mTOR Complex 1 Signaling Regulates the Generation and Function of Central and Effector Foxp3⁺ Regulatory T Cells

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The mechanistic/mammalian target of rapamycin (mTOR) has emerged as a critical integrator of signals from the immune microenvironment capable of regulating T cell activation, differentiation, and function. The precise role of mTOR in the control of regulatory T cell (Treg) differentiation and function is complex. Pharmacologic inhibition and genetic deletion of mTOR promotes the generation of Tregs even under conditions that would normally promote generation of effector T cells. Alternatively, mTOR activity has been observed to be increased in Tregs, and the genetic deletion of the mTOR complex 1 (mTORC1)–scaffold protein Raptor inhibits Treg function. In this study, by employing both pharmacologic inhibitors and genetically altered T cells, we seek to clarify the role of mTOR in Tregs. Our studies demonstrate that inhibition of mTOR during T cell activation promotes the generation of long-lived central Tregs with a memory-like phenotype in mice. Metabolically, these central memory Tregs possess enhanced spare respiratory capacity, similar to CD8⁺ memory cells. Alternatively, the generation of effector Tregs (eTregs) requires mTOR function. Indeed, genetic deletion of *Rptor* leads to the decreased expression of ICOS and PD-1 on the eTregs. Overall, our studies define a subset of mTORC1^{hi} eTregs and mTORC1^{lo} central Tregs. *The Journal of Immunology*, 2018, 201: 481–492.

egulatory T cells (Tregs) play a pivotal role in controlling immune responses and maintaining peripheral tolerance. Defined by the canonical transcription factor Foxp3, natural Tregs emerge from the thymus, whereas inducible Tregs can differentiate from naive CD4⁺ T cells (1). It is clear that the precise expression profile of Tregs varies greatly depending upon their tissue localization. For example, PPARy, an important transcription factor that promotes adipocyte differentiation, plays a critical role in regulating genetic programs for Tregs that reside in adipose tissue (2). Likewise, Tregs also express canonical effector Th cell transcription factors, such as T-bet, GATA-3, Bcl6, and IRF4 and have been shown to be necessary for optimal suppression of the corresponding T helper subsets (3-11). Recently, Tregs from secondary lymphoid organs, such as the spleen and lymph nodes, have been divided into two subgroups based on their CD44 and CD62L expression: CD44^{lo} CD62L^{hi} central Tregs (cTregs) or CD44^{hi} CD62L^{lo} effector Tregs (eTregs) (12). These

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subsets have been suggested to play differential roles in maintaining homeostasis in secondary lymphoid organs and distant tissue sites.

Initially, Tregs were described as CD25⁺ T cells emerging from the thymus that could inhibit the development of systemic organ-specific autoimmunity (13-16). Studies involving the autoimmune-proned scurfy mouse strain led to the identification of Foxp3 as a critical transcription factor of Tregs (17, 18). Furthermore, it was found that Foxp3⁺ Tregs could readily be generated in vitro by activating naive CD4⁺ T cells in the presence of TGF-B and IL-2 or retinoic acid (19-24). Subsequent studies revealed that the efficiency of Treg generation both in vitro and in vivo could be markedly enhanced by the allosteric mechanistic/mammalian target of rapamycin (mTOR) inhibitor rapamycin (25-29). These observations were followed up by studies that demonstrated that the genetic deletion of components of the mTOR signaling pathway in T cells led to the enhanced generation of Tregs (30-32). That is, stimulation of mTOR-deficient T cells under normal activating conditions (in the presence of Th1- or Th2-skewing cytokines) can lead to the generation of Tregs.

These observations supported a model whereby Ag recognition by CD4⁺ T cells in the absence of mTOR signaling leads to the generation of Tregs. However, additional studies revealed that the role of mTOR signaling in regulating Tregs was more complex. Paradoxically, it was observed that mTOR activity was increased in human Tregs and that mTOR supports Treg proliferation (25, 26, 33). Likewise, in a study using mice in which the mTOR complex 1 (mTORC1) adaptor protein Raptor was deleted in Tregs, the mice developed systemic autoimmunity, suggesting that mTORC1 activity was necessary for Treg function (34).

Previously, our group and others have shown that mTOR activation plays an important role in promoting $CD8^+$ T cell effector function (35, 36). Likewise, it has been shown that the inhibition of mTOR either with the small molecule inhibitor rapamycin or by genetic deletion leads to enhanced generation of memory $CD8^+$

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Abbreviations used in this article: *Cpt1a*, carnitine palmitoyltransferase 1a; cTreg, central Treg; ECAR, extracellular acidification rate; eTreg, effector Treg; *Hif1a*, hypoxia inducible factor 1, α subunit; *Hk2*, hexokinase 2; iTreg, induced Treg; mTOR, mechanistic/mammalian target of rapamycir; mTORC1, mTOR complex 1; OCR, oxygen consumption rate; *Pfkp*, phosphofructokinase, platelet; SRC, spare respiratory capacity; SRM, selected reaction monitoring; Treg, regulatory T cell; *t*-SNE, *t*-distributed stochastic neighbor embedding; WT, wild-type.

T cells (35–37). In this article, we employ both genetic and pharmacologic approaches to more precisely clarify the role of mTOR in Treg differentiation and function. To this end, we hypothesized that mTORC1 activation played a similar role in regulating Treg effector and memory T cell differentiation and function. Our studies reveal that cTregs and eTregs show distinct levels of mTORC1 activation. eTregs demonstrate increased mTORC1 activity and a concomitant increase in glycolytic metabolism. Moreover, *Rptor*-deficient eTregs have reduced expression of effector molecules such as CTLA-4 and ICOS and are less potent suppressors. Alternatively, the T-*Rptor*-deficient mice demonstrate an increase in Tregs with central markers (CD62L and CD25). Furthermore, the generation of Tregs in the presence of rapamycin leads to cells with increased cTreg markers that demonstrate robust longevity in vivo.

Materials and Methods

Mice

Six- to eight-week-old male or female mice were used for performing all the experiments in this study. All mouse procedures were approved by the Johns Hopkins University Institutional Animal Care and Use Committee. C57BL/6, Cd4-Cre, $Rag2^{-/-}$, Cd90.1, and mice with loxP-flanked *Rptor* alleles were initially obtained from The Jackson Laboratory. The Foxp3-GFP mice (C57BL/6-Tg(Foxp3-GFP)90Pkraj/J) were originally generated by Dr. P. Kraj and were kindly provided by Dr. C. Drake (Columbia University).

