antibodies were used for flow cytometry and included CD4 (clone RPA-T4), CD8 (RPA-T8), CD14 (MSE2), CD19 (HIB19), CD25 (M-A251), CD45RA (HI100), PD-1 (eBioJ105) and Lag-3 (3DS223H) were purchased from BD Pharmingen or eBioscience (San Diego, CA). Anti-Foxp3 (clone 249D) was from BioLegend (San Diego, CA) and stains were performed with the Foxp3 staining kit (BioLegend, 421403) per manufacturer recommendations. Acquisition was performed using a FACScalibur or LSRII (BD Bioscience, San Jose, CA) and data were analyzed using FlowJo software (TreeStar Inc., Ashland, OR).

Microarray analysis

Cell pellets (1–2×10^6 cells) were frozen in RNAlater at harvest. 100 ng of total RNA extracted using the RNeasy Mini Plus Kit with on-column DNase digestion (Qiagen) was amplified using Ambion WT expression kit (Life Technologies) according to manufacturer's instructions. mRNA was reverse transcribed into cDNA and a second strand cDNA was generated, then used as a template for in vitro transcription for cRNA synthesis. Following purification, the cRNA was converted into cDNA and excess RNA was removed by RNase H treatment. cDNA was fragmented by restriction digestion and end terminal labeled with GeneChip WT Terminal Label kit (Affymetrix). Samples were hybridized to Human Gene ST 1.0 Arrays (Affymetrix) in a GeneChip Hybridization Oven 640 >16 hours, 45°C, 60 rpm, stained on a GeneChip Fluidics Station 450, and scanned by GeneChip Scanner (Affymetrix).

Bioinformatic Analysis

Fluorescent intensity data (CEL files) were imported and Robust Multi-array Average ('RMA') normalized using the Bioconductor 'oligo' package. Probesets were annotated using the Affymetrix annotation file and the Bioconductor 'biomart' package. Normalized fluorescent intensities were filtered using a variance cutoff of 0.3 with the Bioconductor 'genefilter' package. Differential gene expression thresholds included a log2 fold-change cutoff of 0.5 and significance threshold of 0.05 using a moderated T-statistic corrected for multiple hypothesis testing (Bioconductor 'limma' package). Gene modules were identified using the WGCNA package.

Gene-set enrichment analysis (GSEA) and its single-sample variant were performed using gene sets from the MSigDB. Single sample GSEA to assess correlation of in vitro expanded naïve tTreg and CD4 Teff with ImmSig gene sets comparing Treg and conventional CD4 T cells isolated from: mouse adipose tissue (GEO accession code 7852), a composite of mouse adipose/spleen/lymph node (GEO accession code 7852), or human fetus (GEO accession code 25087). Single sample GSEA to assess correlation of in vitro expanded naïve tTreg and CD4 Teff with ImmSig gene sets comparing acute and chronically stimulated T cells, including: CD4+ T cells isolated from mice infected with the clone 13 vs Armstrong variant of LCMV (GEO accession code 30431), a composite of mouse and human transcriptomic-derived exhaustion-specific genes (GEO accession code GSE96578) or human CD8+ T cells isolated from patients with a progressing HIV infection versus those with controlled HIV infection (GEO accession code 25087).

Statistical analyses

Data were analyzed by one-way analysis of variance (ANOVA) or Student *t* test. Probability (*P*) values less than or equal to 0.05 were considered statistically significant.

Results

Sort-purified naïve tTreg maintain Foxp3 and suppressive function after 5 repetitive stimulations over 50-day time period.

Chronic activation of human or murine T cells leads to exhaustion associated with hypofunction and shortened longevity[33,35,42]. To assess culture expansion effects on human naïve tTreg and non-Treg CD4 T cell populations, naïve tTreg (CD4+25++127–45RA+) and control naïve CD4 T cells (CD4+25–127+45RA+) were sort-purified from the same donors and expanded in parallel (see Figure S1 for details). Naïve tTreg yield from PBMC following sorting was 0.08±0.05% tTreg can be distinguished from iTreg or pTreg by hypo-methylation of the conserved non-coding sequence 2 located in the first intron of the *Foxp3* gene, known as the Treg-specific demethylated region (TSDR)[43,44]. Since *Foxp3* is on the X-chromosome, male donors were selected for TSDR analysis. Foxp3 expression in the sorted population (85±7%) correlated with TSDR demethylation (77±5%) (Figures S1D, E).

Sort-purified naive CD4 T cells and naïve tTreg were separately expanded using KT64/86 cells[27,41]. tTreg without rapamycin (tTreg-No Rapa) were re-stimulated after returning to resting size (day 12), when Treg are highly receptive to TCR signals and hence expansion[16] (Figure S2). CD4+25–127+45RA+ T-cells (referred to as CD4 Teff) increased $3.9\pm0.2\times10^4$-fold after 2 stimulations, while tTreg expanded ~3.5-fold less, at $1.1\pm0.7\times10^4$-fold under the same conditions (Figure 1A). Such tTreg maintained high Foxp3 (75±2% and 69±10% for 1 and 2 stimulations, respectively) and concomitant TSDR demethylation (Figure 1B and C, respectively). We have previously shown that tTreg cultures stimulated a third time in the absence of rapamycin have greatly decreased %Foxp3+[16]. Because a decrease in %Foxp3+ cells was observed in naïve tTreg cultures stimulated a third time in the absence of rapamycin (Figure S2C), this condition was excluded from further analyses.

To determine whether a far greater expansion of tTreg would retain the phenotype observed with 2 stimulations as used in our clinical trials[24,27], a portion of the naïve tTreg sorted from the same donor was stimulated a total of 5 times (Figure S2D). In these longer-term cultures, the addition of rapamycin suppressed the proliferation of Teffs and favored tTreg expansion and stability[16]. Despite known rapamycin anti-proliferative properties, naive tTreg cultured in rapamycin and stimulated for a total of 5 times expanded $1.0\pm0.6\times10^7$-fold (Figure 1D and S2E), >1,000-fold more than twice-stimulated tTreg without rapamycin. All cultures remained ≥69% CD127-Foxp3+, and averaged 86±5% CD127-Foxp3+ (Figure 1E). Compared to one stimulation, Foxp3 mean fluorescent intensity progressively increased in tTreg+Rapa (relative Foxp3 expression stimulated 4 or 5 times was 168% or 211%, respectively) (Figure 1F). To compare suppressive function of all culture conditions without potential assay variations, frozen Treg aliquots were thawed and concurrently assayed in a CFSE-based, anti-CD3 mAb-driven CD8+ T cell proliferation assay at tTreg:PBMNC ratios from 1:2–1:8. All cultures had ≥ 42% suppressor function at 1:4 (Treg:PBMNC). While the average suppression of Treg stimulated 5 times in the presence of rapamycin was lower, it was not statistically significant, and these cultures were still highly suppressive (Figure 1G).

tTreg expanded >10-million-fold maintained tTreg-associated gene expression.

