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Rapamycin-sensitive signals control TCR/CD28-driven *Ifng*, *Il4* and *Foxp3* transcription and promoter region methylation

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The mammalian target of rapamycin (mTOR) controls T-cell differentiation in response to polarizing cytokines. We previously found that mTOR blockade by rapamycin (RAPA) delays the G1-S cell cycle transition and lymphocyte proliferation. Here, we report that both mTOR complex 1 and mTOR complex 2 are readily activated following TCR/CD28 engagement and are critical for early expression of *Ifng*, *Il4* and *Foxp3*, and for effector T cell differentiation in the absence of polarizing cytokines. While inhibition of mTOR complex 1 and cell division were evident at low doses of RAPA, inhibition of mTOR complex 2, *Ifng*, *Il4* and *Foxp3* expression, and T-cell polarization required higher doses and more prolonged treatments. We found that while T-bet and GATA3 were readily induced following TCR/CD28 engagement, administration of RAPA delayed their expression, and interfered with the loss of DNA methylation within *Ifng* and *Il4* promoter regions. In contrast, RAPA prevented activation-dependent DNA methylation of the *Foxp3* promoter favoring *Foxp3* expression. As a result, RAPA-cultured cells lacked immediate effector functions and instead were enriched for IL-2$^+$ cells. We propose that mTOR-signaling, by timing the expression of critical transcription factors and DNA methylation of proximal promoter regions, regulates transcriptional competence at immunologically relevant sites and hence lymphocyte differentiation.

Key words: Cell differentiation · Cellular activation · Gene expression · T helper cells

Supporting Information available online

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Introduction

TOR (target of rapamycin) integrates environmental cues, including amino acid and nutrient availability, energy stores and growth factor signaling, and subsequently directs cell growth and proliferation in yeast and mammals. Mammalian (m)TOR exists in two complexes called mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [1]. mTORC1 comprises mTOR, Raptor, mLST8 and PRAS40 while the functionally distinct mTORC2 contains mTOR, mLST8 and the unique regulatory proteins Rictor, mSIN1 and PROTOR (reviewed in [2]). mTORC1 controls transcription and translation in response to nutrient and amino acid levels, growth factors and cytokines through phosphorylation of the p70 S6 kinase (p70S6K) and the initiation factor 4E-binding protein 1 (4EBP1), and is strongly sensitive to inhibition by the naturally occurring compound rapamycin (RAPA). While the function of mTORC2 remains largely uncharacterized, it has been shown to regulate aspects of the actin cytoskeleton, and to directly phosphorylate Akt (Ser473), PKC-α (Ser657) and SGK1 (Ser422). While mTORC2 is insensitive to transient RAPA administration, prolonged RAPA treatment hinders mTORC2 assembly, thus blocking mTORC2 function in some cells [2].

In T cells, mTOR appears to regulate several aspects of lymphocyte biology and to lie at the crossroads of T-cell proliferation and tolerance [3, 4]. T-cell-specific deletion of *Frap1* (encoding for mTOR) hinders Th1 and Th2 differentiation in response to polarizing cytokines favoring differentiation of $Foxp3^+$ cells [5]. In contrast, deletion of Rictor abrogates Th1 and Th2 cell differentiation without, however, diverting the cells into a $Foxp3^+$ phenotype [6].

RAPA was originally identified as an antifungal agent and was later shown to be able to inhibit the proliferation of many mammalian cell types and to have immunosuppressive activity. RAPA delays IL-2-induced G1-S cell-cycle transition by preventing Cdk2 and Cdc2 kinase activation [7] and p27Kip downregulation [8]; it favors the establishment of T-cell unresponsiveness [9, 10] and prevents its reversal by IL-2 [11]. This drug has been extensively used as an immunosuppressant in solid organ, as well as pancreatic islet, transplantation [12]. In mouse models, RAPA prevents graft rejection, graft-versus-host disease and graft versus leukemia, but also induces an autoimmune-like syndrome when administered as single agent [13]. We and others have also reported the ability of T cells to overcome RAPA-mediated cell cycle arrest, and the existence of RAPA-insensitive lymphocyte proliferation [14–16], thus questioning the interpretation for the immunosuppressive activity of the drug. More recently, it was found that RAPA influences the development of $CD4^+$ $CD25^+$ regulatory T cells in vitro [17–19], and T-cell migration [20] and the accumulation of central memory-like T cells in vivo [21].

Here we investigated the impact of RAPA on TCR/CD28-driven T-cell activation, expansion and differentiation in the absence of polarizing cytokines. We report that TCR/CD28 engagement evokes mTORC1- and mTORC2-dependent signaling, both sensitive to RAPA, and that these are critical for proper expression of T-bet and GATA3, of *Ifng, Il4* and *Foxp3* genes, and impact on the control of the DNA methylation state of promoter regions of genes important for cell fate determination. These data provide new insights into the molecular mechanism at the basis of the immunoregulatory activity of RAPA.

Results

TCR/CD28 induce both mTORC1- and mTORC2-dependent signaling

To test the notion that mTOR contributes to initial T-cell activation, we analyzed TCR/CD28-induced mTOR-dependent signaling in purified primary $CD4^+$ T cells. Phosphorylation of p70S6K, its target S6, of 4EBP1, and of AKT on Ser473 were used as indicative of mTORC1 and mTORC2 activity respectively [22]. TCR/CD28 stimulation caused the rapid phosphorylation of p70S6K, S6 and 4EBP1 and of AKT on both Ser 473 (target of mTORC2) and Thr308 (target of PI3K) (Fig. 1). While p70S6K and 4EBP1 were acutely sensitive to RAPA even at low doses (1 nM) (Fig. 1A), S6 phosphorylation was only partially sensitive to the drug (Fig. 1A), and abrogated by the combination of RAPA and SL0101 or UO126, respectively inhibitors of RSK and MAPK (our unpublished data), as previously found in $CD8^+$ T cells [23]. In the case of AKT, phosphorylation at Ser473 was detectable by 30 min, peaked by 1–2 h (Fig. 1B) and declined thereafter. In several independent experiments, AKT phosphorylation at Ser473 was favored by RAPA by 1 h of activation, while the drug inhibited it by 4 h (Fig. 1B). While the former supports the notion that the mTORC1-negative feedback loop [24] is also active in T cells, the latter reveals that mTORC2 is also sensitive to RAPA, but at later times. This was further proven by the finding that AKT phosphorylation in T cells activated for 24 h (Fig. 1C) and 6 days (Fig. 1D) in the presence of RAPA was drastically reduced when compared with that of control cells.

Having found that both mTORC1 and mTORC2 are activated in response to TCR/CD28 and are sensitive to RAPA, we studied the functional consequences of mTOR inhibition, by analyzing the CFSE dilution profile and IFN-γ and Foxp3 expression of control and RAPA-cultured cells. As expected [16], proliferation was readily sensitive to the drug, being already effective at early time points (day 2) at the lowest dose analyzed (Fig. 2A). In contrast, increasing doses of RAPA revealed the ability to interfere with the competence to secrete IFN-γ while favoring Foxp3 expression (Fig. 2B). Thus, while mTORC1 and T-cell proliferation reveal ready sensitivity to the drug, mTORC2 signaling and effector cell differentiation are best inhibited at the higher non-toxic dosage. Together these data support the notion that TCR/CD28 triggers both mTORC1- and mTORC2-dependent events, mostly sensitive to RAPA, and critical for $CD4^+$ T-cell fate determination.