Flow cytometry and cell sorting reagents

Abs against the following proteins were purchased from BD Biosciences: CD4 (RM4-5), CD69 (H1.2F3), CD90.1 (OX-7), phospho-STAT5^{Y694} (C71E5), and CD90.2 (53-2.1). Abs against the following proteins were purchased from eBioscience: CD44 (IM7), CD98 (RL388), ICOS (7E.17G9), IRF4 (3E4), Ki-67 (SolA15), CD39 (24DMS1), KLRG1 (2F1), and Foxp3 (FJK-16s). Abs against the following proteins were purchased from BioLegend: CD4 (RM4-5), CD45 (30-F11), CD62L (MEL-14), Trom Biolegend. CD+ (1914-5), CD is (21-2) (225 (PC61), and Bcl2 CTLA-4 (UC10-4F10-11), PD-1 (29F.1A12), CD25 (PC61), and Bcl2 (BCL/10C4). Normal rabbit IgG (2729) and anti-phospho-S6^S (5364) were purchased from Cell Signaling Technology. Goat anti-rabbit Alexa Fluor 647 secondary Ab was purchased from Invitrogen. Fc Block (2.4G2) and anti-CD28 (37.51) were purchased from Bio X Cell. Stimulatory anti-CD3 (2C11) was purified from hybridoma supernatants prepared in-house. Fixable viability dye eFluor780 was purchased from eBioscience. MitoTracker Deep Red dye was purchased from Invitrogen. Flow cytometry experiments were performed on a FACSCalibur, LSR II, or FACSCelesta (BD Biosciences) and analyzed using FlowJo software (v.10.3; Tree Star) or FCS Express (v. 6; De Novo Software). Cell sorting was performed on a FACSAria II or FACSAria Fusion (BD Biosciences).

Immunoblot analysis

Sorted Tregs were flash frozen and lysed in radioimmunoprecipitation lysis buffer supplemented with protease and phosphatase inhibitor mixtures. Protein was quantified with Pierce Coomassie Plus (Bradford) Assay (Thermo Fisher Scientific), and equal protein was separated on 4–12% gradient gels (Invitrogen). The following Abs were purchased from Cell Signaling Technology: anti–phospho-S6^{S240/244} (5364), anti–phospho-mTOR^{S2448} (2971), anti–phospho-4EBP^{T37/46} (236B4, 2855), anti–β-actin (D6A8, 8457), and anti-rabbit HRP (7074). Proteins were detected with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific). All images were captured with the UVP Biospectrum 500 imaging system (UVP).

Real-time PCR analysis

Tregs were sorted from the spleen and lymph nodes of naive Foxp3-GFP mice. Total RNA was isolated by using TRIzol reagent (Life Technologies) and following the manufacturer's protocol. RNA (800 ng) was then converted to cDNA with ProtoScript II Reverse Transcriptase (New England BioLabs). Real-time PCR was performed using EagleTaq Universal Master Mix (Roche). Real-time PCR primers and probes were obtained from Applied Biosystems: carnitine palmitoyltransferase 1a (*Cpt1a*; liver, Mm01231183_m1); hypoxia inducible factor 1, α subunit (*Hif1a*; Mm00468869_m1); hexokinase 2 (*Hk*2; Mm00443385_m1); and phosphofructokinase, platelet (*Pfkp*; Mm00444792_m1). Values of $\Delta\Delta$ cycle

threshold were normalized to the housekeeping gene 18s rRNA (Life Technologies) and further normalized to the control group. Experiments were performed on an OneStepPlus 96-well instrument (Applied Bio-systems).

In vitro suppression assay

Tregs were isolated from the spleen and lymph nodes of naive mice with the CD4⁺ CD25⁺ Regulatory T Cell Isolation Kit (Miltenyi Biotec) or by cell sorting to isolate cTregs and eTregs. Naive CD4⁺ T cells from the spleen and lymph nodes of congenically distinct mice (CD90.1⁺) were isolated with Naive CD4⁺ T Cell Isolation Kit (Miltenyi Biotec) and were labeled with proliferation dye eFluor 450 (5µM) (eBioscience) following the manufacturer's protocol. Different ratios of Tregs (suppressor) and naive CD4⁺ T cells (responder) were cocultured with soluble anti-CD3 (1 µg/ml) and irradiated APCs. The proliferation of the CD4⁺ CD90.1⁺ T cells was measured by eFluor 450 dilution by flow cytometry. The percentage of suppression was calculated by using the following formula:

% Suppression =
(% of proliferated responders with no Treg
$$-$$
 % of proliferated responders) $\times 100$
% of proliferated responders with no Treg

In vivo administration of rapamycin

Mice were injected i.p. with vehicle or rapamycin (300 μ g/kg; LC Laboratories) daily for 6 d. Rapamycin was first reconstituted in DMSO and then diluted with Kolliphor EL (Cremophor; Sigma-Aldrich) and sterile water to a final 1:1:4 (DMSO/rapamycin: Cremophor:sterile water) ratio.

In vitro Treg generation

Naive CD4⁺ T cells were isolated from the spleen and lymph nodes of wild-type (WT) mice and were activated with plate-bound anti-CD3 (3 µg/ml) and soluble anti-CD28 (2 µg/ml) in the presence of murine IL-2 (10 ng/ml; PeproTech) and TGF- β (10 ng/ml; PeproTech). In some instances, cells were activated with additional DMSO (vehicle) or rapamycin (100 nM; LC Laboratories). Cells were harvested at the indicated time point for subsequent analysis.

Metabolic assay

Tregs were prepared as described above. Activated cells (3×10^{5}) were plated per well on poly-D-lysine (50 µg/ml; Sigma-Aldrich)–coated Seahorse XF96 Cell Culture Microplate in XF Assay Medium Modified DMEM supplemented with 25 mM glucose, 2 mM L-glutamine, and 1 mM sodium pyruvate. The basal extracellular acidification rate (ECAR) was calculated based on the average of the initial readouts before the addition of oligomycin. Spare respiratory capacity (SRC) was determined by subtracting the basal oxygen consumption rate (OCR) from the maximal OCR (detected following FCCP administration). Experiments were performed using XF 96 Extracellular Flux Analyzer (Agilent Technologies). The following were injected at the indicated time interval: oligomycin (1 µM; Sigma-Aldrich), FCCP (1.5 µM; Sigma-Aldrich), rotenone (2 µM; Cayman Chemical), and antimycin A (1 µM; Sigma-Aldrich).