To ascertain the impact of expansion on the expression of Treg-associated genes after multiple stimulations, microarray profiling was conducted on RNA purified from CD4 Teff or tTregs (tTreg-No Rapa) expanded 1 or 2 times and tTreg expanded with 1, 2, or 5 times in rapamycin (tTreg+Rapa) (n=3 donors for each group).

Principal component analysis (PCA) showed significant clustering of gene expression patterns by cell type and by number of stimulations (Fig. 2A; see also Table 1), indicating individual groups of cells propagated under these discrete conditions were distinct. To discern the transcripts most closely associated with each sample type, we employed single-sample gene-set enrichment analysis (ssGSEA)[45] with special attention to the extent to which each population maintained tTreg-associated gene expression. Gene expression differences in expanded cell populations were analyzed with previously curated sets of Treg-associated genes expressed in Tregs purified from three different sources, PB[46], cord blood[47,48], and adipose tissues[46]. To interrogate genes directly regulated by Foxp3, we also performed ssGSEA against a published murine CHIPseq dataset that identifies genes directly bound by FOXP3[49]. *In vitro* expanded tTreg vs Teff showed enrichment in each of these four canonical tTreg gene sets (Figure 2B).

Unlike CD4 Teff, tTreg repetitively stimulated in rapamycin do not upregulate genes associated with exhaustion.

Exhaustion was initially described as a condition in which T cells chronically activated by virus displayed decrease proliferation and effector function[35]. Gene expression profiling of acute vs. chronically activated T cells has now identified a core group of exhaustion-related genes[40,42,50]. Multiply re-stimulated tTreg maintained both proliferative and suppressive function (Figure 1D and G, respectively), providing important functional evidence against tTreg exhaustion. To further evaluate exhaustion, we performed ssGSEA to compare our transcriptome data to three published gene sets. The first gene set was identified as distinguishing exhausted CD4 and CD8 gene expression patterns from mice infected with lymphochoriomeningitis virus (LCMV) clone 13 that causes chronic viral T cell stimulation and exhaustion vs LCMV Armstrong that causes acute infection and does not result in T cell exhausion[40]. The other two gene sets were identified by comparing human CD8 T cell expression patterns from HIV+ patients able to control their infection (“controllers”, putatively non-exhausted) in contrast to “progressors” non-controllers who developed AIDS (putatively exhausted)[42,50] (Figure 3). Genes differentially expressed in CD4 Teffs stimulated once versus twice, corresponding to an increase in expansion from ~400-fold to ~40,000-fold, respectively, showed significant enrichment for genes associated with exhausted murine CD4 and CD8 T cells and human CD8 T-cells from patients who progressed to AIDS (Figure S3A; false discovery rate (FDR)=0.001). Similarly, gene expression changes associated with tTreg stimulated once or twice without rapamycin (tTreg-No Rapa) showed enrichment for gene sets associated with murine CD4 and CD8, and human CD8 T-cell exhaustion (Figure S3B; FDR=0.001). In contrast, no significant enrichment in exhaustion-related genes) was observed in tTreg+Rapa, even though expansion with 5 rounds of stimulation was 100,000-fold higher than one stimulation (Figure S3C; FDR=0.18). This lack of an exhaustion signature suggests that tTreg highly

expanded in rapamycin may have greater *in vivo* therapeutic advantages than tTreg stimulated without rapamycin.

tTreg+Rapa repetitively stimulated 3 or 5 times develop a gene signature distinct from tTreg cultured under other conditions.

Supervised hierarchical clustering and expression analyses were performed comparing naïve CD4 T cells (expanded with 1 or 2 stimulations), to naïve tTreg (expanded with 1 or 2 stimulations *without* rapamycin) and to naïve tTreg (expanded with 1, 2, 3 or 5 stimulations in rapamycin). A total of 4359 differentially expressed genes (FDR $p<0.05$) were identified, distributing into 7 independent clusters (Figure 4A). The cluster color-coded 'red' identified genes enriched in all tTreg groups, and included canonical Treg genes such as Foxp3 and Helios, and other genes shown to be differentially expressed in expanded human tTreg (e.g. UTS2, SLC14A1, CDKN2B, Eos, PLCL1, SOCS2, CD80, GPR55, LSR)[51]. The cluster color-coded 'lilac' was associated with tTreg undergoing one or two rounds of stimulation ± rapamycin, and included a set of previously reported tTreg-related genes (e.g. RTKN2, CASK, SELP, MYO5C, CD27, DGKA, and CCR6)[51].

The cluster color-coded 'purple' associated with tTreg expanded with 3 or 5 rounds of stimulation in rapamycin, and included a separate set of tTreg-related genes (including IL1R1, NIPAL2, IL2RA)[51,52]. Furthermore, several genes in the purple cluster are known to be differentially expressed in follicular regulatory T cells (Tfr) including LRRC32 (GARP), CTLA4, IRF4, and TIGIT[53,54], and this cluster also included two additional genes (EPHB1, INHA) that may encode potential Tfr effector molecules[55,56]. EPHB1 inhibits Tfollicular helper recruitment and germinal center retention[56], while INHA antagonizes INHBA-supported Tfollicular helper differentiation[55]. In agreement with flow phenotyping, the purple cluster was enriched in IL2Rα. Aptly, Pim-2, a functional Akt homolog which confers rapamycin resistance in Treg[25], was associated with the purple cluster. While the purple cluster could simply represent genes regulated by rapamycin, tTreg expanded in rapamycin receiving more limited stimulation (1–2 stimulations) did not associate with the purple cluster. Moreover, ssGSEA showed that tTreg+Rapa expanded with 3 or 5 stimulations did not preferentially associate with several defined rapamycin-dependent gene sets (Figure S4). The gold and gray clusters were not defined by specific cell subsets.

Expanded CD4 Teff have increased expression of multiple inhibitory co-receptors compared to tTreg expanded with or without rapamycin.

The cluster color-coded 'green', associated with CD4 Teff populations, and was comprised of a large set of genes enriched in murine and human CD4 (non-Treg) cells including: IL-7Rα (CD127) and Themis[51,57], as well as several genes involved in effector (e.g. TBX21, ID2, CD40L) and exhaustion (Eomes, TOX) differentiation[40,42,50]. Co-expression of multiple inhibitory receptors which regulate TCR signaling represent a molecular hallmark of exhausted CD4 and CD8 T-cells[40,42]. Studies indicate that the degree of exhaustion was not linked to expression of any single inhibitory receptor, but to the aggregate number of individual receptors co-expressed on a given cell, typically including PD-1, Lag-3, Tim-3, CD160 and CTLA4[40]. More recently, TIGIT and KLRG1 were shown to be upregulated in exhausted human CD8 T cells[42]. In agreement with ssGSEA, the green cluster contained

several inhibitory receptors previously correlated with exhaustion (Lilrb3, PD-L1, PD-L2, Tim-3, CD274, KLRG1, CD96 and LAIR1)[58] as well as several Killer Inhibitory Receptors (KIR) genes, both inhibitory (KIR2DL2, KIR2DL3, KIR2DL4, KIR3DL3, KIR2DL5B) and activating (KIR2DS2, KIR2DS3, KIR2DS4), associated with a late memory CD4 T-cell phenotype[59]. Two inhibitory receptors (CTLA4, TIGIT), which are typically expressed on tTreg, were associated with the purple cluster which defined tTreg stimulated 3–5 times in rapamycin.