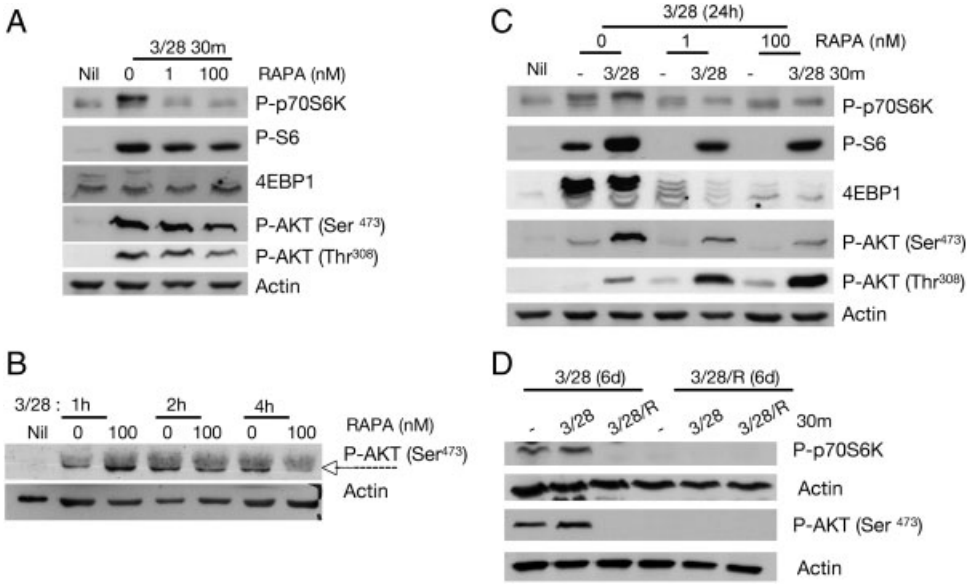

Figure 1. TCR/CD28 elicit both mTORC1 and mTORC2 signaling. $CD4^+$ T cells were purified from LN pools of BALB/c mice ($n = 3–5$) and (A and B) left untreated (Nil) or stimulated with immobilized anti-CD3/CD28 mAb in the absence (0) or in the presence of RAPA (1 and 100 nM) for the indicated times. In (C and D) cells were left untreated (Nil) or activated on immobilized anti-CD3/CD28 mAb for (C) 24 h and (D) 6 days in the absence (3/28) or in the presence of RAPA (3/28/R), and then lysed (–) or re-stimulated with CD3/CD28 mAb for 30 min. SDS-PAGE analyses were performed as detailed in *Materials and methods* with the indicated Ab. Actin was used as loading control. In each panel, one of three to five independent experiments is depicted.

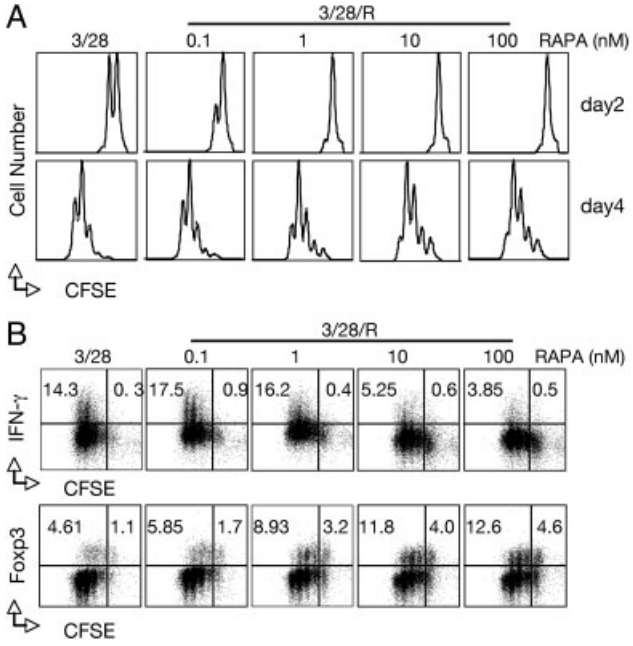

Figure 2. RAPA exerts dose-dependent control of T-cell proliferation and differentiation. LN cells were labeled with CFSE and stimulated with immobilized anti-CD3/CD28 mAb in the absence (3/28) or in the presence of the indicated concentration of RAPA (3/28/R) for 2 and 4 days. (A) Cells were stained with anti-CD4 mAb and analyzed for CFSE content by flow cytometry. Histograms depict the CFSE profile of viable TOPRO-3^- $CD4^+$ T cells. (B) Four-day cultured cells were left untreated (data not shown) or restimulated with PMA and Ionomycin for 4 h, the last 2 h in the presence of Brefeldin A. Thereafter, cells were surface stained with anti-CD4 mAb, and then permeabilized to measure IFN-γ and Foxp3 levels. The experiment was repeated three times with comparable results.

RAPA influences TCR/CD28-induced *Ifng*, *Il4* and *Foxp3* gene expression

TCR-, CD28- and IL-2/IL-2R-driven activation drives the initial transcription of the *Ifng* and the *Il4*-, *Il5*- and *Il3*-linked genes in the absence of polarizing cytokines [25]. We thus investigated whether RAPA interferes with initial cytokine gene transcription. Real-time PCR analysis of purified $CD4^+$ T cells revealed that IL-2, IFN-γ and IL-4 mRNAs were readily induced following CD3/CD28 activation when compared with unstimulated cells (Fig. 3). In the presence of RAPA, *Il2* expression was inhibited early on (24 h), accounting for suboptimal IL-2 secretion at this time (our unpublished data), but not significantly at later times. In contrast, *Ifng* and *Il4* expression was severely impaired at both 24 and 48 h, and did not recover at later times (Fig. 3). Provision of conditioned medium of control CD3/CD28-activated T cells could not bypass RAPA inhibition, excluding that an initial block in cytokine production might prevent further expression of the cytokine genes (Supporting Information Fig. 1A). Furthermore, the impact of RAPA on cytokine gene expression was dose-dependent, being mostly effective at higher concentrations (Supporting Information Fig. 1B).

We also examined Foxp3 levels. When compared with naïve cells, *Foxp3* was induced by CD3/CD28 activation. While Foxp3 mRNA (Fig. 4A and B) and protein (Fig. 4C) levels declined by 72 h in control cells, they were sustained in the presence of the drug. To investigate the origin of RAPA-induced $Foxp3^+$ cells and exclude that RAPA might preferentially expand pre-existing $Foxp3^+$ cells, we purified (Fig. 4D) and CFSE-labeled $CD4^+$ $CD25^-$ ($Foxp3^-$) cells and cultured them in the absence or in the

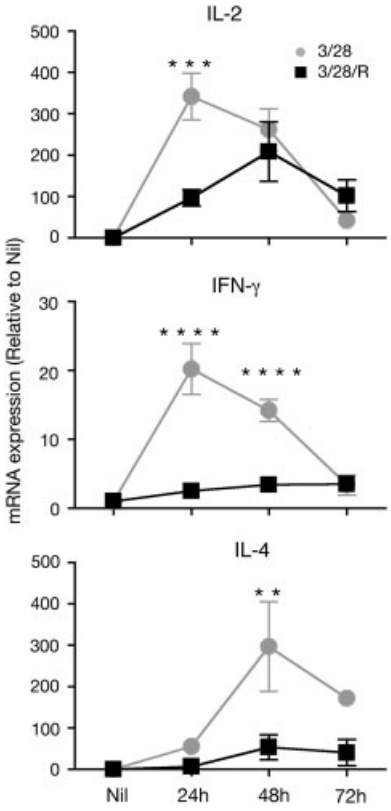

Figure 3. TCR/CD28/IL-2 drive RAPA-sensitive cytokine transcription. $CD4^+$ T cells were purified from LN pools of five BALB/c mice and left untreated (Nil) or stimulated with immobilized anti-CD3/CD28 mAb in the absence (3/28) or in the presence of RAPA (3/28/R) for 24, 48 or 72 h. IL-2, IFN-γ and IL-4 mRNA expression was quantified by RT-real time PCR in $n = 5$–7 (24/48 h) and $n = 3$ (72 h) independent experiments. Cytokine mRNAs were first normalized to the GAPDH housekeeping gene, and then expressed relatively to unstimulated cells (Nil) according to the ΔΔCT method (±SEM). Statistical significance was analyzed by non-parametric ANOVA analysis (**$p<0.01$, ***$p<0.001$, ****$p<0.0001$).