Targeted metabolite analysis with liquid chromatographytandem mass spectrometry

Targeted metabolite analysis was performed with liquid chromatographytandem mass spectrometry. Metabolites from sorted cells were extracted with 80% (v/v) methanol solution equilibrated at -80°C, and the metabolite-containing supernatants were dried under nitrogen gas. Dried samples were resuspended in 50% (v/v) acetonitrile solution, and 4 µl of each sample was injected and analyzed on a 5500 QTRAP triple quadrupole mass spectrometer (AB Sciex) coupled to a Prominence UFLC system (Shimadzu). The instrument was operated in selected reaction monitoring (SRM) with positive and negative ion-switching mode as described below. This targeted metabolomics method allows for analysis of over 200 metabolites from a single 25-min liquid chromatography-mass spectrometry acquisition with a 3-ms dwell time. The optimized mass spectrometry parameters were as follows: electrospray ionization voltage was +5000 V in positive ion mode and -4500 V in negative ion mode, dwell time was 3 ms per SRM transition, and the total cycle time was 1.57 s. Hydrophilic interaction chromatography separations were performed on a Shimadzu UFLC system using an amide column (XBridge BEH Amide, 2.1 imes150 mm, 2.5 µm; Waters). The LC parameters were as follows: column temperature, 40°C; flow rate, 0.30 ml/min. Solvent A, water with 0.1% formic acid; Solvent B, acetonitrile with 0.1% formic acid; a nonlinear



FIGURE 1. cTregs and eTregs can be defined by mTORC1 activity. (**A**) CD44 and CD62L expression was examined among CD4⁺ Foxp3⁺ T cells from spleen (left). cTregs and eTregs were defined as previously described (12). Flow cytometry analysis of Foxp3 expression between cTregs and eTregs is shown on the right. (**B** and **C**) cTregs and eTregs are examined by (B) size and (C) p-S6^{5240/244}, CD98, and IRF4. Geometric mean fluorescence intensity is shown in the plots. Dotted line in (**C**) represents isotype control. (**D**) Immunoblot analysis of the (*Figure legend continues*)

gradient from 99% B to 45% B in 25 min with 5 min of postrun time. Peak integration for each targeted metabolite in SRM transition was processed with MultiQuant Software (v2.1; AB Sciex). The preprocessed data with integrated peak areas were exported from MultiQuant and reimported into MetaboAnalyst software for further data analysis, including statistical analysis, fold change, principle components analysis, and relative expression in a heatmap.

For the $[U^{-13}C]$ glucose tracing experiment, a 20% (w/v) solution of $[U^{-13}C]$ glucose (Cambridge Isotope Laboratories) in PBS was sterilefiltered, and 100 µl of this solution were injected into the tail veins of restrained mice without anesthesia at 15 min intervals. After three injections, mice were sacrificed, and spleens were isolated for further purification of Tregs. Isolated Tregs were then flash frozen and processed as described above.

Adoptive transfer

Tregs were generated as described above, and 1×10^6 cells were adoptively transferred retro-orbitally into congenically distinct WT host. At day 14 posttransfer, cells isolated from spleen and lymph nodes were analyzed separately by flow cytometry.

B16-F10 tumor model

The B16-F10 melanoma cell line was purchased from American Type Culture Collection. The cell line was tested and found to be mycoplasma free by using a MycoAlert Mycoplasma Detection Kit (Lonza) every 6 mo. Cells were cultured in RPMI 1640 media supplemented with 10% FBS, 10 mM HEPES (Corning), and antibiotics (Corning). Tumor cells (2×10^5) were injected s.c. in the flank of the mice at day 0. Fourteen days after tumor implantation, tumors were harvested from mice and digested in 2 mg/ml collagenase I (Life Technologies) with DNase I (Roche) in RPMI 1640 supplemented with 2% FBS. Cells were then stained with Abs for subsequent flow analysis.

B16-F10 tumor model in $Rag2^{-/-}$ mice

Sorted naive CD4⁺ and CD8⁺ T cells were mixed with sorted splenic eTregs from WT or T-*Rptor*^{-/-} in a 1:0.5:0.02 ratio, respectively. Then, a total of 7.5×10^5 cell mixture was injected retro-orbitally into $Rag2^{-/-}$ host. Two days post–cell transfer, 2×10^5 B16-F10 melanoma cells were injected s.c. into flanks of the mice. Tumor growth was monitored.

Statistical analysis

All graphs and statistical analysis were performed using GraphPad Prism software (v. 7). A p value <0.05 was considered statistically significant.

Results

eTregs have increased mTORC1 signaling compared with cTregs

As defined by Smigiel et al. (12), Tregs in the spleen can be divided into cTreg and eTreg based on their CD44 and CD62L expression. Consistent with these findings, we observed CD44¹⁰ CD62L^{hi} (cTregs) and CD44^{hi} CD62L^{lo} (eTregs) populations with no difference in Foxp3 expression in the spleen (Fig. 1A) of naive WT C57BL/6 mice. In as much as mTOR activity has been shown to regulate memory and effector CD8⁺ T cells, we hypothesized that mTOR activity might be differentially regulated in Tregs as well. To test this hypothesis, we compared cell size in the cTregs and eTregs. Cell size is regulated by mTOR, and mTORC1 controls the phosphorylation of S6 as well as the expression of CD98 (38, 39). Indeed, we observed increased cell size, p-S6, and CD98 expression in the eTregs as compared with the cTregs (Fig. 1B, 1C). Furthermore, IRF4 has been shown to play a role in Treg function (4, 5, 9). Indeed, eTregs have higher IRF4 expression than cTregs (Fig. 1C). In addition, immunoblot analysis showed increased phosphorylation of mTOR at S2448, demonstrating increased mTOR activity in eTregs compared with cTregs (Fig. 1D). Correlating to the flow analysis (Fig. 1C), eTregs also showed enhanced p-S6 and p-4EBP, both of which are downstream of mTORC1 (Fig. 1D). Thus, similar to effector CD8⁺ T cells, eTregs display enhanced mTORC1 activity as compared with cTregs.