To determine inhibitory receptor protein expression in CD4 Teff and tTreg cultures, aliquots were concurrently thawed and stained with antibodies to CTLA4, TIGIT, PD-1 and Lag-3. As expected, ≥70% of tTreg+Rapa cultures expressed CTLA4 and TIGIT after 1 stimulation (Figure 5A and B). CTLA4 did not further increase with up to 5 stimulations. CTLA4 and TIGIT expression on naïve and memory CD4+ T cells (CD45RA+ and CD45RA−, respectively) was compared to tTreg directly analyzed *ex vivo*. CTLA4 expression was higher in both naïve and memory tTreg than in CD4+ T cells (Figure 5A). Consistent with activation-induced expression of inhibitory receptors, a higher % of memory CD4+ T cells expressed TIGIT compared to naïve CD4+ T cells (Figure 5B). Compared to CD4+ T cell subsets, a higher % of naïve and memory tTreg expressed TIGIT and memory tTreg had greater TIGIT expression than naïve tTreg.

PD-1 was not identified in the cluster color-coded ‘black’ in Figure 4 and was expressed in only ~5% of tTreg+Rapa stimulated 5 times (Figure 5C). Memory CD4 T cells and tTreg expressed higher PD-1 levels than naïve counterparts and, unlike what was seen following *in vitro* expansion, a higher % of memory tTreg expressed PD-1 than memory CD4 T cells. Lag-3 was present in the black cluster and associated with cultures undergoing the greatest fold-expansion, increasing to ~30% Lag3+ cells in tTreg+Rapa after 5 stimulations (Figure 5D). While Lag-3 has been shown to inhibit Teff cells, Treg with an activated phenotype have been shown to express this inhibitory receptor, and Lag-3 expressing Treg specifically inhibit IL-1ß and IL-23 expression by CX3CR1+ macrophages[60,61]. Flow cytometry showed decreasing TIGIT expression in tTreg+Rapa cells stimulated 2–5 times (61±4% to 51±3%; $p<0.05$) and a paucity (≤4%) of tTreg+Rapa stimulated 5 times co-expressed PD-1 and Lag-3 (Figure 5E and F). As tTregs stimulated 5 times had potent suppressor function (Figure 1G) and no evidence of an exhaustion signature (Figure 3), in aggregate. these data are consistent with tTreg not becoming exhausted despite substantial expansion rates.

Transcription factor WGCNA identifies differentially expressed ‘tTreg locking’ transcription factors in tTreg undergoing 1 or 2 vs. 3 or 5 stimulations.

To interrogate the transcriptional programs driving Treg specification and rapamycin effects using an unsupervised statistical approach, we applied weighted gene correlation network analysis (WGCNA)[62] to construct a gene co-expression network[63,64]. To identify TF that might drive expression of target genes associated with differentially cultured Treg, we focused the network analysis on the top-1000 most variant TFs identified by gene array. To improve statistical power for network analysis, we grouped our samples into CD4 Teff (1 and 2 stimulations), tTreg-No Rapa (1 and 2 stimulations), tTreg+Rapa (1 and 2 stimulations), and tTreg+Rapa (3 and 5 stimulations). WGCNA for TFs identified 3

discrete self-assembling modules, illustrated by a new cluster color schema (with modules color-coded ‘green’; ‘violet’; ‘yellow’) (Figure 6A). CD4 Teff cultures were associated with the green module (Figure 6B) enriched for Teff TF including Tbet (Tbx21), Eomes, and BATF. This module also includes 52 separate histone genes, shown to increase greatly during S-phase in CD4 T-cells[65]. Correspondingly, flow phenotyping showed that CD4 Teff had significantly higher expression of Ki-67, a proliferation marker most highly expressed during S-phase, than tTreg grown in the presence or absence of rapamycin (not shown).

tTreg-No Rapa and tTreg+Rapa (1 and 2 stimulations each) cultured cells associated with the violet module contained many canonical Treg-associated TF, including Foxp3. Importantly, while Foxp3 gene expression was more enriched in the violet-associated tTreg-No Rapa and tTreg+Rapa (1 and 2 stimulations) populations, flow cytometry phenotyping demonstrated that Foxp3 protein was highest in the tTreg+Rapa (3 and 5 stimulations) cultures, suggesting post-transcriptional regulation of Foxp3 is also likely involved (Figure 1C,S5A). Helios, an *Ikaros*-family transcription factor and Foxp3 co-factor that is required to maintain Foxp3 expression in a chronic inflammatory microenvironment[66,67], was also present in the violet module, consistent with flow cytometry data showing increased protein expression in tTreg cultures with 1 or 2 stimulations (Figure S5B). Eos, another *Ikaros*-family TF that binds to and maintains Foxp3 expression, preventing Treg reprogramming into Teff[68], was also significantly enhanced in the violet TF module.

Using a computational algorithm [the context likelihood of relatedness (CLR)[69]] to predict TF that contribute to the Treg signature, a recent study identified five TFs (Eos, IRF4, Satb1, Lef1, GATA-1), in addition to Helios and Xbp1, that act in synergy with Foxp3 in a redundant manner to ‘lock in’ the Treg cell signature[28,70]. The violet and yellow modules were enriched in specific subsets of these ‘Treg locking factors. The violet module contained three of the ‘Treg locking factors’, (Eos, Lef1 and Satb1)[71]. We previously showed that Bach2 has a similar ‘Treg-locking’ function and maintains Treg stability by repressing effector gene expression[30,72]. Bach2 was present in the violet transcription factor module and associated with tTreg groups stimulated 1–2 times.

tTreg stimulated 3 or 5 times in rapamycin was associated with the yellow module (Figure 5B), enriched in IRF4 and GATA1. IRF4 is a positive regulator of Treg homeostasis and, by upregulating PRDM1 expression (also present in the yellow module) and IL-10 production, controls differentiation of effector Treg which undergo stimulus-specific differentiation to Treg helper types[73,74]. IRF4 expression is enhanced by TCR stimulation and IRF4 mRNA expression was further increased when Treg were activated in the presence of the mTOR inhibitors Torin 1 or PP242[75]. IRF4 increases Pim-2, the rapamycin-independent Akt functional homolog[25]. One of the top Treg connections for GATA1 identified by CLR analysis was CD25[28], consistent with tTreg+Rapa (3 and 5 stimulations) having the highest CD25 expression (Figure 1B). GATA1 also potently down-regulates IFNγ expression[76], consistent with the lack of enrichment for this effector cytokine in tTreg cultures in rapamycin, providing supporting evidence for preservation of a tTreg signature.