presence of RAPA. Results depicted in Fig. 4E indicate that following CD3/CD28 stimulation a fraction of $CD25^-$ $Foxp3^-$ $CD4^+$ cells up-regulated both CD25 and Foxp3, and this was favored in the presence of RAPA (Fig. 4E upper panels). Furthermore, both dividing and non-dividing cells were found to gain Foxp3 expression (Fig. 4E lower panels). Thus, together our data indicate that CD3/CD28 activation of $CD25^-$ $Foxp3^-$ $CD4^+$ cells induces de novo Foxp3 up-regulation and this is favored by RAPA. Of note, RAPA-treated cells failed to reveal suppressive activity (data not shown). This might be in keeping with the finding that repeated Ag exposure in the presence of the drug is needed to acquire suppressive functions [17], or with the fact that cells were cultured in the absence of TGF-β, which alone and together with RAPA further favors the $Foxp3^+$ phenotype ([26] and Supporting Information Fig. 2). These results thus support the possibility that TCR/CD28-IL-2-dependent RAPA-sensitive signaling, by inhibiting *Ifng* and *Il4* expression and transiently favoring *Foxp3* expression, limits TCR/CD28-driven cell autonomous differentiation.

RAPA inhibits expression of T-bet and GATA3 target genes

TCR/CD28-induced IL-2 production initiates Th-cell polarization via the signal transducer and activator of transcription 5 (STAT5) and entry into the cell cycle. Thereafter, lineage choice is largely determined by the cytokine milieu that, via the recruitment of other STAT family members such as STAT1, induce expression of lineage-specific transcription factors, such as T-bet and GATA3. We thus investigated STAT1 and STAT5, T-bet and GATA3 expression following TCR/CD28 activation of primary cells. Both STAT1 and STAT5 proteins were induced upon CD3/CD28 activation and phosphorylated to similar extents in both control and RAPA cells (Fig. 5A and B). STAT5 phosphorylation was evident in about 50% of the cells by 24 h of activation and peaked by 48 h (Fig. 5B) in most of control and RAPA CD3/CD28-activated T cells. Thus, TCR/CD28-driven activation favours STAT family members' phopshorylation regardless of RAPA administration. T-bet and GATA3 were undetectable in control naïve cells and induced by 24 h of CD3/CD28 activation (Fig. 5C and D). Expression was preserved at 48 h (Fig. 5C and D) and by 72 h started to decrease, and returned to undetectable levels by 96 h (data not shown). RAPA hindered T-bet and GATA3 expression both at the population (Fig. 5C) and at the single-cell level (Fig. 5D), at least in part by lowering gene expression (Fig. 5E and G). Although protein expression in the presence of RAPA was partially recovered by 48 h of activation, both T-bet (Fig. 5F) and GATA3 (Fig. 5H) target genes failed to be properly induced. Thus, together these data support the notion that mTOR blockade by RAPA impedes the expression and transcriptional activity of T-bet and GATA3.

RAPA-sensitive signaling controls methylation of *Ifng*, *Il4* and *Foxp3* promoter regions

Epigenetic marks control transcription of genes important in T-cell fate determination. In particular, gene expression associates with increased histone acetylation within both the *Ifng* and the *Il4* loci [27–29]. Acquisition and fixation of DNA methylation patterns determine the final commitment to given T-helper subsets. Previous reports found that differentiation towards Th1 cells results in progressive CpG demethylation [30] with the acquisition of de novo methylation in Th2 cells [31–33], extensive *Il4* promoter methylation that is lost in Th2 but not Th1 conditions [34, 35] and moderate *Foxp3* promoter methylation in $CD4^+$ T cells unless they are natural Tregs [36, 37]. In the case of *Ifng*, T-bet was found to contribute to the remodeling of the *Ifng* locus [38]. Having found that RAPA interferes with proper T-bet and GATA3 expression, we analyzed the influence of mTOR-blockade on promoter methylation using methylated DNA immunoprecipitation (MeDIP), a technique in which the DNA is sonicated to yield 300–600 bp fragments, precipitated with an Ab recognizing methylated CpG dinucleotides and analyzed by real-time PCR. We designed primers within proximal promoter

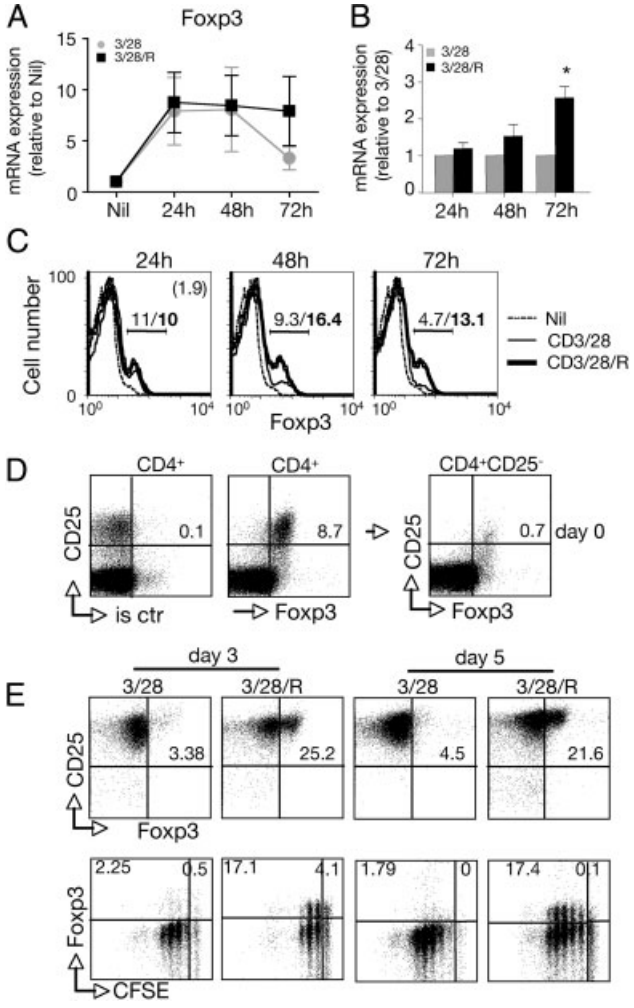

Figure 4. mTOR blockade favors Foxp3 expression. $CD4^+$ T cells were purified from LN pools of three to five BALB/c mice and stimulated with immobilized anti-CD3/CD28 mAb in the absence (3/28) or in the presence of RAPA (3/28/R) for the indicated times. (A and B) Foxp3 mRNA level was quantified by real-time PCR in four (24/48 h) and three (72 h) independent experiments. Foxp3 mRNA expression was normalized to the GAPDH housekeeping gene, and then expressed relatively to (A) unstimulated cells (Nil) or (B) the 3/28 control group within each time point according to the ΔΔCT method (+SEM). Statistical analysis in (B) was obtained by the Student's *t*-Test ($^*p<0.05$). (C) At the indicated times cells were recovered and analyzed by flow cytometry after staining with anti-CD4, anti-CD25 and Foxp3 or isotype control mAb. $CD4^+$ events are depicted. Naïve cells were used as control (dotted line). Data are representative of two independent experiments. The frequency of naïve cells is reported in brackets. The frequencies of control and RAPA-cultured cells are indicated in plain and bold text respectively. (D and E) $CD4^+$ $CD25^-$ cells were bead-purified, CFSE-labeled and cultured for 3 or 5 days on immobilized anti-CD3/CD28 mAb. (D) Dot plots depict relative CD25 and Foxp3 levels. Numbers refer to $CD25^+Foxp3^+$ events. (E) Upper panel: relative CD25 and Foxp3 levels; lower panels: Foxp3 expression levels by dividing and non-dividing cells. The plots are representative of four independently performed experiments.

