T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR

Stephan Sauer*, Ludovica Bruno*, Arnulf Hertweck*, David Finlay†, Marion Leleu*, Mikhail Spivakov*, Zachary A. Knight‡, Bradley S. Cobb*, Doreen Cantrell†, Eric O'Connor§, Kevan M. Shokat‡, Amanda G. Fisher*, and Matthias Merkenschlager*¶

*Lymphocyte Development Group and §Flow Cytometry Facility, Medical Research Council Clinical Sciences Centre, Imperial College London, Du Cane Road, London W12 0NN, United Kingdom; †Division of Cell Biology and Immunology, College of Life Sciences, University of Dundee, Dundee DD1 5EH, United Kingdom; and ‡Department of Cellular and Molecular Pharmacology, Howard Hughes Medical Institute, University of California, San Francisco, CA 94143

Edited by Christophe Benoist, Joslin Diabetes Center, Boston, MA, and approved April 7, 2008 (received for review January 29, 2008)

Regulatory T (Treg) cells safeguard against autoimmunity and immune pathology. Because determinants of the Treg cell fate are not completely understood, we have delineated signaling events that control the *de novo* expression of Foxp3 in naive peripheral CD4 T cells and in thymocytes. We report that premature termination of TCR signaling and inibition of phosphatidyl inositol 3-kinase (PI3K) p110α, p110δ, protein kinase B (Akt), or mammalian target of rapamycin (mTOR) conferred Foxp3 expression and Treg-like gene expression profiles. Conversely, continued TCR signaling and constitutive PI3K/Akt/mTOR activity antagonised Foxp3 induction. At the chromatin level, di- and trimethylation of lysine 4 of histone H3 (H3K4me2 and -3) near the *Foxp3* transcription start site (TSS) and within the 5′ untranslated region (UTR) preceded active Foxp3 expression and, like Foxp3 inducibility, was lost upon continued TCR stimulation. These data demonstrate that the PI3K/Akt/mTOR signaling network regulates Foxp3 expression.

Specialized cell types in multicellular organisms are defined by distinct patterns of gene expression (1). During their differentiation from hematopoietic stem cells, developing T cells undergo progressive restriction of their lineage potential. After the CD4/CD8 lineage choice in the thymus, CD4 lineage cells remain able to adopt a naive or regulatory cell fate, and naive CD4 T cells can opt for a range of Th lineages or, alternatively, become regulatory T (Treg) cells after activation (2, 3). The choice of Th lineage is important for effective immune responses to specific pathogens, and the balance between effector and regulatory cells is critical to ensure immune competence while avoiding immune pathology and autoimmunity. Thymus-derived Treg cells are generated via a TGFβ independent pathway that requires costimulatory signals (2–4) and typically express the signature transcription factor Foxp3, which confers regulatory T cell function (7–10). Differences between the TCR repertoires of conventional and regulatory CD4 T cells attest to the importance of MHC/peptide recognition and TCR signaling in conventional versus regulatory T cell differentiation (11, 12). Adaptive Treg cells can arise from naive peripheral CD4 T cells, for example by immunisation with low dose antigen and limited costimulation (13). TGFβ is a potent inducer of Foxp3 expression *in vitro* (14) and *in vivo* (15–17) and immunosuppressive drugs, such as rapamycin (18–20), act by as yet undefined mechanisms to induce Foxp3 expression (18) or to expand preexisting Treg cells (19, 20). To clarify the determinants of the Treg cell fate choice, we set out to identify signaling events that control Foxp3 expression. We show that activation of CD4 lineage thymocytes and peripheral T cells confers competence for the *de novo* expression of Foxp3 in a pathway that is independent of TGFβ and is instead controlled by phosphatidyl inositol 3 kinase (PI3K), protein kinase B (Akt), and mammalian target of rapamycin (mTOR). The competence for Foxp3 induction is limited by TCR stimulation itself, and continued stimulation results in the loss of permissive chromatin modifications from the *Foxp3* TSS and 5′ UTR.