Discussion

Our goals were two-fold: to determine whether naive tTreg maintain expression of tTreg-associated genes after multiple rounds of in vitro expansion, and whether such a technique would result in an exhausted phenotype as can be seen in human Teff cells expanded for cancer immunotherapy[71]. Using a bioinformatics approach, we report that *in vitro* expanded tTreg maintained a tTreg signature, even after ten million-fold expansion. While a single re-stimulation of CD4 Teff resulted in a significant increase in the expression of mRNAs present in exhaustion-related gene sets, no increase was observed in tTreg stimulated 5 times in the presence of rapamycin. One of the hallmarks of exhaustion is the accumulated expression of a group of inhibitory receptors. Hierarchical clustering found a group of 13 inhibitory receptor genes that were preferentially associated with CD4+ Teff cultures but not in the highly expanded tTreg. The 3 inhibitory receptors (CTLA4, TIGIT and Lag-3) with increased expression on expanded Treg compared to CD4 Teff have each been shown to mark Treg with enhanced suppressive function[77]. The ability of this stimulation protocol to generate such robust expansion without significant signs of exhaustion or activation induced cell death are likely multifactorial. These may include the conditions and use of a cell-based artificial antigen presenting cell (aAPC) for stimulation, choice of aAPCs expressing co-stimulatory molecules (including 4.1BBL and TRAIL), surface expression of CD58 that binds to CD2 on T cells to strengthen cell adhesion, and the timing of stimulation. With respect to the latter, we have previously reported that allowing Tregs to return to a resting size prior to additional stimulation increases their expansion which we ascribe to avoiding restimulation during a period of relative TCR signaling refractoriness.

This bioinformatic approach also found that, while all expanded tTreg express a core group of genes (that includes Foxp3 and Helios), one key difference between tTreg expanded with 1 or 2 rounds versus 3 or 5 rounds of stimulation was the differential expression level of 5 previously defined ‘Treg locking genes’ (Eos, Lef1, Satb1, IRF4, GATA1). tTreg expanded with 1 or 2 stimulations had increased expression of Eos, Lef1 and Satb1 (as well as the more recently defined Bach2 and TCF1), whereas tTreg+Rapa cells expanded 3 or 5 times had increased expression of 2 of the 5 Treg locking signature genes, IRF4 and GATA1, associated with effector tTreg[74]. To our knowledge, this is the first example of an expansion-based, differential expression of these TF for human Tregs, suggesting an ordered approach to the transcriptional control of Treg stability and function.

tTreg receiving 3 or 5 rounds of stimulation in rapamycin also developed an independent gene expression pattern that did not overlap with genes previously demonstrated to be regulated by rapamycin suggesting that tTreg had intrinsic properties that favored a locking Treg signature and disfavored the acquisition of an exhaustion signature. The link between this cluster of genes and Tfr-associated genes is intriguing and is reminiscent, especially in the context of differential Treg-locking gene expression, of the recently described connection between murine resting Treg and Tfr associated with the activated Treg subset[32]. Expression of Lef1 and TCF1 can be used to divide Treg into resting (TCF1+LEF1+), activated (TCF1+LEF1+/lo), and effector (TCF1-LEF1-) subsets[32]. Similar to our data, this report found, in addition to Lef1 and Tcf1, resting Treg had higher expression of Satb1 and

Bach2. Additionally, Tfr cells were almost exclusively derived from the activated Treg pool that uniquely, amongst the three subsets, expressed both GATA1 and IRF4.

In conclusion, this study has shown that, in addition to maintaining Foxp3 expression and suppressive function, large-scale *in vitro* expanded tTreg maintained a canonical tTreg transcriptional profile and did not develop a signature consistent with exhaustion. The presence of increased inhibitory receptor expression on memory tTreg analyzed directly *ex vivo* suggests clinical products would best be started with naïve tTreg, which also maintain higher Foxp3+ expression after expansion than memory tTreg[78], even though this would reduce initial yield. In regards to potential clinical banking, a typical apheresis unit of 12×10^9 cells would yield ~10×10^6 purified naïve tTreg which, after 10×10^6-fold expansion from 5 rounds of stimulation in the presence of rapamycin, would generate a bank of ~1×10^{14} cells. Since CD45RA expression on CD4 T cells decreases with age[79], and our starting tTreg population was only 14±3% CD45RA+, bank size could be further increased by restricting donor age or by using UCB tTreg, which are uniformly CD45RA+[80]. While, admittedly, many additional factors will play into how many patients a bank of this size could treat, it is interesting to note that, at the 100×10^6/kg dose our recent clinical trial with UCB tTreg showed almost completely ameliorated GVHD, the upper limit would be 10,000 patients receiving a single tTreg infusion. These data pave the way for consideration of generating tTreg banks of multiply re-stimulated cells to provide an off-the-shelf product, especially given that our prior data[24,81]and that of others[82] indicate that related or unrelated donor Treg need not be more than HLA 3/6 matched with the possibility that HLA-matching requirements would be limited if at all. These studies provide relevant insights on the mechanisms of Treg-mediated protection from GVHD and support for the use of third-party Treg in clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Disclosure of Conflicts of Interest statement

BRB is a founder of Tmunity Therapeutics, serves as an advisor for and receives research support from BlueRock Therapeutics, and, along with KLH holds patents for the production and use of Treg for clinical trials.

LSK is on the scientific advisory board for HiFiBio. She reports research funding from Bristol Myers Squibb, Kymab Limited, Magenta Therapeutics, BlueBird Bio, and Regeneron Pharmaceuticals. She reports consulting fees from Equillium, FortySeven Inc, Novartis Inc, EMD Serono, Gillead Sciences and Takeda Pharmaceuticals. She has a patent "Method to prevent relapse after transplant" which is pending, and a patent "Method to prevent GVHD after transplant" with royalties paid.