regions previously defined to be regulated by epigenetic means. β-*actin* and *Xist*, respectively representative of a hypo-methylated gene and of a gene that is fully methylated in one of the two copies, were used as references to determine fractional methylation for the genes of interest (Fig. 6A). Following CD3/CD28 activation, fractional methylation of both *Ifng* and *IL4* promoter regions decreased relative to that found in naïve cells (Fig. 6B and C). In contrast, in the presence of RAPA, methylation of these regions was preserved and remained comparable with that found in naïve T cells (Nil in Fig. 6B and C). Comparable trends were observed at later times (data not shown). Interestingly, the analysis of the *Foxp3* promoter revealed an opposite behavior, with a massive increase in DNA methylation upon CD3/CD28 stimulation, which was nearly completely inhibited by the presence of RAPA (Fig. 6B and C). Of note, RAPA-sensitive changes in DNA methylation correlated with the RAPA-sensitive changes in *Ifng*, *IL4* (Fig. 3) and *Foxp3* (Fig. 4A) expression, suggesting they could be casually linked. Thus, taken together, our data support a role for RAPA-sensitive events controlling methylation of promoter regions within genes critical for T-cell polarization.

RAPA-cultured cells are enriched for IL-2-secreting cells

Next, we analyzed the phenotype of $CD4^+$ T cells allowed to proliferate under the pressure of RAPA. $CD4^+$ T cells from BALB/c mice (Fig. 7) and from DO11.10 and OT-II/$Rag^{-/-}$ TCR transgenic mice (Supporting Information Figs. 3 and 4) were used to compare polyclonal and antigen-driven responses. In spite of an initial delay, by 5–7 days RAPA-cultured cells had undergone several cycles of cell division (Fig. 7A), even upon daily refreshment of the drug (data not shown). However, while control T-cell cultures contained sizeable frequencies of cells capable of IFN-γ (Fig. 7A and B and Supporting Information Fig. 3) and IL-4 (Supporting Information Fig. 3) expression upon re-stimulation, these were much less represented among RAPA-treated cultures (Fig. 7 and Supporting Information Fig. 3). IL-2-producing cells, in contrast, were greatly enriched among RAPA-cultured cells (Fig. 7B). Finally, we analyzed the impact of RAPA in the presence of Th1 and Th2 polarizing cytokines. Also, in this case, both control and RAPA-cultured cells underwent several cell divisions. Of note, RAPA did not prevent differentiation of IFN-γ-competent cells (Fig. 7C and D) and it hindered the generation of IL-4-producing cells (Fig. 7E and F). As in the case of Th0 conditions, RAPA-cultured cells were enriched for IL-2-secreting lymphocytes, supporting the notion that activation in the context of mTOR blockade might preserve the cells in a less differentiated state.

Discussion

mTOR regulates several aspects of lymphocyte biology including T-cell proliferation and tolerance, and the acquisition of effector, memory or regulatory features [3, 5, 6, 21]. Here, we report that mTOR-dependent signaling orchestrates TCR/CD28-driven

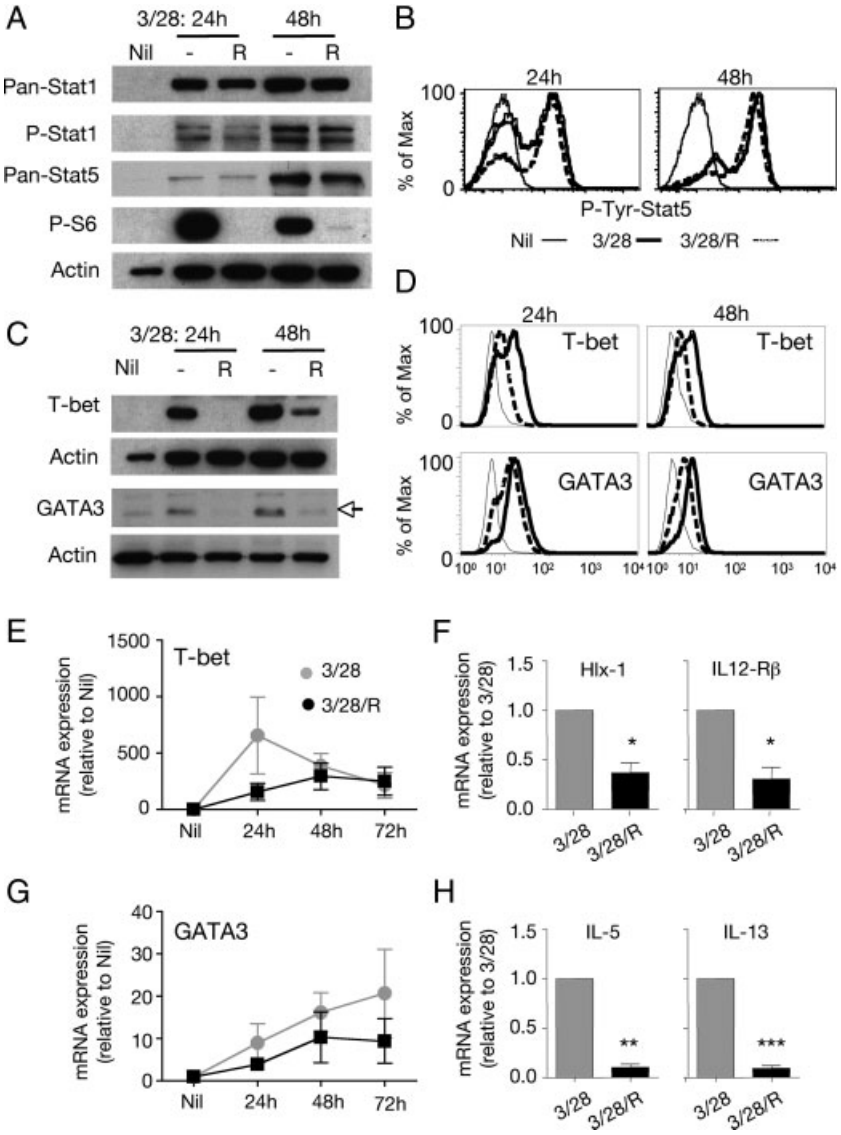

Figure 5. mTOR blockade hinders T-bet and GATA3 expression and transcriptional activity. $CD4^+$ T cells purified from BALB/c LN were left untreated (Nil) or stimulated with immobilized anti-CD3/CD28 mAb in the absence (–) or in the presence of RAPA (R) for the indicated times. Protein and mRNA levels were determined by (A and C) SDS-PAGE, (B and D) flow cytometry and (E–H) RT-real-time PCR. (A and C) One representative experiment of three is depicted. (B and D) Cells were stained for intracellular p-Tyr STAT5, T-bet and GATA3 contents (Nil: thin line; 3/28; thick line; 3/28/R; dotted line). (E–G) mRNA levels of the indicated genes were normalized to GAPDH and then expressed (E and G) relative to unstimulated cells (Nil) or (F and H) to the 3/28 control group within the 48 h time point according to the ΔΔCT method (+SEM). (E and G) Data are representative of five (24/48 h) and three (72 h) independent experiments. Differences were not statistically different by two-way ANOVA. (F and H) Data are representative of four independent experiments. Statistical significance was determined by the two-tailed Student's *t*-Test. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

activation of naïve T cells and controls initial *Ifng*, *Il4* and *Foxp3* transcription and their promoter regions' methylation.