We have shown thus far that we can define cTregs and eTregs based on mTORC1 activity as well their CD44 and CD62L expression. To further define these distinct populations of Tregs, we took a multidimensional, unbiased approach. A two-dimensional t-distributed stochastic neighbor embedding (t-SNE) plot was generated based on the expression levels of the effector (p-S6, IRF4, ICOS, KLRG1, PD-1, and CTLA-4) and central markers (CD62L and CD25) from splenic Tregs. We then overlaid the geometric mean fluorescence intensity of these individual molecules onto the existing t-SNE plot to investigate their distribution patterns. Not to our surprise, Tregs that express effector molecules all clustered together on the t-SNE plot (Fig. 1E). Alternatively, the central molecules such as CD62L and CD25 clustered together on the t-SNE plots (Fig. 1F). Of note, the eTregs that express the highest expression of all effector molecules further cluster away from the CD62L expression population. Utilizing a different flow cytometry panel, we reconstructed the t-SNE analysis using different eTreg (CTLA-4, KLRG1, CD98, and Ki-67) and cTreg (CD62L, CD25, and Bcl-2) markers. We observed similar cell clustering with Ki-67 and CD98 clustering together with CTLA-4-expressing cells (Supplemental Fig. 1A), whereas Bcl-2 clusters with CD62L-expressing cells (Supplemental Fig. 1B).

Based on the unbiased *t*-SNE analysis, we decided to apply an unbiased *k*-mean clustering to define two distinct groups of Tregs. The *k*-mean clustering algorithm again produced the distinct population of Tregs based on their CD44 and CD62L expression. Cluster 1 encompassed all the CD62L^{hi} cTregs, whereas cluster 2 encompassed all the CD62L^{lo} Tregs (Fig. 1G). Furthermore, the relative expression of cTreg and eTreg markers in these two clusters correlates with our previous findings (Fig. 1E, 1F, 1H). Taken together, these analyses demonstrate that the two populations of Tregs defined by CD62L expression can also be reconstructed based on mTORC1 activity.

Because we observed differential mTOR activity among these two populations of Tregs, we wondered if their suppressive function might be different upon TCR stimulation. However, both cTregs and eTregs suppressed equally well in an in vitro suppression assay, suggesting there are no dramatic functional differences in response to direct TCR stimulation (Fig. 1I).

eTregs have increased glycolysis compared with cTregs

Recently, the role of metabolic reprogramming in promoting T cell differentiation and function has been revealed (40). Along these lines, mTORC1 specifically has been shown to be crucial in promoting the metabolic reprogramming (39, 41, 42). Furthermore, the transcription factor IRF4 also regulates the glycolytic metabolism and differentiation of CD8⁺ T cells (43, 44). As such, we wondered if there were differences in metabolic programming

mTOR pathway, as measured by p-mTOR^{S2448}, p-S6^{S240/244}, and p-4EBP^{T37/46} between cTreg and eTreg from spleen. β -Actin served as loading control. (**E**) Heatmap overlay of eTreg molecules onto a *t*-SNE plot generated from splenic Tregs in WT mice. (**F**) Heatmap overlay of cTreg molecules from splenic Tregs. (**G**) CD44 and CD62L expression of cluster 1 and 2 generated from an unbiased *k*-mean clustering algorithm. (**H**) Heatmap of the expression of molecules from the cluster 1 and 2 populations. (**I**) Treg suppression assay from sorted cTregs and eTregs. Two-way ANOVA with Bonferroni multiple comparisons test (I). Data are representative of at least three independent experiments. **p < 0.005.



FIGURE 2. cTregs and eTregs have distinct metabolic requirements. (**A**) Tregs from Foxp3-GFP mice were sorted based on CD44 and CD62L expression. Comparison of mRNA expression of metabolic genes (*Hif1a*, *Hk2*, *Pfkp*, and *Cpt1a*) between sorted cTregs and eTregs. (**B**) Mitochondrial mass was compared between WT splenic cTregs and eTregs. A summary plot is shown on the right. (**C** and **D**) Targeted metabolic analysis was performed, and (C) a PCA plot and (D) heatmap showing glycolysis and TCA cycle

between cTregs and eTregs based on the differential mTORC1 activity and IRF4 expression illustrated from Fig. 1. To further examine the glycolytic programming of cTreg and eTregs, splenic Tregs were isolated from spleen and lymph nodes of Foxp3-GFP mice and sorted based on GFP, CD44, and CD62L expression. Then, mRNA was extracted from these two subsets. eTregs demonstrated an increase in the expression of Hifla when compared with cTregs (Fig. 2A). Moreover, eTregs also showed enhanced mRNA expression of Hk2 and Pfkp, two important enzymes in the initial steps of the glycolysis (Fig. 2A). Thus, eTregs have increased glycolytic machinery as compared with cTregs. As previously shown, Tregs preferentially use fatty acid oxidation as a major source of energy production compared with effector CD4⁺ T cells (45). We observed no differences between the two subsets with regard to Cpt1a expression (Fig. 2A). Additionally, an increase in mitochondrial mass has been associated with long-lasting memory cells (46). Thus, we employed Mito-Tracker Deep Red dye to assess mitochondrial mass in cTregs and eTregs. Interestingly, we observed an increase in mitochondrial mass in the cTregs as compared with the eTregs (Fig. 2B).

To further investigate the metabolic status differences between cTregs and eTregs, we sorted cTregs and eTregs from the spleen and subjected these cells to targeted metabolomics analysis. Principle component analysis of the metabolites accounts for 68% of differences between the two subsets and clearly distinguishes these two subsets of Tregs (Fig. 2C). That is, the observed differences in mTORC1 activity correlates to distinct differences in metabolic programming. We further focused our analysis on glycolysis and TCA cycle metabolites. Interestingly, cTregs show a reduction in both pyruvate and lactate levels and an increase in TCA cycle intermediates compared with eTregs (Fig. 2D). Overall our data demonstrate that eTregs and cTregs display differential mtORC1 activity, which regulates differential metabolic reprogramming.