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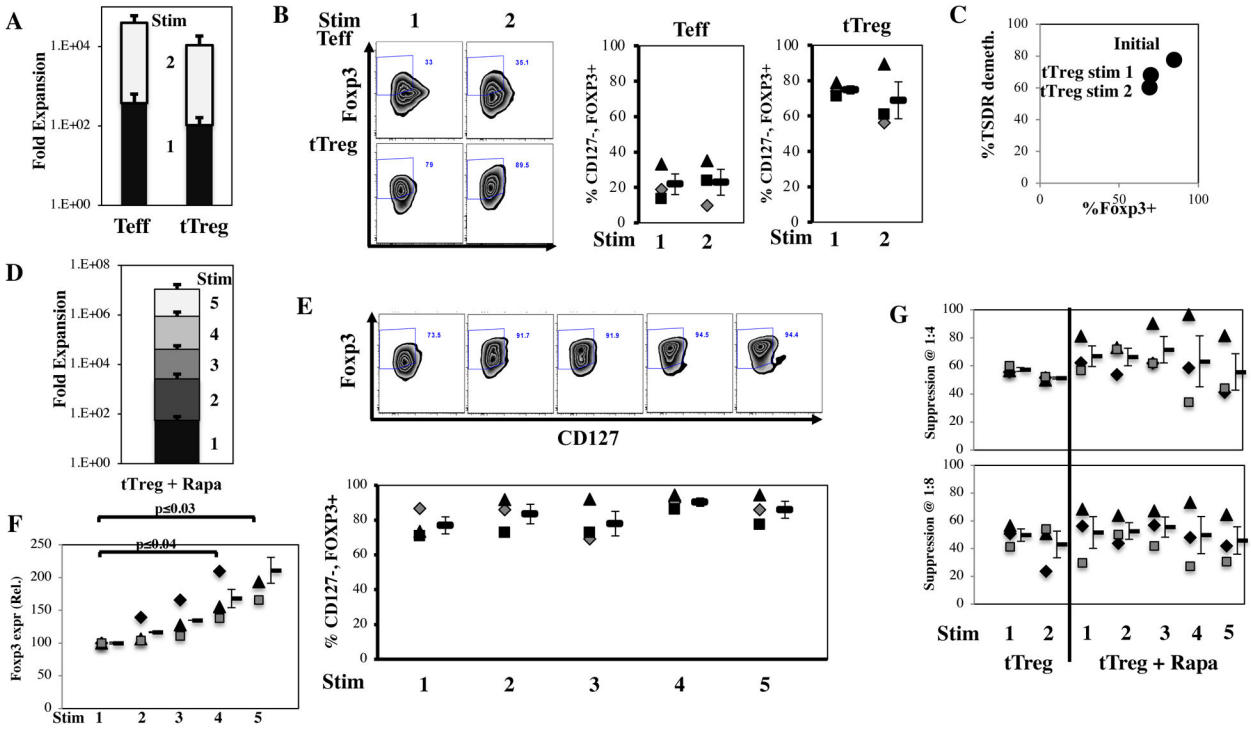


Figure 1: Sort-purified naïve tTreg maintain Foxp3 and suppressive function after multiple stimulations. In vitro expansion of naïve human tTreg and naive CD4 T cells.

Naïve PB CD4+ T cells and tTreg were sort-purified (CD4+25–127+45RA+ and CD4+25hi127−45RA+, respectively) and expanded with anti-CD3 mAb-loaded KT64/86 using two rounds of stimulation. tTreg were also stimulated 5 times in the presence of rapamycin. (A) Average expansion for CD4 T cells (Teff) and tTreg-No Rapa. (B) Representative example (left) and average percent CD127−Foxp3+ (CD4-gated). (C) Average (n=3) %TSDR demethylation of naïve tTreg stimulated once or twice in the absence of rapamycin compared with Foxp3 expression. (D) Average expansion for naïve tTreg stimulated 1–5 times in the presence of rapamycin. (E) Representative example (above) and individual data points along with average percent CD127−Foxp3+ (CD4-gated) for tTreg+Rapa stimulated 1–5 times as indicated. Mean ± 1 SEM is shown. (F) Average relative Foxp3 expression in naïve tTreg expanded in rapamycin with the indicated number of stimulations normalized to 1 stimulation. (G) Percent suppression of in vitro CD8 T cell proliferation tTreg/PBMNC ratios of 1:2, 1:4 and 1:8 for tTreg cultures stimulated the indicated number of times in the presence or absence of rapamycin. Data shown are mean ± SEM. No significant differences were observed in suppression. All results are from 3–4 independent experiments.

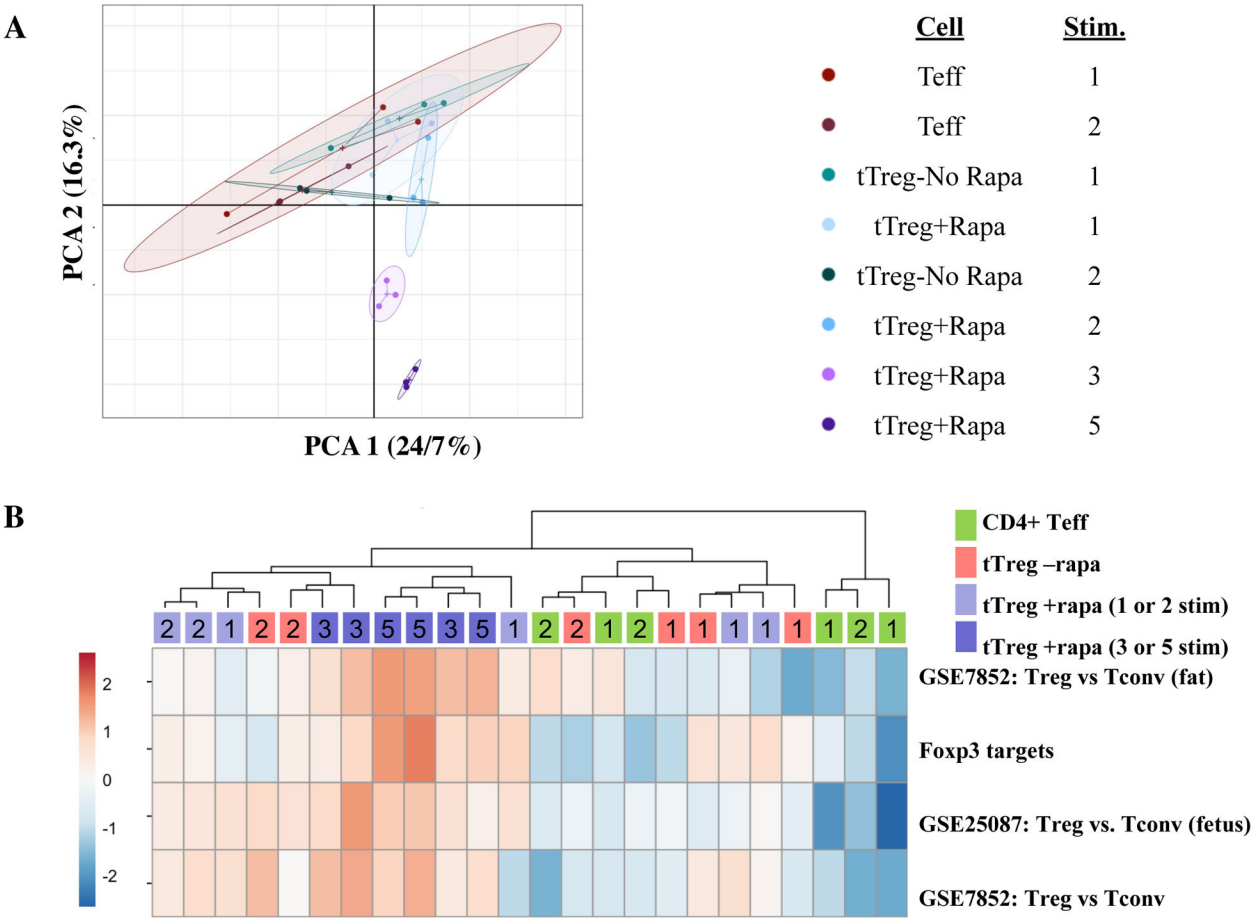


Figure 2: Thymic Treg expanded >10-million fold maintain expression of tTreg-associated genes.