Studies mostly performed on Th1 and Th2 $CD4^+$ T cells have proposed that the TCR-, CD28- and IL-2/IL-2R elicit the early transcription of the *Ifng* and the *Il4*, *Il5*, and *Il13* linked genes [25], and favor histone acetylation in both the *Ifng* and the *Il4* loci [27, 28]. As differentiation proceeds, acquisition and fixation of new DNA methylation patterns determine the final commitment to a given T-helper subset [29]. In the case of *Ifng*, naïve $CD4^+$ T cells are 'poised' to have low expression of *Ifng* mRNA shortly after activation, which correlates with the absence of marks of activity at the *Ifng* promoter. Differentiation towards Th1 cells results in progressive CpG demethylation and polarized histone acetylation patterns [30], and the acquisition of DNAse I hypersensitivity within the *Ifng* promoter and multiple *cis*-regulatory regions [33]. T-bet, rapidly induced following T-cell activation, also facilitates chromatin looping at the *Ifng* locus [39], further supporting the possibility that early signaling events determine heritable IFN-γ production. The mechanisms and molecules responsible for such events remain yet to be largely defined. Our data indicate that TCR/CD28-signaling, in the absence of polarizing cytokines, initiates such events through the

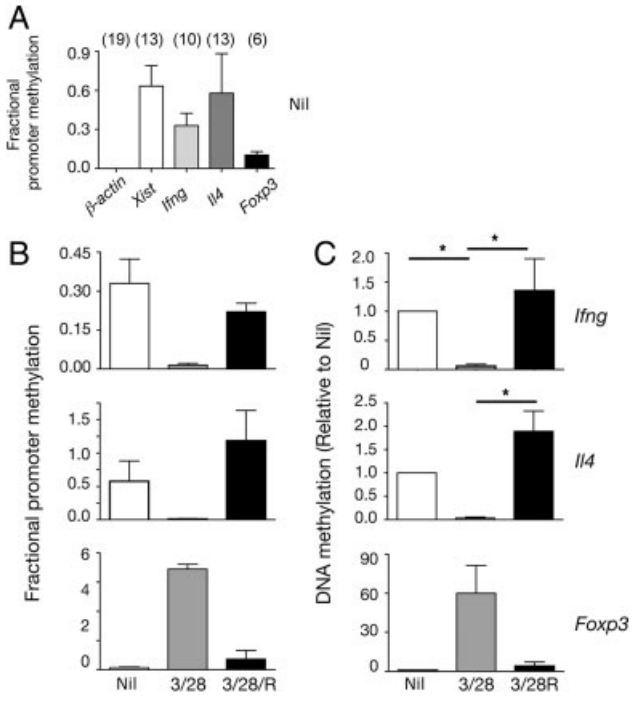

Figure 6. mTOR-dependent signaling controls DNA methylation. $CD4^+$ T lymphocytes were purified from BALB/c LNs and left untreated (Nil) or stimulated with immobilized anti CD3/CD28 mAb in the absence (3/28) or in the presence of RAPA (C/28/R) for 48 h. Genomic DNA was obtained and analyzed by MeDIP assays with a mouse monoclonal anti-5-methylcytidine Ab (see *Materials and methods*). (A) Fractional methylation was expressed relative to input ($2^{-CTIp-CTinput} = \Delta CT \pm SEM$). Numbers in brackets represent CpG hits in the regions of interest. (B and C) Changes in promoter region methylation following activation, either absolute (B; $2^{-\Delta CT}$) or relative to unstimulated T cells (Nil) (C; $2^{-\Delta\Delta CT}$ calculated as follows $\Delta\Delta CT = (\Delta CTSample - \Delta CTNil$; mean + SEM) are depicted. Data are representative of five (*Ifng*), four (*Il4*) and three (*Foxp3*) independent determinations. Trends depicted in the figure were found in all of the experiments. Statistical significance was determined by one-way ANOVA (*$p<0.05$).

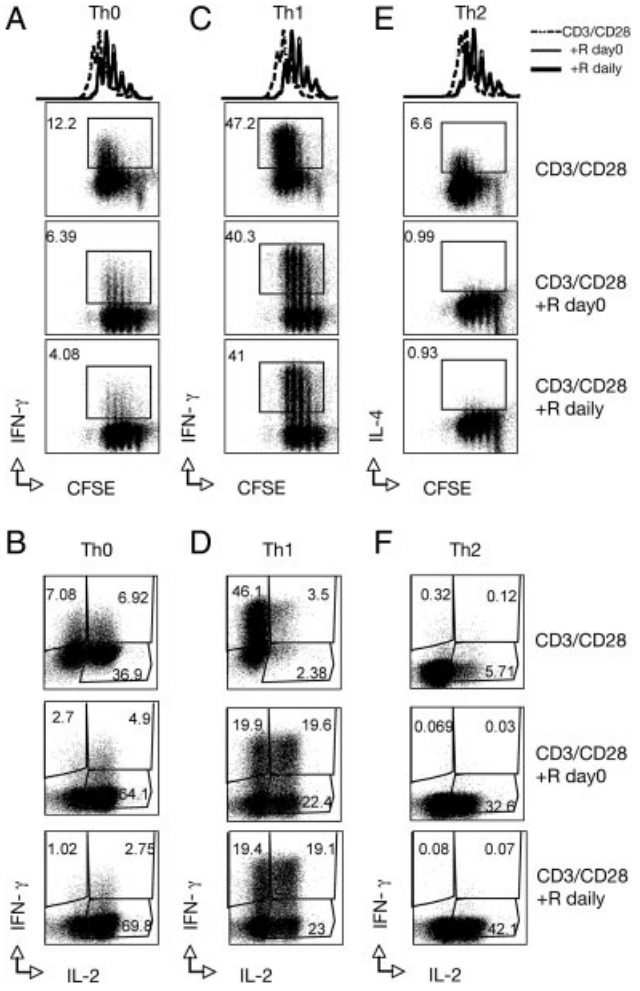

Figure 7. RAPA impacts on $CD4^+$ T-cell differentiation in Th0, Th1 and Th2 polarizing conditions. LN cells of BALB/c origin were labeled with CFSE and cultured on immobilized anti-CD3/CD28 mAb in (A and B; Th0) the absence or (C and D) the presence of Th1 or (E and F) Th2 skewing conditions (see *Materials and methods*). RAPA was added to a set of cultures at day 0 or refreshed daily. After 5 days, cells were harvested, stimulated with PMA and Ionomycin for 4 h (last 2 h in the presence of Brefeldin A), surface stained with anti-CD4 mAb, and analyzed for IL-2, IFN-γ and IL-4. Events are shown after gating on viable $CD4^+$ cells. (A) Overlays reflect the CFSE profile of $CD4^+$ T cells activated on CD3/CD28 mAb in the absence (dotted line) or in the presence of RAPA provided at day 0 (thin line) or daily (thick line). Data are representative of three independent experiments.

involvement of mTOR-dependent signaling. This appears to be critical for proper expression of the lineage-determining transcription factors T-bet and GATA3, and in the control of DNA methylation in *Ifng* and *Il4* promoter regions.

We found that TCR/CD28-mediated activation involves mTORC1 and mTORC2, and that these are both sensitive to RAPA inhibition. RAPA hindered both mTORC1 and mTORC2 signaling and delayed the expression of T-bet and GATA3. In a recent report, Lee et al. have shown that Rictor (mTORC2) deficient cells failed to upregulate T-bet and GATA3, and this could be restored by expression of active mutants of AKT and PKC-θ [5, 6]. Thus, inhibition of mTORC2, revealed by defective phosphorylation of AKT, might account for delayed T-bet and GATA3 expression and consequently *Ifng* and *Il4* transcription under the pressure of RAPA. In addition, RAPA-sensitive events also impacted on the state of DNA methylation of proximal promoter regions previously shown to be critical for gene expression. While DNA methylation of *Ifng* and *Il4* promoter regions decreased following activation, it was preserved to the level found in naïve T cells under the pressure of RAPA. This possibly explains why, although T-bet and GATA3 levels appeared to be mostly recovered in RAPA cultured cells by 48 h of activation, target genes remained undetectable. We speculate that preserved DNA methylation of proximal cytokine promoter regions might prevent T-bet and GATA3 binding and by that gene transcription.