The role of mTORC1 in defining the cTreg and eTreg subsets

Our data thus far suggest that cTreg and eTregs have different mTORC1 activity that correlates with differential metabolic programs. Next, we wanted to test the effect of mTORC1 inhibition on the two subsets. We treated WT or Foxp3-GFP mice with either vehicle or rapamycin (300 µg/kg) daily for 6 d. On day 6, we harvested the spleen of the mice for further analysis. First, we sorted splenic cTregs and eTregs and confirmed mTORC1 inhibition by measuring the phosphorylation of S6 at S240/244, and 4EBP at T37/46 via immunoblotting (Supplemental Fig. 2A). To address the phenotypic changes that occurred as a consequence of mTORC1 inhibition, we again generated t-SNE plots from splenic Tregs employing the markers defined in Fig. 1E for cTregs and eTregs. We then overlaid the Treg populations from mice that were treated with either vehicle or rapamycin (Fig. 3A). The plot reveals phenotypic changes in the Treg populations derived from mice that were treated with rapamycin. We then applied an unbiased k-mean transformation to cluster the Tregs into two populations. Each cluster has a distinct CD44 and CD62L profile, with cluster 1 containing most of the CD62L^{hi} population and cluster 2 containing most of the CD62L^{lo} population (Fig. 3B). We then

metabolites from sorted cTregs and eTregs. Two-way ANOVA with Bonferroni multiple comparisons test [(A), left], Mann–Whitney U test [(A), right], and paired t test (B). Data are representative of either two (C and D) or at least three (A and B) independent experiments. *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001. ns, not significant. PCA, principle component analysis.

analyzed the changes in both clusters after rapamycin treatment. The percentage of cluster 1 Tregs increased with a concomitant decrease in cluster 2 Tregs upon mTORC1 inhibition (Fig. 3C). Not surprisingly, the ratio between cTregs and eTregs increased in mice that were treated with rapamycin, suggesting a requirement for mTORC1 activity in eTregs (Fig. 3D). Of note, Ki-67 was reduced in both cTregs and eTregs upon rapamycin treatment (Supplemental Fig. 2B).

Furthermore, we also sought out metabolic changes resulting from treatment with rapamycin. We sorted splenic cTregs and eTregs from rapamycin-treated mice and subjected the metabolite extracts to targeted metabolic analysis. There were marked differences in glycolysis and TCA cycle intermediates in rapamycintreated eTregs (Fig. 3E). Similar to what we observed in the mTOR¹⁰ cTregs (Fig. 2), glycolysis was reduced in eTregs treated with rapamycin (Fig. 3E). Additionally, rapamycin-treated eTregs demonstrated increased TCA cycle intermediates (Fig. 3E). Thus, from a metabolic perspective, the rapamycin-treated eTregs phenocopied the mTOR¹⁰ cTregs. Taken together, these data suggest that mTOR signaling is important in regulating the homeostasis and metabolic status of both cTregs and eTregs.

mTORC1 deficiency mitigates eTreg function

Thus far we have demonstrated that high mTORC1 activity is associated with eTregs and promotes glycolysis, whereas low mTORC1 activity is associated with cTregs and leads to increased mitochondrial mass and TCA cycle intermediates (Fig. 2). To further discern the role of mTOR in regulating Tregs, we employed mice in which the mTORC1 adaptor protein Raptor is selectively deleted in T cells (T-*Rptor*^{-/-}). First, we noted that the splenic cTreg and eTreg ratio is similar between T-*Rptor*^{-/-} and WT littermate control mice based on CD44 and CD62L expression (Fig. 4A). On the other hand, T-*Rptor*^{-/-} eTregs demonstrated decreased levels of the proliferation marker Ki-67 and decreased levels of the effector molecules ICOS, CD69, CTLA-4, CD39, and PD-1 (Fig. 4B). Furthermore, T-*Rptor*^{-/-} eTregs have decreased IRF4 expression (Fig. 4C).

Interestingly, although the eTregs from the T- $Rptor^{-/-}$ mice had reduced cell surface expression of effector molecules, these cells expressed increased levels of cTreg markers such as CD25 and Bcl-2 compared with WT eTregs (Fig. 4D). The relative amount of phosphorylated STAT5 is also increased in the $T-Rptor^{-/-}$ eTregs and cTregs when compared with WT littermate control mice (Fig. 4D, Supplemental Fig. 3A). In addition, the prosurvival factor Bcl-2 was increased at protein and mRNA level in T-Rptor^{-/-} cTreg than WT cTregs (Supplemental Fig. 3B, 3C). Furthermore, CD25 expression was further enhanced in T-*Rptor*^{-/-} cTregs (Supplemental Fig. 3D). We</sup>also performed an in vivo [U-13C]glucose tracing experiment to examine glycolysis and TCA cycle intermediate metabolites in WT and T-Rptor^{-/-} mice contributed from glucose. Tregs isolated from $T-Rptor^{-/-}$ mice showed a reduction of labeled [¹³C]glucose into glycolytic metabolites such as glucose-6-phosphate (G6P) and lactate, as well as the TCA cycle intermediates succinate and malate (Supplemental Fig. 3E). Overall, the mTORC1 signaling deficient T-Rptor^{-/-} eTregs have decreased proliferation and expression of eTreg molecules. This is juxtaposed with a concomitant increase in the expression of molecules associated with cTregs. Likewise, the T- $Rptor^{-/-}$ Tregs were overall less glycolytic, further demonstrating the important role of mTORC1 in glycolytic programming in Tregs.