Results

Premature Withdrawal of TCR Signals and Inhibitors of the PI3K/mTOR Pathway Induce Foxp3 Expression in Activated CD4 T Cells. Naive CD62L^{hi}CD4^{+}CD25^{-} LN T cells were isolated by flow cytometry and labeled with CFSE. Residual Foxp3 expression was minimal as judged by intracellular staining (Fig. 1*a*, post sort) and remained unchanged after 18 h of activation with plate bound anti-TCR and anti-CD28 (Fig. 1*a*, 18h anti-TCR, anti CD28) and after another 36 h with anti-TCR (Fig. 1 *a–d*, with TCR signaling). However, Foxp3 RNA and protein were markedly up-regulated when the same cells were activated for 18 h with plate bound anti-TCR and anti-CD28 and then maintained without TCR stimulation for 36 h (Fig. 1 *a–d*, no TCR signaling). Hence, the continued availability of TCR signals appeared to control Foxp3 expression in newly activated CD4 T cells. TCR/CD28 engagement triggers multiple signaling pathways (21). To investigate which of these control Foxp3 expression, we screened small molecule inhibitors of enzymes involved in signal transduction. No increase in Foxp3 expression was seen when inhibitors of calcineurin/NFAT (cyclosporin A and FK-506), mitogen activated kinases (SB203580, PD98059), protein kinase-C (UCN-1028c, calphostin C, Myr-N-FARKGALRQ-NH2, Gö6976, Ro-32-0432, Ro-31-8220), glycogen synthase kinase-3 (SB21673), PPARδ (GW501516), and γ-secretase/Notch (L-685458; data not shown) were added to 18 h activated CD4 T cells. By contrast, the PI3K inhibitor LY294002 potently induced Foxp3 in this assay (Fig. 1 *a–c*, LY). Rapamycin, an inhibitor of the protein kinase mTOR, which lies in the same signaling pathway (25), also induced Foxp3 (Fig. 1 *a–c*, rapa). The combination of LY294002 and rapamycin induced Foxp3 in $\approx$75% of CD4 T cells (Fig. 1 *a–c*, rapa+LY) and synergized with TGFβ, resulting in >90% Foxp3 induction in the absence of exogenous cytokines (Fig. 1 *a–c*, TGFβ+rapa+LY). CFSE labeling ruled out the selective expansion of preexisting Foxp3^{+} cells (Fig. 1*a*), and cell counts showed a substantial net increase in Foxp3^{+} cell numbers (Fig. 1*c*).

De novo induction of Foxp3 by PI3K and mTOR inhibitors was formally demonstrated by using AND TCR transgenic *Rag1*$^{-/-}$ CD62L^{hi}CD4^{+}CD25^{-} LN T cells, which are devoid of preexisting Foxp3^{+} cells [Fig. 1*d* and supporting information (SI) Fig. S1*a*].

IMMUNOLOGY

Author contributions: M.M. designed research; S.S., L.B., A.H., D.F., B.S.C., E.O., and M.M. performed research; Z.A.K., D.C., and K.M.S. contributed new reagents/analytic tools; S.S., L.B., A.H., M.L., M.S., Z.A.K., A.G.F., and M.M. analyzed data; and M.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: Gene expression data have been deposited in the ArrayExpress database, www.ebi.ac.uk/ArrayExpress (accession no. E-MIMR-1241).

¶To whom correspondence should be addressed. E-mail: matthias.merkenschlager@csc.mrc.ac.uk.

This article contains supporting information online at www.pnas.org/cgi/content/full/0800928105/DCSupplemental.