Inhibition of mTORC1 and mTORC2 by RAPA also favored Foxp3 expression. In contrast to the *Ifng* and *Il4* genes, following CD3/CD28 stimulation the *Foxp3* promoter underwent activation-dependent methylation, previously linked to *Foxp3* transcription [40]. We found that *Foxp3* methylation could be

inhibited by mTOR blockade and correlated with prolonged *Foxp3* mRNA and protein expression. While previous reports indicate that RAPA favors the selective expansion and the de novo generation of T cells with regulatory functions [17–19, 41, 42] by counteracting molecular brakes on Foxp3 induction [5, 43], in our hands cells cultured for a week in the presence of RAPA transiently expressed Foxp3 but did not acquire T regulatory activity (data not shown). We speculate that this could be due to the absence of TGF-β in our cultures [26] or to the need for repetitive Ag encounters in the presence of RAPA for the acquisition of suppressive capacities [17].

The challenge will now be to identify the mechanisms by which RAPA impacts on TCR/CD28/IL-2 controlled promoter region methylation. Lineage-specific DNA methylation occurs at promoter proximal and distal sites of immunological relevant genes [44]. Specific patterns of DNA methylation are maintained during DNA replication by the DNA methyltransferases (Dnmts), and presumably lost when the DNA methylation sites in newly synthesized DNA are inaccessible. While Dnmt1 is the main Dnmt responsible for preserving the pattern of the parental-strand methylation in the newly synthesized daughter strand during DNA replication, Dnmt3a and Dnmt3b are involved in the de novo introduction of methyl groups on cytosine residues [45]. Defective Dnmt1 expression or function causes passive demethylation of defined regions and results in transcriptional upregulation of previously suppressed cytokine genes and autoimmune manifestations [46, 47]. Dnmt3a is induced following TCR engagement and controls *Ifng* and *Il4* promoter methylation [48]. We found that expression of Dnmt1, Dnmt3a and Dnmt3b was increased following TCR/CD28 stimulation, and that this was not sensitive to RAPA (our unpublished data). Although their recruitment to the DNA in mTOR sufficient and deficient conditions remains to be investigated, we speculate that their binding to *Ifng* and *Il4* promoter regions might be facilitated in the absence of T-bet and GATA3. Finally, although the existence of DNA demethylases is a matter of intense debate [49], in some cases demethylation was observed soon before DNA replication [50]. Thus, mTOR might also influence DNA methylation by controlling expression/activity of yet to be defined DNA demethylases or of histone acethylases/deacetylases/methylases, also involved in epigenetic changes. In this respect it is of interest that TOR was linked to the Esa1 histone acetylase pathway in yeast [51].

Results reported here, although in line with those obtained with Frap-, Rictor- and Raptor-deficient T cells, reveal some differences between genetic inactivation and pharmacological inhibition of mTOR. For instance, while Frap- and Rictor-deficient lymphocytes failed to differentiate into IFN-γ-secreting cells in Th1-polarizing conditions [5, 6] this was not the case for cells activated in the presence of RAPA, which retained both IFN-γ and IL-2 expression. This discrepancy might be due to the failure of RAPA to fully inhibit mTORC2 or the ability of IL-12 to control *Ifng* expression via mTOR-dependent RAPA-insensitive mechanisms. Likewise, while mTOR-deficient T cells diverted into a $Foxp3^+$ phenotype, RAPA-cultured cells did not, and instead remained sensitive to Ag for further clonal expansion and differentiation (Supporting Information Fig. 4). Whether this is due to the fact that mTOR deficiency more than RAPA treatment confers different sensitivity to TGF-β remains to be understood. Regardless, these differences reinforce the notion that genetic inactivation of single pathways might not always be predictive for the outcome of pharmacological inhibitors.

Together our data support a model whereby mTOR could time the expression of factors able to regulate transcription and chromatin-remodeling events [52, 53] with cell cycle progression. These might have to be concomitant for proper T-cell differentiation [54]. In the absence of mTOR signaling T cells undergo several rounds of cell division, but fail to acquire effector function, and instead gain surface and functional features of memory cells [21]. Thus, in vivo RAPA might exert immunosuppressive/immunoregulatory functions by hindering differentiation of pathogenic T cells early on, and allowing the expansion of cells with regulatory functions over long-term treatments. Solving these issues in appropriate in vivo animal models would be of benefit for transplanted patients undergoing prolonged immunosuppressive regimens.

Materials and methods

Mice

BALB/c mice were obtained from Charles River (Calco). DO11.10 mice ($H\text{-}2^d$) express a transgenic αβ TCR specific for the CD4-restricted chicken OVA-derived peptide ($OVA_{323–339}$) on the majority of the $CD4^+$ T cells [55]. The Ethical Committee of the San Raffaele Scientific Institute approved the animal usage.

Primary T-cell cultures

$CD4^+$ T cells were purified by negative selection using anti-CD8 (clone KT1.5) and anti-$I\text{-}A^{b\text{-}d}$/I-E (clone B21-22) rat Abs and sheep anti-rat-coated magnetic beads (Dynal Biotech, UK) to a purity of >95%. Cells were then stimulated on immobilized anti-CD3 and anti-CD28 mAbs (0.05 μg/mL and 5 μg/mL, respectively) or with $OVA_{323–339}$ (0.25 μM) and irradiated syngeneic splenocytes in the absence or in the presence of RAPA (0.1–100 nM). Where indicated cells were cultured in the presence of rIL-12 (0.5 ng/mL, Bender MedSystem, Milan, Italy), and neutralizing anti-IL-4 mAb (5 μg/mL) (Th1 conditions), or rIL-4 (1 ng/mL; Bender MedSystem) and neutralizing anti-IL-12 mAb (5 μg/mL, BD Pharmingen) (Th2 conditions). After 3–7 days cells were stimulated with PMA (0.05 μg/mL) and Ionomycin (1 μg/mL) for 4 h, of which the last 2 in the presence of Brefeldin A, and intracellular cytokine staining was performed as previously described [16] and evaluated on a BD Biosciences FACSCanto cytometer using Flowjow acquisition

analysis software. CFSE-labeling was performed also as previously described [16].

Western blot

SDS-PAGE was performed as previously described [16]. Abs were all from Cell Signaling Technology (Milan, Italy), except the ones against mouse T-bet, GATA3 and actin, which were obtained from Santa Cruz Biotechnology, and anti H3K27-me3 histone, which was obtained from Millipore.

Real-time PCR

RNA was extracted with RNeasy Mini Kit (Qiagen). Reverse-transcription was performed with oligo-$(dT)_{12–18}$ primers and M-MLV Reverse Transcriptase (Invitrogen). Real-time amplification was performed with Taqman primers and chemistry (Applied Biosystems). Specific mRNA expression was normalized by housekeeping genes GAPDH or TBP and expressed as ΔΔCT fold increase over untreated cells (Nil). Where indicated CD3/CD28-activated control and drug-treated cells were directly compared within each time point by the ΔΔCT method, using control cells as reference.

MeDIP

MeDIP assays were carried out as previously described [56]. Briefly, DNA was sonicated to yield fragments ranging in size between 300 and 600 bp. Sonicated DNA was then used as a whole-genome reference for comparative analysis (Input DNA) or immunoprecipitated with a mouse monoclonal anti-5-methyl-cytidine Ab from Eurogentec (♯BI-MECY-1000). The methylation status of *Ifng*, *Il4* and *Foxp3* genes was analyzed by real-time quantitative PCR by employing primers targeting proximal promoter regions (*Ifng*: left-163: AACATGCCACAAAACCATAGC; right-25: CACCTCTCTGGCTTCCAGTT; *Il4*: left-226 GGTCCTCTGGAAGAGCAACA; right-96 CTTCGGTGCTGCAGA-TACAA; *Foxp3* left-454 CCTCCAACGTCTCACAAACA; right-300 CCCCTCACCACAGAGGTAAA). Real-time PCR was performed using the Sybr Green technique (LightCycler 480 SYBR Green I Master). Fractional methylation was expressed relative to input (CT_{Ip}-CT_{input} = ΔCT). To compare different experiments data were expressed relative to control cells (Nil) as follows: $2^{-(\Delta CT-\Delta CT)}_{Nil}$. Unmethylated and methylated housekeeping genes (β-*actin* and *Xist* respectively) were used as controls. Data were analyzed by one-way ANOVA.