The decrease in effector molecules in the absence of mTORC1 activity suggested that Tregs from the T-*Rptor*^{-/-} mice would be less effective in suppressing T cell function. To this end, we



FIGURE 3. cTregs and eTregs are both sensitive to mTOR inhibition. WT mice were subjected to vehicle (Veh) or rapamycin (Rapa, 300 μ g/kg) treatment for 6 d. Mice were sacrificed, and the spleens were harvested. (**A**) *t*-SNE plot of splenic Tregs from mice treated with or without rapamycin. (**B**) Relative expression of markers in Tregs defined by clusters 1 and 2 generated from *k*-mean transformation. (**C**) Population changes of clusters 1 and 2 upon rapamycin treatment. (**D**) Ratio of cTreg and eTreg in the spleen. (**E**) Heatmap showing targeted metabolite differences in eTregs between vehicle and rapamycin treatment groups. Two-way ANOVA with Bonferroni multiple comparisons test (C) or Mann–Whitney *U* test (D). Data are representative of two (E) or at least three (A–D) independent experiments. **p < 0.005.

performed an in vitro suppression assay using sorted Tregs from the spleen and lymph nodes of $T-Rptor^{-/-}$ mice and WT littermate control mice. Indeed, the $T-Rptor^{-/-}$ Tregs were less



FIGURE 4. mTORC1 is crucial in maintaining the eTreg phenotype. (**A**) Percentage of splenic eTregs and cTregs was examined between WT and T- $Rptor^{-/-}$ mice. (**B**) Flow cytometry comparison of effector molecule expression between WT and T- $Rptor^{-/-}$ eTregs from spleen. (**C**) Flow cytometry comparison of IRF4 between WT and T- $Rptor^{-/-}$ eTregs from spleen. (**D**) Flow cytometry comparison of cTreg molecules between splenic eTregs among WT and T- $Rptor^{-/-}$. (**E**) WT and T- $Rptor^{-/-}$ Tregs were isolated from spleen and titrated in a different ratio to responder cells to assess the ability of Tregs to suppress in vitro. Two-way ANOVA with Bonferroni multiple comparisons test. Data are representative of at least three independent experiments. *p < 0.05, **p < 0.005.

effective in suppressing proliferation when compared with the WT Tregs (Fig. 4E). Notably, the $T-Rptor^{-/-}$ Tregs can still suppress effector T cell proliferation, just not as efficiently as WT Tregs.

Next, we wanted to determine the role of mTORC1 activity in regulating eTreg function in vivo. To this end, T-*Rptor*^{-/-} mice and WT littermate control mice were inoculated s.c. with 2×10^5 B16-F10 melanoma cells. On day 14, the tumors were harvested and evaluated for tumor-infiltrating lymphocytes. We did not

observe any differences in tumor area or mass between the WT and T-*Rptor*^{-/-} mice at this time point (Fig. 5A). Regardless of the similarity in tumor size, we observed a significant decrease in the overall percentage of tumor-infiltrating Foxp3⁺ Tregs in the T-*Rptor*^{-/-} mice when compared with the WT mice (Fig. 5B). Moreover, the tumor-infiltrating Tregs in the T-*Rptor*^{-/-} mice consistently demonstrated significantly decreased cell surface expression of the Treg effector molecules ICOS, CLTA-4, and PD-1 (Fig. 5C–E).



FIGURE 5. mTORC1-deficient Tregs have decreased effector molecule expression. B16-F10 melanoma cells (2×10^5) were inoculated s.c. in WT or T-*Rptor*^{-/-} mice. (**A**) Tumor area and mass of B16-F10 tumors were measured 14 d after inoculation. (**B**) Percentage of tumor-infiltrating Tregs between WT and T-*Rptor*^{-/-} mice was examined. Summary is shown on the right. (**C**–**E**) Summary of the examination of effector molecule expression between WT and T-*Rptor*^{-/-} Tregs in (C) ICOS, (D) CTLA-4, and (E) PD-1 tumors. (**F**) Sorted splenic WT or T-*Rptor*^{-/-} eTregs were combined with conventional CD4⁺ and CD8⁺ T cells and then adoptively transferred to $Rag2^{-/-}$ mice. Two days post–cell transfer, mice were implanted s.c. with B16-F10 melanoma cells. Tumor growth was monitored over time. Mann–Whitney *U* test. Data are representative of at least three (A–E) or two independent experiments (F). *p < 0.05, **p < 0.005, **p < 0.005.

To further address the intrinsic role for *Rptor*-deficient Tregs in antitumor immunity, we used an adoptive transfer model that was previously shown to address Treg function in a tumor setting (47). In short, sorted conventional CD4⁺ and CD8⁺ T cells were mixed with either splenic WT or $T-Rptor^{-/-}$ eTregs and then adoptively transferred into $Rag2^{-/-}$ hosts. Two days later, 2×10^5 B16-F10 melanoma cells were injected into the flanks of the $Rag2^{-/-}$ mice. Tumor growth was monitored as a means of evaluating the ability of the transferred eTregs to suppress the antitumor immune response. Mice that received no T cells demonstrated the most rapid tumor growth (Fig. 5F). Mice that received WT conventional CD4⁺/CD8⁺ T cells and WT eTregs demonstrated slightly slower tumor growth. On the other hand, mice that received WT CD4+/ $CD8^+$ T cells and T-*Rptor*^{-/-} eTregs had the slowest tumor growth. That is, the T-*Rptor*^{-/-} eTregs were less able to inhibit the antitumor response of the conventional T cells.

Inhibition of mTORC1 enhances generation of cTregs

Activation of naive CD4⁺ T cells (with anti-CD3 and anti-CD28) in the presence of exogenous TGF- β and IL-2 leads to the robust generation of Foxp3⁺ Tregs (19, 20, 22, 23) (Fig. 6A). However, this method of promoting Treg generation also results in robust mTORC1 activation. Indeed, the vast majority of Tregs in such cultures display an eTregs phenotype CD44^{hi} CD62L^{lo} (Fig. 6B). Furthermore, as previously shown in vivo, these CD62L^{lo} Tregs demonstrate increased mTORC1 activity compared with CD62L^{hi} Tregs (Fig. 6C). Note that in Fig. 6, the cells in nonpolarizing conditon are primarily CD4⁺ Foxp3⁻ cells, and the mTORC1 activity in this population of "effector" cells is comparable to the Tregs (Fig. 6C). Thus, utilizing the standard method of activating CD4⁺ T cells in the presence of exogenous TGF- β leads to the immediate generation of mTORC1^{hi} eTregs.

Next, we stimulated naive CD4⁺ T cells in the presence of TGF- β and IL-2 for 3 d with or without rapamycin. Under these Treg promoting conditions, there were similar percentages of Foxp3⁺ Tregs both in the presence and absence of rapamycin (Fig. 6D). However, the presence of rapamycin markedly enhanced the generation of the CD62L^{hi} cTregs (Fig. 6E) with decreased p-S6 (Fig. 6F). Metabolically, the rapamycin-treated Tregs showed a significantly lower basal glycolytic rate, as measured by ECAR, than nontreated Tregs (Fig. 6G). Conversely, the rapamycin-treated Tregs possessed a higher OCR and an increased SRC (Fig. 6H).