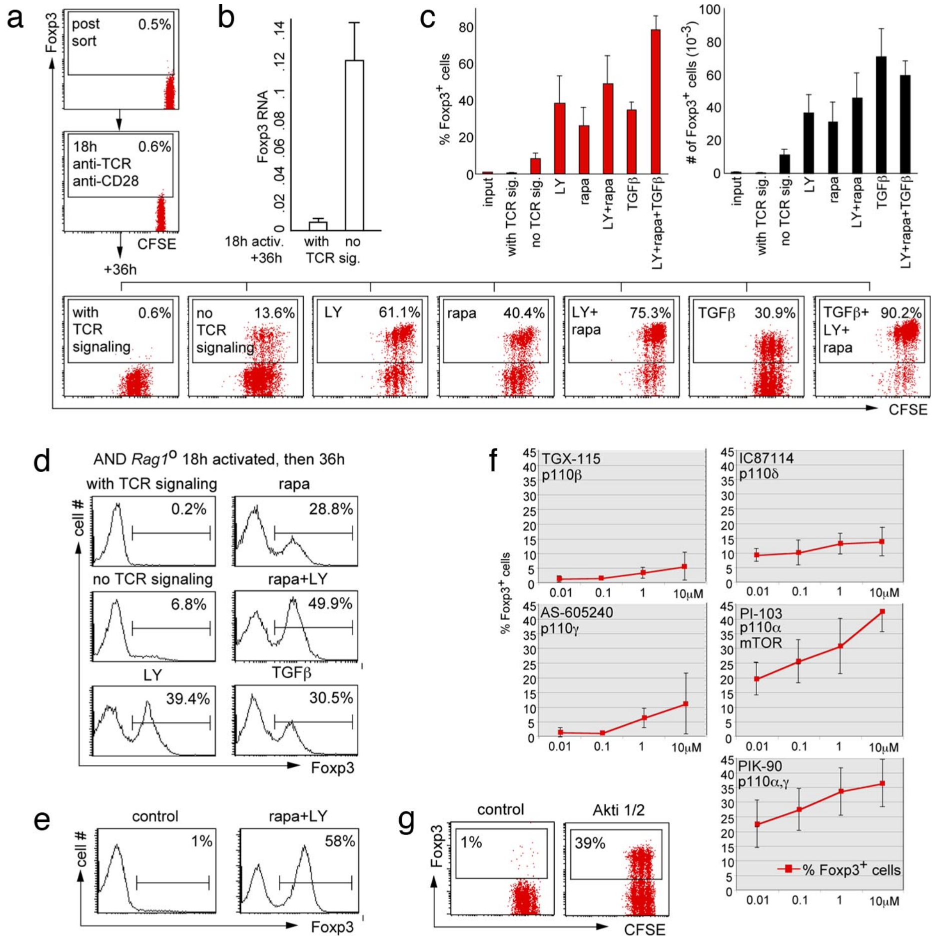


Fig. 1. TCR signal deprivation and inhibitors of the PI3K/mTOR pathway induce Foxp3 expression in newly activated CD4 T cells. (*a*) Naive CD62L^{hi}CD4^{+}CD25^{-} LN T cells were isolated by flow cytometry, labeled with CFSE, and activated for 18 h with anti-TCR and anti-CD28. After this, the cells were cultured for 36 h under various conditions: with plate-bound anti-TCR (with TCR signaling); without anti-TCR (no TCR signaling); and with the PI3K inhibitor LY294002 (LY, 10 μM), the mTOR inhibitor rapamycin (rapa, 25 nM), and/or TGFβ (1 ng/ml). Foxp3 expression was assessed by intracellular staining. (*b*) Naive CD4 T cells were activated for 18 h as in *a* and then cultured for 36 h with anti-TCR (with TCR signaling) or without anti-TCR (no TCR signaling). The expression of *Foxp3* RNA was assessed by real time RT-PCR (mean $\pm$ SD, n = 3). (*c*) Naive CD4 T cells were activated for 18 h and aliquots of 10^5 cells (input) were transferred to the conditions described in *a*. After 36 h, cells were counted, and Foxp3 expression assessed by intracellular staining. Bars show the percentage (red) and the number (black) of Foxp3^{+} cells. (*d*) AND TCR transgenic *Rag1*$^{-/-}$ CD62L^{hi}CD4^{+}CD25^{-} LN T cells were activated for 18 h as in *a* and then cultured for 36 h with anti-TCR (with TCR signaling); without anti-TCR (no TCR signaling); and with LY294002 (LY, 10 μM), rapamycin (rapa, 25 nM), or TGFβ (1 ng/ml). See Fig. S1 for percentages and numbers of Foxp3^{+} cells. (*e*) CD4^{+}CD8^{-}CD25^{-} thymocytes were activated for 18h as in *a* and then cultured for 36 h with anti-TCR (control) or with LY294002 and rapamycin without anti-TCR (LY+rapa). (*f*) Naive CD4 T cells were activated for 18 h as in *a* and cultured for 36 h with the indicated PI3K inhibitors without anti-TCR. The percentage of Foxp3^{+} cells is shown after subtracting Foxp3^{+} cells generated in cultures without anti-TCR (mean $\pm$ SD, n = 4–12). The p110 isoform specificity of each inhibitor is summarized in Table S1. (*g*) Naive CD4 T cells were activated for 18 h and cultured for 36 h with plate-bound anti-TCR (control) or with the allosteric Akt inhibitor Akti1/2 (0.3 μM) without anti-TCR.

Activation with peptide-pulsed antigen presenting cells also conferred competence for Foxp3 induction by PI3K inhibitors (Fig. S1*b*).

TCR/CD28 activation followed by PI3K/mTOR inhibition induced Foxp3 not only in peripheral CD4 T cells, but also in CD4^{+}CD8^{-}CD25^{-} thymocytes (Fig. 1*e*).

Differential Role of p110 Isoenzymes in Foxp3 Regulation. Selective PI3K isoenzyme inhibitors have recently been characterized at the biochemical, structural, and biological level (22–25) (Table S1). We utilized such compounds to define the role of specific PI3K catalytic subunits in Foxp3 regulation. TGX115 did not affect Foxp3 expression at concentrations that selectively inhibit p110β (0.1 μM; 23), indicating that p110β does not control Foxp3 in this setting. Similarly, the p110γ inhibitor AS-605240 induced Foxp3 expression only at concentrations far in excess of those required to inhibit p110γ (0.008 μM; ref. 22 and Fig. 1*f*). IC-87114 is highly selective for p110δ (23) and induced Foxp3 in 10–15% more cells than TCR deprivation alone, demonstrating a contribution of p110δ to the regulation of Foxp3. PI-103 strongly induced Foxp3 at concentrations below its *in vitro* IC_{50} for mTOR (0.02 μM) and around the *in vitro* IC_{50} for p110α (0.008 μM) (23). PIK90 strongly induced Foxp3 at 0.1 μM (Fig. 3*a*), where it inhibits p110α (and p110γ, which did not induce Foxp3; see above). Higher concentrations of PI-103 and PIK90 affect p110δ (23), but Foxp3 induction by PI-103 and PIK90 exceeded that of full p110δ inhibition by IC-87114 (Fig. 1*f*). These data identify p110α as a dominant isoenzyme and the hierarchy of p110$\alpha > \delta \ggg \beta/\gamma$ may set Foxp3 regulation apart from lymphocyte activation, where p110δ appears dominant (25, 26).