Statistical analysis

Data were analyzed by one-way or two-way ANOVA (respectively with the Dunns or the Bonferroni post test) or the two tailed Student's *t*-Test, as specified in figure legends. Statistical significance: $^{*}p<0.05$; $^{**}p<0.005$; $^{***}p<0.001$.

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References

1 **Wullschleger, S., Loewith, R. and Hall, M. N.,** TOR signaling in growth and metabolism. *Cell* 2006. **124**: 471–484.

2 **Guertin, D. A. and Sabatini, D. M.,** The pharmacology of mTOR inhibition. *Sci. Signal.* 2009. **2**: pe24.

3 **Mondino, A. and Mueller, D. L.,** mTOR at the crossroads of T cell proliferation and tolerance. *Semin. Immunol.* 2007. **19**: 162–172.

4 **Powell, J. D. and Delgoffe, G. M.,** The mammalian target of rapamycin: linking T cell differentiation, function, and metabolism. *Immunity* 2010. **33**: 301–311.

5 **Delgoffe, G. M., Kole, T. P., Zheng, Y., Zarek, P. E., Matthews, K. L., Xiao, B., Worley, P. F. et al.,** The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. *Immunity* 2009. **30**: 832–844.

6 **Lee, K., Gudapati, P., Dragovic, S., Spencer, C., Joyce, S., Killeen, N., Magnuson, M. A. and Boothby, M.,** Mammalian target of rapamycin protein complex 2 regulates differentiation of Th1 and Th2 cell subsets via distinct signaling pathways. *Immunity* 2010. **32**: 743–753.

7 **Morice, W. G., Wiederrecht, G., Brunn, G. J., Siekierka, J. J. and Abraham, R. T.,** Rapamycin inhibition of interleukin-2-dependent p33cdk2 and p34cdc2 kinase activation in T lymphocytes. *J. Biol. Chem.* 1993. **268**: 22737–22745.

8 **Nourse, J., Firpo, E., Flanagan, W. M., Coats, S., Polyak, K., Lee, M. H., Massague, J. et al.,** Interleukin-2-mediated elimination of the p27Kip1 cyclin-dependent kinase inhibitor prevented by rapamycin. *Nature* 1994. **372**: 570–573.

9 **Powell, J. D., Lerner, C. G. and Schwartz, R. H.,** Inhibition of cell cycle progression by rapamycin induces T cell clonal anergy even in the presence of costimulation. *J. Immunol.* 1999. **162**: 2775–2784.

10 **Vanasek, T. L., Khoruts, A., Zell, T. and Mueller, D. L.,** Antagonistic roles for CTLA-4 and the mammalian target of rapamycin in the regulation of clonal anergy: enhanced cell cycle progression promotes recall antigen responsiveness. *J. Immunol.* 2001. **167**: 5636–5644.

11 **Colombetti, S., Benigni, F., Basso, V. and Mondino, A.,** Clonal anergy is maintained independently of T cell proliferation. *J. Immunol.* 2002. **169**: 6178–6186.

12 **Street, C. N., Lakey, J. R., Shapiro, A. M., Imes, S., Rajotte, R. V., Ryan, E. A., Lyon, J. G. et al.,** Islet graft assessment in the Edmonton Protocol: implications for predicting long-term clinical outcome. *Diabetes* 2004. **53**: 3107–3114.

13 **Blazar, B. R., Korngold, R. and Vallera, D. A.,** Recent advances in graft-versus-host disease (GVHD) prevention. *Immunol. Rev.* 1997. **157**: 79–109.

14 **Slavik, J. M., Lim, D. G., Burakoff, S. J. and Hafler, D. A.,** Rapamycin-resistant proliferation of CD8+ T cells correlates with p27kip1 down-regulation and bcl-xL induction, and is prevented by an inhibitor of phosphoinositide 3-kinase activity. *J. Biol. Chem.* 2004. **279**: 910–919.

15 **Fox, C. J., Hammerman, P. S. and Thompson, C. B.,** The Pim kinases control rapamycin-resistant T cell survival and activation. *J. Exp. Med.* 2005. **201**: 259–266.

16 **Colombetti, S., Basso, V., Mueller, D. L. and Mondino, A.,** Prolonged TCR/CD28 engagement drives IL-2-independent T cell clonal expansion through signaling mediated by the mammalian target of rapamycin. *J. Immunol.* 2006. **176**: 2730–2738.

17 **Battaglia, M., Stabilini, A. and Roncarolo, M. G.,** Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood* 2005. **105**: 4743–4748.

18 **Valmori, D., Tosello, V., Souleimanian, N. E., Godefroy, E., Scotto, L., Wang, Y. and Ayyoub, M.,** Rapamycin-mediated enrichment of T cells with regulatory activity in stimulated CD4+ T cell cultures is not due to the selective expansion of naturally occurring regulatory T cells but to the induction of regulatory functions in conventional CD4+ T cells. *J. Immunol.* 2006. **177**: 944–949.

19 **Strauss, L., Whiteside, T. L., Knights, A., Bergmann, C., Knuth, A. and Zippelius, A.,** Selective survival of naturally occurring human CD4+CD25+ Foxp3+ regulatory T cells cultured with rapamycin. *J. Immunol.* 2007. **178**: 320–329.

20 **Sinclair, L. V., Finlay, D., Feijoo, C., Cornish, G. H., Gray, A., Ager, A., Okkenhaug, K. et al.,** Phosphatidylinositol-3-OH kinase and nutrient-sensing mTOR pathways control T lymphocyte trafficking. *Nat. Immunol.* 2008. **9**: 513–521.

21 **Araki, K., Turner, A. P., Shaffer, V. O., Gangappa, S., Keller, S. A., Bachmann, M. F., Larsen, C. P. and Ahmed, R.,** mTOR regulates memory CD8 T-cell differentiation. *Nature* 2009. **460**: 108–112.

22 **Hay, N. and Sonenberg, N.,** Upstream and downstream of mTOR. *Genes Dev.* 2004. **18**: 1926–1945.

23 **Salmond, R. J., Emery, J., Okkenhaug, K. and Zamoyska, R.,** MAPK, phosphatidylinositol 3-kinase, and mammalian target of rapamycin pathways converge at the level of ribosomal protein S6 phosphorylation to control metabolic signaling in CD8 T cells. *J. Immunol.* 2009. **183**: 7388–7397.

24 **Sarbassov, D. D., Ali, S. M., Sengupta, S., Sheen, J. H., Hsu, P. P., Bagley, A. F., Markhard, A. L. and Sabatini, D. M.,** Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol. Cell* 2006. **22**: 159–168.

25 **Grogan, J. L., Mohrs, M., Harmon, B., Lacy, D. A., Sedat, J. W. and Locksley, R. M.,** Early transcription and silencing of cytokine genes underlie polarization of T helper cell subsets. *Immunity* 2001. **14**: 205–215.

26 **Kopf, H., de la Rosa, G. M., Howard, O. M. and Chen, X.,** Rapamycin inhibits differentiation of Th17 cells and promotes generation of FoxP3+ T regulatory cells. *Int. Immunopharmacol.* 2007. **7**: 1819–1824.

27 **Agarwal, S., Viola, J. P. and Rao, A.,** Chromatin-based regulatory mechanisms governing cytokine gene transcription. *J. Allergy Clin. Immunol.* 1999. **103**: 990–999.