Naïve PB CD4+ T cells and tTreg were sort-purified (CD4+25–127+45RA+ and CD4+25hi127−45RA+, respectively) and expanded with anti-CD3 mAb-loaded KT64/86 using up to 5 rounds of stimulation, and microarray profiling performed. (A) Principle-component analysis (PCA) comparing CD4 Teff, tTreg-No Rapa, and tTreg-Rapa expanded with low (1 or 2) and high (3 or 5) number of stimulations. PCA1, PCA2: principle components 1 and 2. (B) Heatmap of Treg-related gene sets enriched between CD4 Teff, tTreg-No Rapa and tTreg+Rapa stimulated the indicated number of times using ssGSEA. Gene sets were comparisons of Treg vs. conventional T cells isolated from adipose tissue (GSE25087), fetal tissue (GSE25087) and peripheral blood (GSE7852). Foxp3 targets is a gene set identified by Foxp3 ChIP assay.

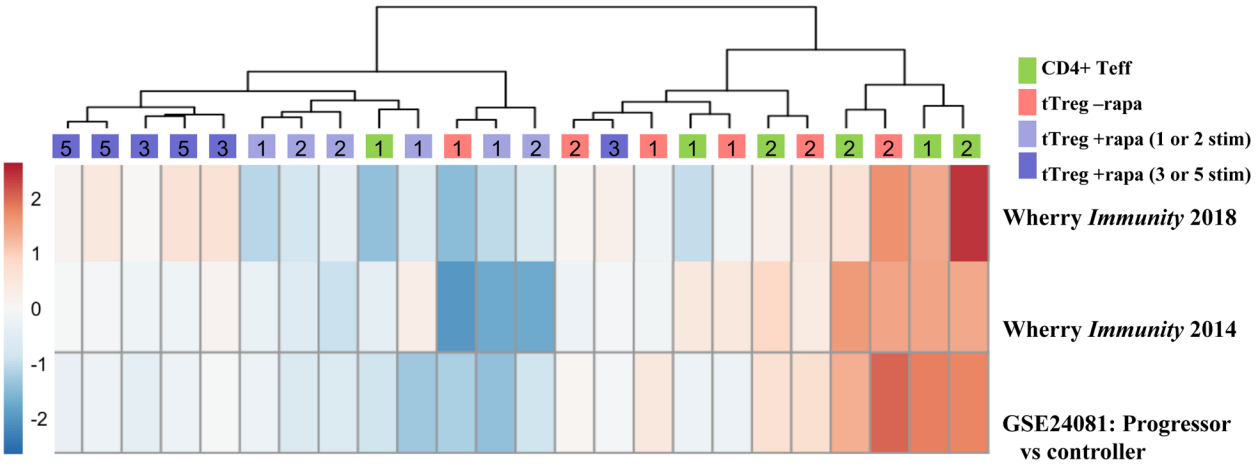
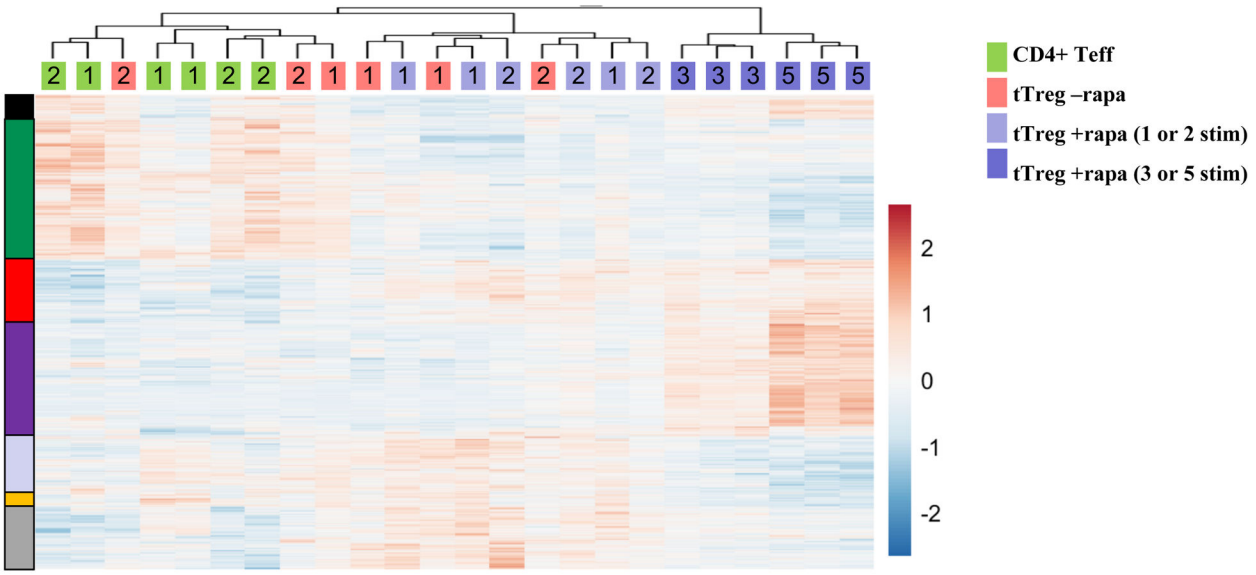


Figure 3: Thymic Treg expanded >10-million do not upregulate genes associated with exhaustion.