TCR Signaling and the PI3K/Akt/mTOR Network. Protein kinases of the Akt/PKB family are components of the PI3K/Akt/mTOR

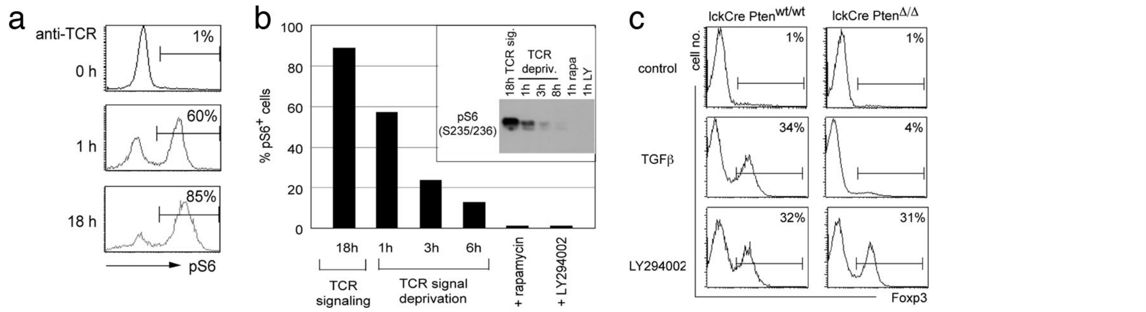


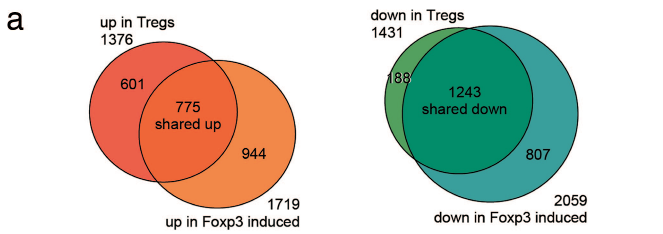
Fig. 2. TCR signaling controls the expression of Foxp3 via PI3K/mTOR/Akt. (*a*) Naive LN CD4 T cells were stimulated with anti-TCR and anti-CD28 for 1 or 18 h or for 1 h with anti CD28 alone (no TCR signaling). S6 phosphorylation was determined by intracellular staining. (*b*) Naive LN cells were activated as in *a*. After 18 h, rapamycin (25 nM) or LY294002 (10 μM) were added for 1 h, or the cells were cultured for the indicated time in the absence of anti-TCR. pS6 levels were determined as in *a*. (*Inset*) Immunoblotting confirmed declining pS6 in response to TCR signal deprivation, rapamycin, and LY294002. (*c*) (*Top*) PTEN-deficient and control CD4 T cells were depleted of preexisting Treg cells and activated with anti-TCR/CD28. (*Middle*) TGFβ (0.3 ng/ml) was added, and Foxp3 expression was assessed 48 h later. (*Bottom*) As a control, the PI3K inhibitor LY294002 was added and cells were deprived of TCR signaling after 18 h of activation.

network, and their activity is regulated by PI3K via PDK1 and by one of the two known mTOR-containing complexes, mTORC2 (27). We treated activated CD4 T cells with the allosteric Akt inhibitor Akti-1/2 (28) and found Foxp3 induction at concentrations around its IC50 for Akt1 and Akt2 (58 nM and 210 nM, respectively). This shows that inhibition of Akt and PI3K and mTOR can drive Foxp3 induction (Fig. 1*g*).

T cell activation results in the sustained activation of the PI3K/Akt/mTOR network (21), reflected in the phosphorylation of S6 ribosomal protein (pS6), a direct target of the mTOR-regulated p70 S6 kinase S6K1 (27). Intracellular staining showed that TCR/CD28 signaling (but not anti-CD28 alone) induced and maintained high levels of pS6 (Fig. 2*a*). Upon withdrawal of TCR antibody from 18 h activated T cells, S6 phosphorylation declined only gradually (Fig. 2*b*; confirmed by immunoblotting in Fig. 2*b Inset*). LY294002 and rapamycin abrogated S6 phosphorylation much more rapidly (Fig. 2*b*), correlating with their ability to enhance Foxp3 induction.