28 **Fields, P. E., Kim, S. T. and Flavell, R. A.,** Cutting edge: changes in histone acetylation at the IL-4 and IFN-gamma loci accompany Th1/Th2 differentiation. *J. Immunol.* 2002. **169**: 647–650.

29 **Avni, O., Lee, D., Macian, F., Szabo, S. J., Glimcher, L. H. and Rao, A.,** T(H) cell differentiation is accompanied by dynamic changes in histone acetylation of cytokine genes. *Nat. Immunol.* 2002. **3**: 643–651.

30 **Messi, M., Giacchetto, I., Nagata, K., Lanzavecchia, A., Natoli, G. and Sallusto, F.,** Memory and flexibility of cytokine gene expression as separable properties of human T(H)1 and T(H)2 lymphocytes. *Nat. Immunol.* 2003. **4**: 78–86.

31 **Young, H. A., Ghosh, P., Ye, J., Lederer, J., Lichtman, A., Gerard, J. R., Penix, L. et al.,** Differentiation of the T helper phenotypes by analysis of the methylation state of the IFN-gamma gene. *J. Immunol.* 1994. **153**: 3603–3610.

32 **Winders, B. R., Schwartz, R. H. and Bruniquel, D.,** A distinct region of the murine IFN-gamma promoter is hypomethylated from early T cell development through mature naive and Th1 cell differentiation, but is hypermethylated in Th2 cells. *J. Immunol.* 2004. **173**: 7377–7384.

33 **Schoenborn, J. R., Dorschner, M. O., Sekimata, M., Santer, D. M., Shnyreva, M., Fitzpatrick, D. R., Stamatoyannopoulos, J. A. and Wilson, C. B.,** Comprehensive epigenetic profiling identifies multiple distal regulatory elements directing transcription of the gene encoding interferon-gamma. *Nat. Immunol.* 2007. **8**: 732–742.

34 **Agarwal, S. and Rao, A.,** Modulation of chromatin structure regulates cytokine gene expression during T cell differentiation. *Immunity* 1998. **9**: 765–775.

35 **Bird, J. J., Brown, D. R., Mullen, A. C., Moskowitz, N. H., Mahowald, M. A., Sider, J. R., Gajewski, T. F. et al.,** Helper T cell differentiation is controlled by the cell cycle. *Immunity* 1998. **9**: 229–237.

36 **Floess, S., Freyer, J., Siewert, C., Baron, U., Olek, S., Polansky, J., Schlawe, K. et al.,** Epigenetic control of the foxp3 locus in regulatory T cells. *PLoS Biol.* 2007. **5**: e38.

37 **Baron, U., Floess, S., Wieczorek, G., Baumann, K., Grutzkau, A., Dong, J., Thiel, A. et al.,** DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3(+) conventional T cells. *Eur. J. Immunol.* 2007. **37**: 2378–2389.

38 **Mullen, A. C., High, F. A., Hutchins, A. S., Lee, H. W., Villarino, A. V., Livingston, D. M., Kung, A. L. et al.,** Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. *Science* 2001. **292**: 1907–1910.

39 **Sekimata, M., Perez-Melgosa, M., Miller, S. A., Weinmann, A. S., Sabo, P. J., Sandstrom, R., Dorschner, M. O. et al.,** CCCTC-binding factor and the transcription factor T-bet orchestrate T helper 1 cell-specific structure and function at the interferon-gamma locus. *Immunity* 2009. **31**: 551–564.

40 **Lal, G. and Bromberg, J. S.,** Epigenetic mechanisms of regulation of Foxp3 expression. *Blood* 2009. **114**: 3727–3735.

41 **Sauer, S., Bruno, L., Hertweck, A., Finlay, D., Leleu, M., Spivakov, M., Knight, Z. A. et al.,** T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR. *Proc. Natl. Acad. Sci. USA* 2008. **105**: 7797–7802.

42 **Zeiser, R., Leveson-Gower, D. B., Zambricki, E. A., Kambham, N., Beilhack, A., Loh, J., Hou, J. Z. and Negrin, R. S.,** Differential impact of mammalian target of rapamycin inhibition on CD4+CD25+Foxp3+ regulatory T cells compared with conventional CD4+ T cells. *Blood* 2008. **111**: 453–462.

43 **Haxhinasto, S., Mathis, D. and Benoist, C.,** The AKT-mTOR axis regulates de novo differentiation of CD4+Foxp3+ cells. *J. Exp. Med.* 2008. **205**: 565–574.

44 **Schmidl, C., Klug, M., Boeld, T. J., Andreesen, R., Hoffmann, P., Edinger, M. and Rehli, M.,** Lineage-specific DNA methylation in T cells correlates with histone methylation and enhancer activity. *Genome Res.* 2009. **19**: 1165–1174.

45 **Liang, G., Chan, M. F., Tomigahara, Y., Tsai, Y. C., Gonzales, F. A., Li, E., Laird, P. W. and Jones, P. A.,** Cooperativity between DNA methyltrans-

ferases in the maintenance methylation of repetitive elements. *Mol. Cell Biol.* 2002. **22**: 480–491.

46 **Lee, P. P., Fitzpatrick, D. R., Beard, C., Jessup, H. K., Lehar, S., Makar, K. W., Perez-Melgosa, M. et al.,** A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. *Immunity* 2001. **15**: 763–774.

47 **Makar, K. W. and Wilson, C. B.,** DNA methylation is a nonredundant repressor of the Th2 effector program. *J. Immunol.* 2004. **173**: 4402–4406.

48 **Gamper, C. J., Agoston, A. T., Nelson, W. G. and Powell, J. D.,** Identification of DNA methyltransferase 3a as a T cell receptor-induced regulator of Th1 and Th2 differentiation. *J. Immunol.* 2009. **183**: 2267–2276.

49 **Ooi, S. K. and Bestor, T. H.,** The colorful history of active DNA demethylation. *Cell* 2008. **133**: 1145–1148.

50 **Bruniquel, D. and Schwartz, R. H.,** Selective, stable demethylation of the interleukin-2 gene enhances transcription by an active process. *Nat. Immunol.* 2003. **4**: 235–240.

51 **Rohde, J. R. and Cardenas, M. E.,** The tor pathway regulates gene expression by linking nutrient sensing to histone acetylation. *Mol. Cell Biol.* 2003. **23**: 629–635.

52 **Murphy, K. M. and Reiner, S. L.,** The lineage decisions of helper T cells. *Nat. Rev. Immunol.* 2002. **2**: 933–944.

53 **Schulz, E. G., Mariani, L., Radbruch, A. and Hofer, T.,** Sequential polarization and imprinting of type 1 T helper lymphocytes by interferon-gamma and interleukin-12. *Immunity* 2009. **30**: 673–683.

54 **Richter, A., Lohning, M. and Radbruch, A.,** Instruction for cytokine expression in T helper lymphocytes in relation to proliferation and cell cycle progression. *J. Exp. Med.* 1999. **190**: 1439–1450.

55 **Murphy, K. M., Heimberger, A. B. and Loh, D. Y.,** Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes in vivo. *Science* 1990. **250**: 1720–1723.

56 **Weber, M., Davies, J. J., Wittig, D., Oakeley, E. J., Haase, M., Lam, W. L. and Schubeler, D.,** Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. *Nat. Genet.* 2005. **37**: 853–862.

Abbreviations: **4EBP1:** 4E-binding protein 1 · **Dnmt:** DNA methyltransferase · **MeDIP:** methylated DNA immunoprecipitation · **m:** mammalian · **mTORC1:** mTOR complex 1 · **mTORC2:** mTOR complex 2 · **p70S6K:** p70 S6 kinase · **RAPA:** rapamycin · **STAT5:** signal transducer and activator of transcription 5 · **TOR:** target of rapamycin

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