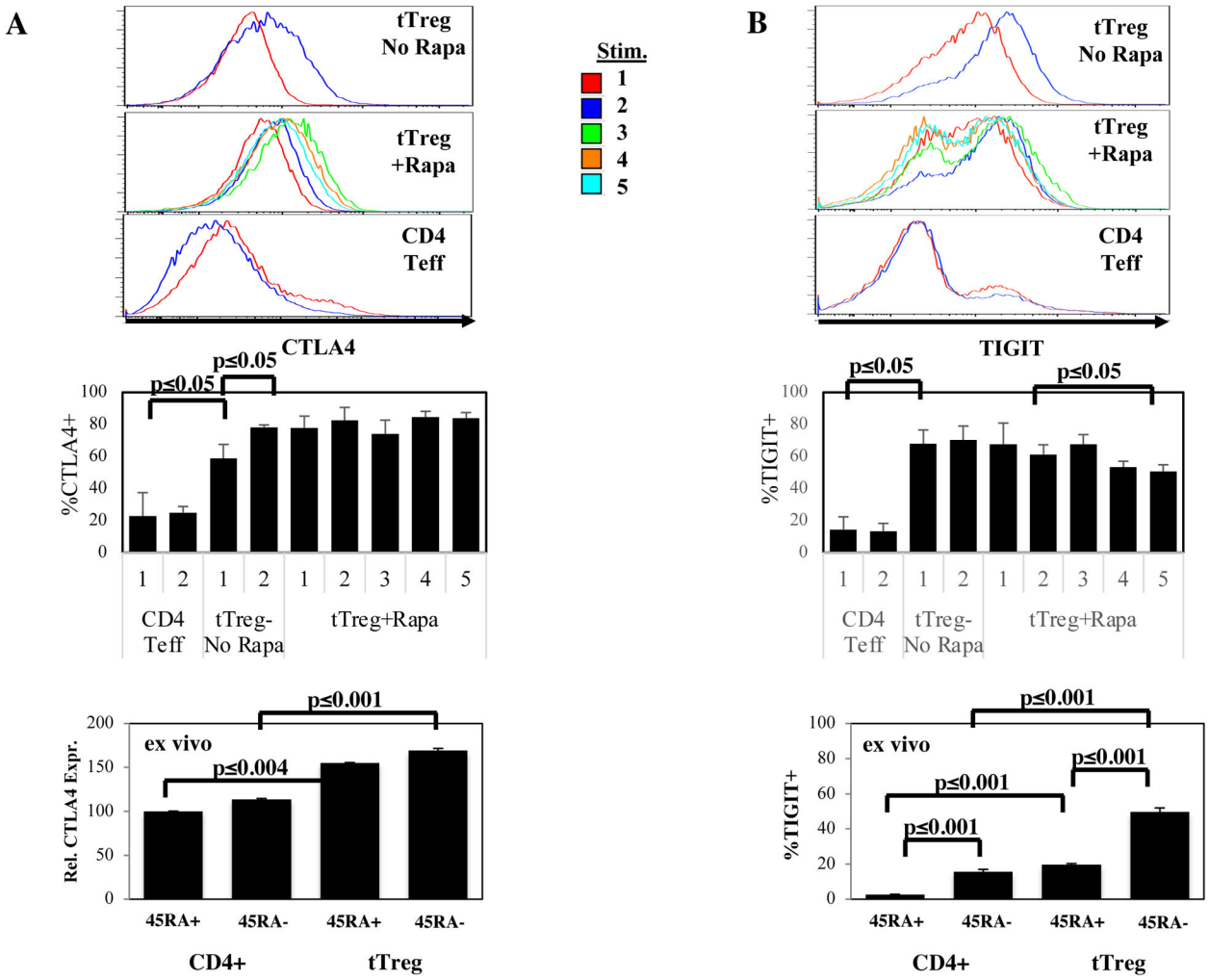
Naïve PB CD4+ T cells and tTreg were sort-purified (CD4+25–127+45RA+ and CD4+25hi127−45RA+, respectively) and expanded with anti-CD3 mAb-loaded KT64/86 using up to 5 rounds of stimulation, and microarray profiling performed. Heatmap of exhaustion-related gene sets enriched between CD4 Teff, tTreg-No Rapa and tTreg+Rapa stimulated the indicated number of times using ssGSEA. Gene sets were murine genes associated with exhaustion in both murine CD4 and CD8 T cells (Wherry *Immunity* 2014), and two gene sets from human CD8 T cell isolated from HIV+ patients able to control their infection (controllers) vs. those who developed AIDS (progressors) (Wherry *Immunity* 2018 and GSE24081).



	Color	Assc. Cells	Representative genes
	Black	Greatest expansion	Lag-3
	Green	CD4 Teff	IL-7RA, Themis, TBX21, ID2, CD40L Eomes, TOX; multiple inhibitory receptors and cytokines
	Red	All tTreg	Foxp3, Helios, UTS2, SLC14A1, CDKN2B, Eos, PLCL1, SOCS2, CD80, GPR55, LSR
	Purple	tTreg +Rapa, 3 or 5 stim.	CD25, IL1R1, NIPAL2, TIGIT, EPHB1 LRRC32/GARP, Pim2
	Lilac	tTreg with 1 or 2 stim., independent of rapa	RTKN2, CASK, SELP, MYO5C, CD27, DGKA, and CCR6
	Gold	Not defined	
	Gray	Not defined	

Figure 4: tTreg+Rapa stimulated 3 or 5 times develop a gene signature distinct from tTreg stimulated 1 or 2 times.

To assess in vitro expansion-related changes in global gene expression between CD4 Teff, tTreg-No Rapa and tTreg+Rapa, RNA was purified from cultured cells and microarray analysis performed. Heatmap of genes differentially expressed between CD4+ Teff and tTreg-No Rapa stimulated once or twice, and tTreg+Rapa stimulated 1, 2, 3 or 5 times (FDR-adjusted P value, <0.05).



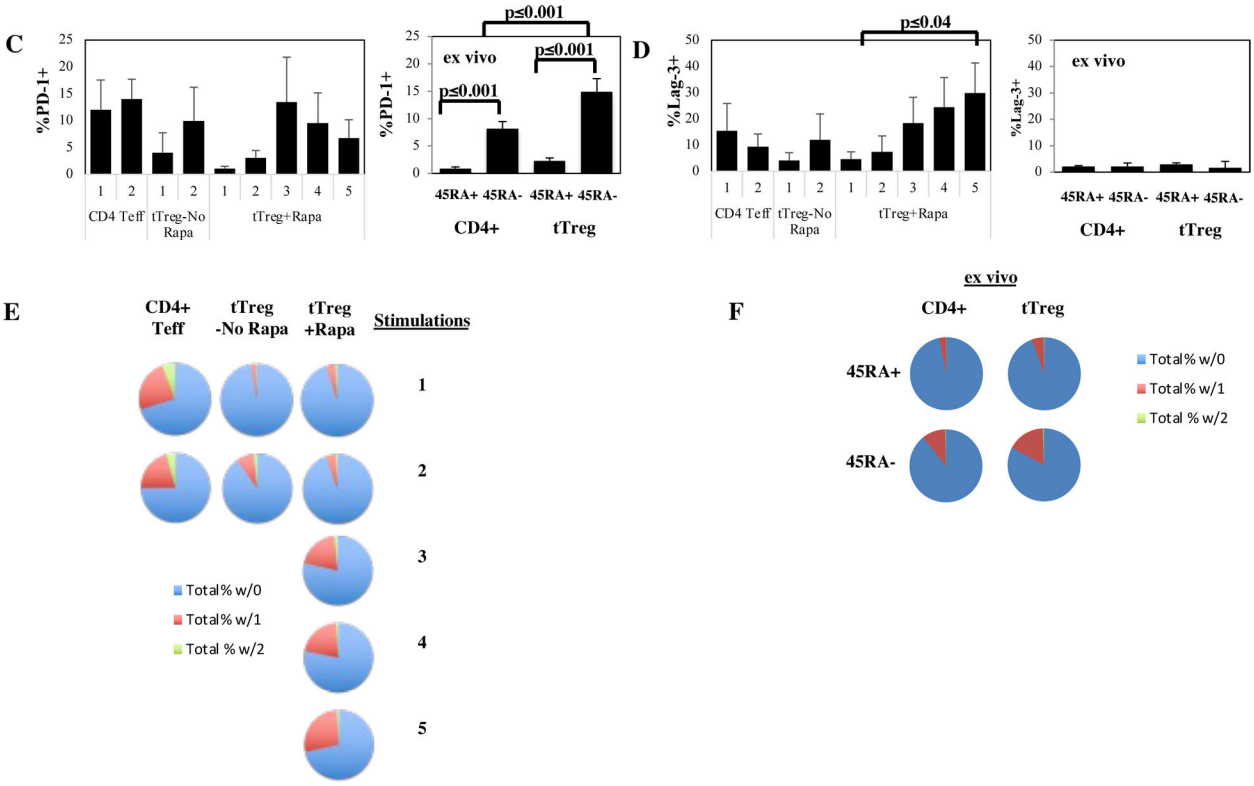
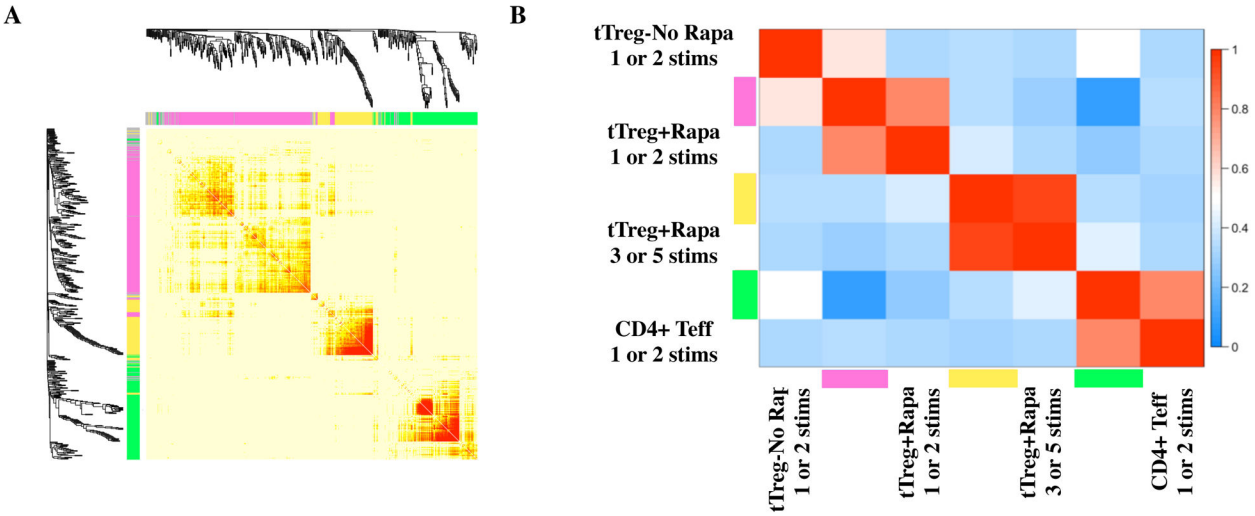