Constitutive Activation of the PI3K/Akt/mTOR Network Antagonises Foxp3 Induction. Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is the major negative regulator of the PI3K/Akt signaling pathway, and its loss results in constitutive Akt activity (29). To test the concept that PI3K/Akt/mTOR signaling controls Foxp3, we compared the inducibility of Foxp3 in TCR/CD28 activated PTEN-deficient and control T cells (29) in response to the classical Foxp3 inducer TGFβ versus PI3K inhibitors. The frequency of TGFβ-induced Foxp3 cells was considerably lower in PTEN-deficient than in control CD4 cells, but the PI3K inhibitor LY294002 restored Foxp3 induction in PTEN-deficient CD4 T cells (Fig. 2*c*). These data add genetic evidence that PI3K/Akt/mTOR signaling controls Foxp3 expression in activated T cells.

PI3K/mTOR Inhibitors Induce Treg-Like mRNA and microRNA Expression Profiles. To address whether Foxp3 was induced in isolation or as part of a Treg-like transcriptional program, we performed cDNA expression arrays 24 h after PI3K/mTOR inhibition. Comparison with control activated T cells showed that, in addition to *Foxp3*, numerous Treg cell markers were up-regulated, including *IL2ra* (3.0x), *Il2rb* (3.0x), and *Ctla4* (2.9x) and members of the suppressor of cytokine signaling (Socs) family *Socs1* (3.1x), *Socs2* (8.3x), and *Socs3* (10.5x). As expected from a Treg-like progam, the lymphokine transcripts *Il2*, *Ifng* and *IL3* were strongly down-regulated (112x, 56x, and 7.8x, respectively). Next, we compared PI3K/mTOR inhibitor-induced cells and freshly isolated Treg cells with naive CD4 T cells and found substantial coregulation: More than half of the transcripts up-regulated in Treg cells were also up-regulated in Foxp3-induced cells (775 of 1376, 56%). Even more strikingly, 87% (1,243 of 1,431) of transcripts that were down-regulated in Treg cells were also down-regulated in response to PI3K/mTOR inhibition (Fig. 3*a*). Functional annotation showed that up-regulated tran-



b

Shared upregulated genes			Shared downregulated genes		
Process	n	p	Process	n	p
cellular metabolism	329	2.1E-16	transcription	136	1.9E-07
macromolecular metabolism	222	2.9E-15	regulation of transcription	132	2.0E-07
cell division	35	3.2E-14	regul. of nucleic acid metabol.	133	1.5E-07
cell cycle	63	2.5E-12			
primary metabolism	304	6.3E-12			
Function			Function		
nucleotide binding	108	3.2E-09	zinc ion binding	132	5.9E-20
electron transporter activity	23	3.4E-05	ubiquitin ligase activity	19	3.4E-01
kinase regulator activity	13	1.0E-04			
protein binding	171	1.3E-04			
Component			Component		
mitochondrion	83	2.0E-12	nucleus	228	1.8E-18
cytoplasm	212	5.8E-10			

Fig. 3. PI3K/mTOR inhibition initiates a Treg-like transcriptional program in newly activated T cells. (*a*) Gene expression differences between Treg cells versus naive CD4 T cells and in 24 h PI3K/mTOR inhibitor-treated cells versus naive CD4 T cells. Shown are the numbers of up- and down-regulated transcripts and the intersection of expression differences between Treg and Foxp3 induced cells. (*b*) Functional annotation of coregulated transcripts in Treg cells and Foxp3 induced cells relative to naive CD4 T cells, using DAVID. (*c*) microRNA expression by Treg cells and Foxp3 induced cells compared by qPCR. miR-142-3p and miR-21 are shown as examples for Treg-like and non-Treg-like microRNA expression by Foxp3 induced cells. The expression profiles of eight other microRNAs are listed.

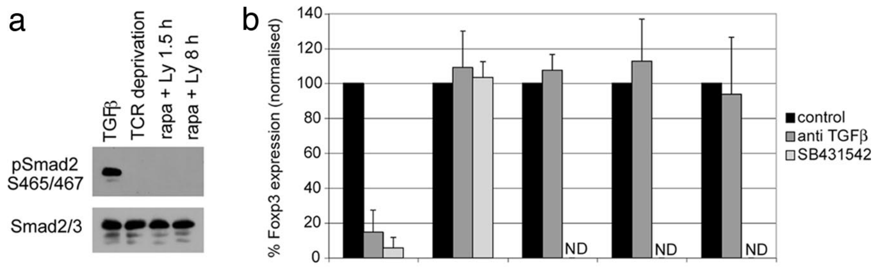


Fig. 4. No apparent TGFβ involvement in Foxp3 induction by PI3K and mTOR inhibitors. (*a*) Naive LN CD4 T cells were activated for 18 h in serum-free AIM-V medium and then exposed to TGFβ (1 ng/ml, 90 min, lane 1); TCR signal deprivation (90 min, lane 2); or TCR signal deprivation, rapamycin, and LY294002 (90 min for lane 3, 8 h for lane 4). Blots were sequentially probed with anti-pSmad2 (S465/467) and anti-Smad2/3. (*b*) Naive LN CD4 T cells activated as in *a* were deprived of TCR signals and TGFβ, and PI3K/mTOR inhibitors were added as indicated. Cultures were supplemented with neutralizing anti TGFβ (3 μg/ml) or the Smad kinase inhibitor SB431542. Foxp3 expression in the presence of anti TGFβ (dark gray bars) or SB431542 (light gray bars) was determined 2 days later and normalized to control cultures (black).