The presence of rapamycin led to the robust generation of cTregs. Furthermore, these cells possessed increased SRC, which is a hallmark metabolic property of memory CD8⁺ T cells (46). Thus, we hypothesized that the Tregs generated in the presence of rapamycin would demonstrate increased persistence upon adoptive transfer. To test this hypothesis, Tregs were generated in vitro in the presence or absence of rapamycin and adoptively transferred into congenically distinct WT mice. Fourteen days after transfer, spleen and lymph nodes were harvested. We observed that the percentage of recovered Tregs from both the spleen and lymph nodes was significantly higher in the mice that received the rapamycin-treated cells (Fig. 7A). Furthermore, most of the recovered cells from both the untreated or rapamycin treated mice showed higher proportions of CD62L^{hi} cells both in the spleen and lymph nodes, indicative of cTregs (Fig. 7B). These results support a model by which differential regulation of mTOR supports the function of effector versus central Tregs (Fig. 8).

Discussion

Based on studies from our own laboratory and others we have proposed a model whereby mTOR signaling integrates cues from the immune microenvironment to regulate T cell activation, differentiation and function (30, 40, 48-50). Moreover, mTOR signaling also directly links environmental cues to modulate metabolism in support for cell growth and proliferation (39-41, 49). In this current work, we further define the role for mTOR in regulating the activation and differentiation of Tregs. Previous studies by Smigiel et al. (12) defined a population of cTreg and eTreg subsets based on selective homeostatic properties. Specifically, they defined cTregs as being CD44^{lo} CD62L^{hi} CCR7^{hi} cells located within the secondary lymphoid tissue and dependent upon access to IL-2 for long term survival. In contrast, eTregs are CD44^{hi} CD62L^{lo} CCR7^{lo} and are found in the peripheral tissues and depend on ICOS signaling. Using this paradigm, we have been able to demonstrate that cTregs are defined by lower mTORC1 activity (Fig. 1C, 1D). Metabolically, cTregs are less glycolytic (Figs. 2, 6G) and have increased SRC (Fig. 6H).

FIGURE 6. Tregs generated under mTOR suppression display a memorylike phenotype. Naive CD4+ T cells were isolated and activated with platebound anti-CD3 (3 µg/ml) and soluble anti-CD28 (2 µg/ml) in the presence or absence of TGF-B (10 ng/ml) and IL-2 (10 ng/ml). Cells were either cultured in the presence or absence of rapamycin (100 nM). (A) Foxp3 expression between CD4+ T cells activated under either nonpolarizing (NT) or Tregpolarizing condition (TGF-B) were analyzed on day 2. (B) Comparison of CD44 and CD62L expression among $CD4^+$ Foxp3⁺ cells from (A). (C) mTORC1 activity among CD62Lhi and CD62L¹⁰ cells gated from CD4⁺ Foxp3⁺ cells. (D) Foxp3 expression of CD4⁺ T cells activated under Treg-polarizing condition (TGF-B) with or without rapamycin treatment on day 2. (E) Comparison of CD44 and CD62L expression among CD4+ Foxp3+ cells from (D). (F) Comparison of p-S6 level in cells generated under TGF-B with or without rapamycin conditions. (G) ECAR and (H) OCR between cells generated under Treg-polarizing condition treated with or without rapamycin were measured. SRC is shown on the right. Mann-Whitney U test. Data are representative of at least three independent experiments. *p < 0.05, **p <0.005. Oligo, oligomycin; R+A, rotenone and antimycin A.



Furthermore, such cells demonstrate robust persistence upon adoptive transfer (Fig. 7). This is analogous to long-lived CD8⁺ memory T cells, which are also mTORC110 and have increased SRC along with increased mitochondrial mass to effector CD8⁺ T cells (35, 36, 46). Conversely, we observed increased mTORC1 activity in eTregs. Metabolically, eTregs demonstrated increased glycolytic programming (Fig. 2), which is a characteristic of effector CD8⁺ T cells (40). Moreover, mice that received rapamycin treatment continuously for 6 d demonstrated increased cTreg to eTreg ratios, suggesting the need of mTORC1 activity for eTreg conversion (Fig. 3D). Furthermore, genetic deletion of Rptor mitigated the suppressor function of the eTregs. Interestingly, the $T-Rptor^{-/-}$ mice demonstrated equivalent percentages of phenotypic (CD44^{hi} CD62L¹⁰) eTregs, but such cells demonstrated decreased ICOS and PD-1 expression (Fig. 4B) consistent with their defective suppressive function (Figs. 4E, 5F). In addition, $T-Rptor^{-/-}$ Tregs showed a reduction in glycolysis and TCA cycle metabolism from glucose (Supplemental Fig. 3E). The functional manifestation of their defects was reflected in decreased tumor infiltration of Tregs from the T-*Rptor*^{-/-} mice in vivo (Fig. 5B). Overall, based on these results, we propose that the CD44^{lo} CD62L^{hi} mTOR^{lo} cells (described as cTregs) represents a pool of central memory Tregs, whereas the

CD44^{hi} CD62L^{lo} mTOR^{hi} cells depict the eTregs (Fig. 8). In the absence of mTORC1 activity, as we demonstrated with T-*Rptor*^{-/-} mice, eTregs fail to upregulate mTOR activity and downstream metabolic reprogramming, whereas cTregs compartment did not change. This further indicates that the differential mTOR activity in regulating the function of the two subsets. Further, although decreased mTORC1 activity promotes the generation of cTreg, if such cells do not have the capability to upregulate mTORC1 activity (for example in the T-*Rptor*^{-/-} cells), then they lose their eTreg functional ability.