Figure 5: Expanded CD4 Teff have increased expression of multiple inhibitory co-receptors genes compared to tTreg expanded with or without rapamycin.

To assess protein expression of specific inhibitory receptors, aliquots of CD4 Teff and tTreg cultured with or without rapamycin undergoing the indicated number of stimulations were concurrently thawed and stained with antibodies to CTLA4, TIGIT, PD-1 and Lag-3. To determine how this expression compares to naïve and memory CD4 T cells and tTreg directly ex vivo, PBMC were also stained for these markers. Representative example and average % of CD4+ Teff, tTreg-No Rapa or tTreg+Rapa that express CTLA4 (A) or TIGIT (B). Summary of PD-1 (C) and Lag-3 expression (D) in CD4+ Teff, tTreg-No Rapa or tTreg+Rapa after expansion or directly ex vivo. (E, F) Pie chart showing Boolean gating for PD-1 and Lag-3 co-expression in CD4+ Teff, tTreg-No Rapa or tTreg+Rapa after expansion (E) or directly ex vivo (F) from a representative experiment. Results from expanded cells are n=3 independent donors and ex vivo data is from n=12 donors.



Cell type	Stim. #	Rapa	Color	Module	Representative TF
CD4 Teff	1/2	NA		Green	Tbx21, Eomes, BATF
tTreg	1/2	-		Violet	Foxp3, Helios, Eos, Satb1, Lef1, Bach2, TCF1
tTreg	1/2	+		Violet	
tTreg	3/5	+		Yellow	IRF4, GATA1, PRDM1

Figure 6: Transcription factor WGCNA shows tTreg stimulated 1– 2 times express different 'tTreg locking' transcription factors than tTreg stimulated 3–5 times.

Naïve PB CD4+ T cells and tTreg were sort-purified (CD4+25– 127+45RA+ and CD4+25hi127−45RA+, respectively) and expanded with anti-CD3 mAb-loaded KT64/86 using up to 5 rounds of stimulation, and microarray profiling performed. (A) Topological overlap matrix plot with hierarchical clustering tree and the resulting gene modules from a weighted network of total CD4 Teff, tTreg-No Rapa and tTreg+Rapa transcripts. (B) Eigengene adjacency heatmap showing module eigengene similarity to CD4 Teff, tTreg-No Rapa and tTreg+Rapa.

Table I:

Global summary of CD4 Teff and tTreg Data

Cell type	Stim#	Rapa	Fold Exp. (Approx.)	Hierarchical clustering		TF WGCNA		Overall conclusions
				Module	Representative genes	Module	Representative TF	
CD4 Teff	1 or 2	NA	**400–40,000**	**Green**	IL-7RA, Themis, TBX21, ID2, CD40L Eomes, TOX; multiple inhibitory receptors and cytokines	**Green**	Tbx21, Eomes, BATF	• T effector/memory gene expression • TOX and inhibitory receptor expression suggest later stage of differentiation.
tTreg	1 or 2	−	**100–1000**	**Red**	Foxp3, Helios, UTS2, SLC14A1, CDKN2B, Eos, PLCL1, SOCS2, CD80, GPR55, LSR	**Violet**	Foxp3, Helios, Eos, Satbl.Lefl, Bach2, TCF1	• Maintain Treg signature • Treg locking gene expression similar to resting tTreg
				Lilac	RTKN2, CASK, SELP, MY05C, CD27, DGKA, and CCR6			
tTreg	1 or 2	+	**50–2500**	**Red**	Foxp3, Helios, UTS2, SLC14A1, CDKN2B, Eos, PLCL1, SOCS2, CD80, GPR55, LSR	**Violet**	Foxp3, Helios, Eos, Satbl, Lefl, Bach2, TCF1	• Maintain Treg signature • Treg locking gene expression similar to resting tTreg
				Lilac	RTKN2, CASK, SELP, MY05C, CD27, DGKA, and CCR6			
tTreg	3 or 5	+	**40,000–10,000,000**	**Red**	Foxp3, Helios, UTS2, SLC14A1, CDKN2B, Eos, PLCL1, SOCS2, CD80, GPR55, LSR	**Yellow**	IRF4, GATA1, PRDM1	• Maintain Treg signature • Treg locking gene expression similar to activated tTreg • May be a precursor to Tfr pool.
				Purple	RTKN2, CASK, SELP, MY05C, CD27, DGKA, and CCR6			