scripts were enriched for processes of cellular, macromolecular and primary metabolism, cell division, and cell cycle and for the functional terms nucleotide binding, electron transporter, and kinase regulatory activity. Down-regulated transcripts represented distinct processes, in particular transcriptional regulation. Only a minority of genes that were coregulated in *ex vivo* Treg cells and Foxp3 induced cells were known genomic targets of Foxp3 (Fig. S2). MicroRNAs are important mediators of posttranscriptional gene regulation and naive CD4 T cells and Treg cells express distinct microRNAs (31). Of the 10 microRNAs we profiled, 7 showed Treg-like expression in Foxp3-induced cells (Fig. 3*c*). Taken together, our analysis suggests that PI3K/mTOR signaling controls not only Foxp3 and its direct targets, but a wider Treg-like transcriptional progam (30).

No Detectable Involvement of TGFβ in Foxp3 Induction by PI3K and mTOR Inhibitors. Because TGFβ is a powerful inducer of Foxp3 expression (14–17) and synergizes with PI3K/mTOR inhibitors (Fig. 1), we addressed its requirement in this system. TGFβ binding induces phosphorylation of receptor-associated Smad2 and Smad3, providing a sensitive indicator of TGFβ signaling. pSmad2 (S465/467) was readily detectable in cells exposed to TGFβ (Fig. 4*a* lane 1) but not in cells subjected to TCR signal deprivation (Fig. 4*a*, lane 2) or PI3K and mTOR inhibition (Fig. 4*a*, lanes 3 and 4). Neutralizing TGFβ antibodies and the Smad kinase inhibitor SB 431542 (32) blocked Foxp3 induction by TGFβ, but did not affect Foxp3 induction by PI3K/mTOR inhibitors (Fig. 4*b*). Hence, TGFβ appears dispensable for Foxp3 induction by TCR signal deprivation and PI3K/mTOR inhibition.

Histone Modifications Mark a Window of Opportunity for Foxp3 Induction by PI3K and mTOR Inhibition. T cell activation was required for Foxp3 induction, and Foxp3 inducibility was maximal in T cells activated for 18 h before PI3K/mTor inhibition. Earlier addition of inhibitors blocked activation (ref. 19 and data not shown), and Foxp3 induction was inefficient at later time points (Fig. 5 *Top Left*). Hence, the competence for Foxp3 expression induced by activation of CD4 T cells is transient and continued TCR signaling antagonises Foxp3 inducibility.

The expression of the *Foxp3* locus is intimately linked to its chromatin structure (33, 34). Permissive posttranslational histone modifications are found in Treg cells at the *Foxp3* promoter, the intronic differentially metylated region 3 (DMR3), and the recently described +2079 to +2198 enhancer (33–35). To explore how continued TCR signaling reduces the competence of CD4 T cells to express Foxp3, we considered that chromatin marks can provide important information not only about the actual expression, but also the potential for the expression of developmentally regulated loci (36). We used ChIP (chromatin immunoprecipitation) to analyze histone modifications at the *Foxp3* locus in male (XY) cells (Foxp3 is X-linked). We compared CD4 cells activated for 18 h (high potential for Foxp3 induction, no Foxp3 expression) to the same cells after 72 h of TCR stimulation (reduced potential for Foxp3 induction, no Foxp3 expression) and CD4 cells activated for 18 h and then exposed to PI3K/mTOR inhibitors (high Foxp3 expression). O*ct4*, which is silent in T cells, and the actively transcribed *Ikzf1* (Ikaros) locus served as controls (Fig. 5). Interestingly, H3K4 di- and trimethylation was found near the *Foxp3* TSS (34) and the 5′ UTR not only in $Foxp3^+$ cells but also in 18-h activated CD4 T cells, which had the potential for Foxp3 induction but did not actually express Foxp3. In contrast, H3K4me2 and -3 were lost after 72 h of continuous TCR signaling (Fig. 5). These data