Observations regarding the regulation of Tregs by mTOR have been complex and at times seemingly paradoxical. Several studies have shown that the strength of TCR signaling affects $CD4^+$ T cell differentiation, particularly differential TCR-induced mTOR activity can modulate Foxp3 expression (51–57). Early studies employing rapamycin demonstrated that mTOR inhibition enhanced the generation of induced Tregs (iTregs) (25–28). Additionally, such findings were further supported by genetic knockout studies of mTOR signaling pathway components (30–32). Specifically, high Akt signaling induced by strong TCR activation can inhibit iTreg differentiation in the periphery (31, 58–60). In this paper, we demonstrated that iTregs generated



FIGURE 7. Tregs generated under mTOR suppression persist longer in vivo. Tregs (1×10^6) generated as described in Fig. 6 were adoptively transferred into congenically distinct hosts. Fourteen days post-adoptive transfer, spleen and lymph nodes (LNs) were harvested. (**A**) Percentage of CD90.1⁺ CD4⁺ T cell recovery and (**B**) CD44 and CD62L expression were examined. One-way ANOVA with Tukey multiple comparison test). Data are representative of at least three independent experiments. *p < 0.05, **p < 0.005, ***p < 0.0005. ns, not significant.

concomitantly with rapamycin showed decreased mTOR activity with an increase in SRC (Fig. 6). These mTOR^{lo} Tregs phenocopied cTregs that were found in the peripheral tissues and were more long-lived in an adoptive transfer model (Fig. 7). This phenomenon is not due to the stability of Foxp3 expression because the recovered T cells from the adoptive transfer experiment displayed equivalent levels of Foxp3 expression (Supplemental Fig. 4). Alternatively, this increased longevity of Tregs can be explained by mTOR regulating the metabolic programming of these cells. The Tregs that were generated under mTOR inhibition resemble that of the cTregs generated from the thymus.

Previous studies observed that mTOR signaling is elevated in peripheral Tregs from human and mice compared with conventional CD4⁺ T cells (33, 34, 61–64). This led to their conclusion that all peripheral Tregs have high mTOR activity and glycolysis. Further supporting the role of mTORC1 activity in promoting Treg function is the observation in mice that $T-Rptor^{-/-}$ Tregs, which lack mTORC1 activity, have been shown to be functionally defective (34). However, previous studies using a mixed population of Tregs cannot reveal the precise role of mTOR in regulating metabolism and function of Tregs. Thus, we demonstrated that there are two distinct mTOR^{hi} and mTOR^{lo} peripheral Tregs with differential metabolic phenotypes. Our revised model posits that mTOR activity is necessary for upregulation of glycolysis and Treg effector molecules in eTregs for their optimal function, whereas inhibition of mTOR promotes the generation of central memory Tregs (Fig. 8). In future studies, it will be interesting to examine mTORC1 activity in freshly isolated eTreg, cTreg, naïve, and memory CD4⁺ CD25⁻ CD45RO⁺ CD127⁺ human T cells.



FIGURE 8. Model for the regulation of Tregs by mTOR.

Likewise, we observed that eTregs are more glycolytic, whereas central memory Tregs possess increased SRC (Figs. 2, 6H). Recently, it has been reported that Tregs with hyper-mTOR activity and increased glycolysis possess decreased suppressive function (65-67). Although these studies seem to contradict our model that mTOR activity and upregulation of glycolysis are important for optimal eTreg function, it is important to note that mTORC1 activity in these studies was induced through TLR stimulation. Under these conditions, these mTOR^{hi} glycolytic Tregs observed in those studies possessed decreased Foxp3 expression, which itself has been shown to maintain a stable Treg signature (65, 68-70). In our model, the expression of Foxp3 is equivalent in the effector and central memory Tregs (Fig. 1A, right). Thus, we propose that robust TCR stimulation in the presence of a suppressive microenvironment (TGF-β) promotes mTORC1^{hi} Treg effector cell generation. In contrast, when robust activation of Tregs occurs in the presence of inflammatory signals (TLR signaling or high PI3K signaling), this leads to decreased Foxp3 expression and consequently decreased suppression capability.

Our data also serve to better define the mechanism by which rapamycin promotes Tregs. Although the addition of rapamycin has been shown to promote Tregs both in vivo and in vitro (25–28), a common technique for the generation of such cells in vitro has been maximal activation of naive CD4⁺ T cells in the presence of TGF- β . Such conditions actually lead to robust mTORC1 activity. In our model, such strong stimulation leads initially to the

maximal generation of eTregs (Fig. 8). Over time, the Tregs remaining in these culture conditions become mTOR¹⁰ memory Tregs (Fig. 8, 5–7 d postactivation). Alternatively, the addition of rapamycin to these culture conditions leads to the generation of central memory Tregs with increased SRC and persistence (Figs. 6, 7). Several studies have demonstrated the utility of adoptively transferring Tregs to inhibit autoimmunity, transplant rejection, or graft-versus-host disease (16, 71–74). From a clinical perspective, our data suggest that mTORC1 inhibition with rapamycin is an effective means of promoting robust memory Tregs for adoptive transfer. Likewise, our data suggest that the use of mTORC1 inhibitors in vivo might also promote the generation of long-lasting central memory Tregs (Fig. 7).

In summary, our work helps to clarify the precise role of mTOR signaling in regulating Treg differentiation and function. Our studies serve to support and extend the model defining the distinct Tregs subsets discovered and defined by the Campbell laboratory by further defining these subsets based on mTOR activity and metabolic programming. In this regard, our results do not define a continuum of mTORC1 activity in activated Tregs but rather define the level of mTORC1 activity within defined populations of Tregs. In our revised model, we propose that Foxp3⁺ Tregs emerging from the thymus possess characteristics phenotypically and metabolically of central memory Tregs (Fig. 8). Such cTregs provide the pool of Tregs that will migrate to the tissues and become eTregs when encountering Ag (under noninflammatory conditions). Similar to effector CD8⁺ T cells, these eTregs are characterized by increased mTOR activity, glycolysis, and effector molecule upregulation after Ag encounter. Furthermore, our model can account for the divergent methods of generating Tregs in vitro. We propose that strong stimulation of naive CD4⁺ T cells leads to the generation of an mTOR^{hi} effector cell pool. Alternatively, after 5-7 d in culture, the remaining Foxp3⁺ cells are now mTOR¹⁰ with decreased glycolysis and will behave like central memory Tregs. Likewise, the generation of central memory T cells in vitro can be markedly enhanced by adding rapamycin to Treg culture conditions.

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Disclosures

The authors have no financial conflicts of interest.

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