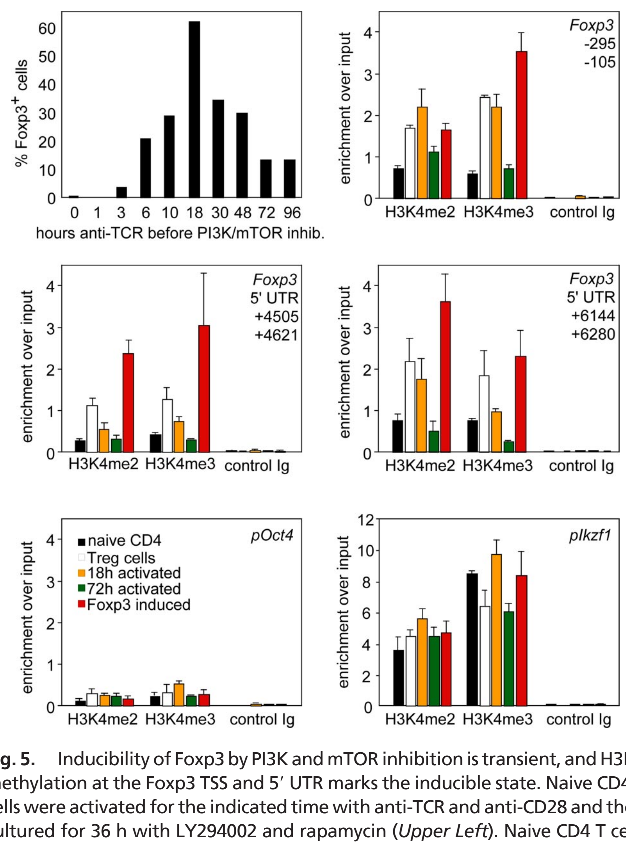


Fig. 5. Inducibility of Foxp3 by PI3K and mTOR inhibition is transient, and H3K4 methylation at the Foxp3 TSS and 5′ UTR marks the inducible state. Naive CD4 T cells were activated for the indicated time with anti-TCR and anti-CD28 and then cultured for 36 h with LY294002 and rapamycin (*Upper Left*). Naive CD4 T cells (black), Treg cells (white), naive CD4 T cells activated for 18 h (orange) or 72 h (green) or induced to express Foxp3 by PI3K/mTOR inhibitors (red) were examined for H3K4me2 and H3K4me3 by ChIP and qPCR near the Foxp3 TSS (−295 to −105) and within the 5′ UTR (+4505 to +4621 and +6144 to +6280). Primer positions are indicated (34). *pOct4* and *pIkzf1* are shown as controls.

link H3K4 methylation to the potential for Foxp3 expression. Conversely, the TCR signaling-induced loss of this permissive chromatin mark correlates with declining competence for Foxp3 expression.

Discussion

We have shown that TCR signaling via PI3K p110α, p110δ, Akt, and mTOR controls Foxp3 expression in activated CD4 lineage thymocytes and peripheral T cells. Inhibition of this signaling network conferred *de novo* expression of Foxp3 and Treg-like mRNA and miRNA profiles. Conversely, constitutive activation of the PI3K/AKT/mTOR network in PTEN-deficient T cells reduced Foxp3 inducibility, which was restored by PI3K inhibition. Foxp3 induction by TCR signal deprivation and PI3K/mTOR inhibition shares features with the development of natural Treg cells. TGFβ, although synergistic, is not required for the induction of Foxp3 expression in the thymus (8, 9) or in response to TCR signal deprivation and PI3K and mTOR inhibition (this article), and both are markedly enhanced by costimulatory signals (refs. 2 and 3 and Fig. S3). Whereas CD28 signals can interfere with adaptive Treg differentiation driven by TGFβ (e.g., 13, 17), cooperation between TGFβ and CD28 was reported for the *Foxp3* enhancer element at +2079 to +2198 (35).

Evidence for an involvement of the PI3K/Akt/mTOR network in Treg differentiation and function has been accumulating: Treg cell numbers increase in the thymi of PI3K p110δ-deficient mice (37), rapamycin can promote Treg cell differentiation in specific settings (18–20), and exciting data published while this manuscript was under review indicate that Akt signaling interferes with Foxp3 expression *in vitro* and *in vivo* (38). Our data provide a rationale for these genetic and pharmacological data by demonstrating that (*i*) TCR signaling controls Foxp3 expression via a signaling network with the key components PI3Kα and δ, Akt, and mTOR, the mammalian target of rapamycin, and (*ii*) the timing of PI3K/Akt/mTOR inhibition relative to TCR signaling is critical for the outcome. Interestingly, Akt signaling appears to be compromised in a PTEN-dependent fashion in established Treg cells (39).

Directing cell fate choice in the immune system by pharmacological means is potentially attractive, and a growing number of suitable compounds are approved for clinical use. The ability of small molecule inhibitors to block PI3K/mTOR/Akt signaling temporarily rather than permanently may be beneficial because constitutive p110δ deficiency promotes the differentiation of Treg cells in the thymus, but impairs their subsequent maintenance in the periphery (26, 37).

In our experiments, the duration of TCR signaling affects the probability of Foxp3 induction. Weak TCR signals and limited costimulation had been linked to Foxp3 induction (13, 17, 40). We find higher Foxp3 induction rates by optimal anti-TCR/CD28 signaling followed by TCR signal deprivation and PI3K/mTOR inhibition, consistent with a recent two-stage model of Treg differentiation in which TCR signals are required for the up-regulation of genes like *Cd25* that predispose to Treg differentiation, but not for the subsequent induction of Foxp3, which occurs in the absence of additional TCR signals (41). Treg development can result from the recognition of tissue-specific antigens expressed by thymic medullallary epithelium (42). Such antigens are expressed in a mosaic fashion (43) and may therefore induce transient rather than sustained TCR signals, which in turn could contribute to the outcome of Foxp3 induction versus negative selection. Our data further show that continued TCR signaling extinguishes the competence for Foxp3 induction in naive CD4 T cells. ChIP analysis identified H3K4me2 and -3 near the *Foxp3* TSS and within the 5′ UTR as a mark for the inducibility of Foxp3, because these permissive histone modifications were induced by the activation of naive CD4 T cells and persisted upon the induction of Foxp3 expression, but declined in parallel with the loss of Foxp3 inducibility in response to continued TCR signaling. The challenge now is to understand how signaling, transcription factors, and chromatin components cooperate to translate the duration of TCR signaling into a determinant of Treg cell fate choice.

Materials and Methods

Cell Sorting and Culture. Animal work was done under the Animals (Scientific Procedures) Act, U.K. Lymph node (LN) cells or thymocytes from wild-type (C57BL/6, BALB/c, or C57BL/6 × 129) AND TCR transgenic (44) *Rag1*$^{-/-}$ or lckCre Pten$^{lox/lox}$ mice (29) were sorted by flow cytometry as described in ref. 31. LN CD4^{+} CD25^{-} CD62L^{hi} T cells or CD4^{+} CD8^{-}CD25^{-} thymocytes were activated with plate bound anti-TCRβ (H57; 200 ng/ml; PharMingen) and anti-CD28 (2 μg/ml, PharMingen). AND TCR transgenic cells were also activated with B10.BR antigen presenting cells and pigeon cytochrome-C peptide 81–104 (44). Intracellular staining for Foxp3 protein was done as advised by the manufacturers (eBiosciences). Anti-pS6 Ser-235/236 (Cell Signaling; catalog no. 2211) was used with the eBioscience Foxp3 staining kit and anti rabbit IgG-FITC or IgG-Cy5 (Jackson ImmunoResearch).

RT-PCR, Northern Blot Analysis, and Immunoblotting. Total RNA was isolated by using RNA-Bee (Tel-Test) and reverse transcribed. Real-time PCR analysis was normalized to the geometric mean of *Ywhaz* and *Ube2L3* as described in ref. 31. The following primer sequences were used: *Ywhaz*, CGTTGTAGGAGCCCG-TAGGTCAT (forward), TCTGGTTGCGAAGCATTGGG (reverse); *Ube2L3*, AG-GAGGCTGATGAAGGAGCTTGA (forward); TGGTTTGAATGGATACTCTGCT-GGA (reverse); and Foxp3, ACTCGCATGTTCGCCTACTTCAG (forward); GGCGGATGGCATTCTTCCAGGT (reverse). Immunoblotting was performed as described in ref. 31. Quantitative real-time RT-PCR of miRNAs used gene specific RT primers and TaqMan MicroRNA Assay Mix (Applied Biosystems) as described in ref. 31.

Chromatin Immunoprecipitation. ChIP was done as described in ref. 45, using 20 μg of chromatin mixed with 80 μg of *Drosophila* S2 chromatin with 2.5 μl of anti-H3K4me2 (Upstate; catalog no. 07-030), anti-H3K4me3 (Abcam; catalog no. ab8580-100), or rabbit anti mouse IgG (Dako Cytomation; catalog no. Z0259). The following primer sequences (5′ to 3′): *Foxp3* −295 to −105 (TSS; numbering according to ref. 34) CATTGATACCTTTTACCTCTGTGGTG (forward), GTGTGTGCTGATAATTGCAGGGT (reverse); Foxp3, 5′ UTR +4505 to +4621 (DMR3) GTTGTGACAACAGGGCCCAG (forward), CACTGTCTGTT-GGGGCGTTC (reverse); *Foxp3*, 5′ UTR +6144 to +6280 (*exon-1*) CAACTTCTC-CTGACTCTGCCTTCA (forward), GGAACTGTGCTAGTGGGAAGTGTACT (reverse); *pOct4* GTGAGCCGTCTTTCCACCAGG (forward), GGGTGAGAAGGC-GAAGTCTGAA (reverse); and *pIkzf1* CCAGTTTCAGGGACTCGGCT (forward), TCGGGGAACACGGGACAC (reverse).

Gene Expression Arrays and Bioinformatics Analysis. Affymetrix mouse genome 430 2.0 arrays were used in duplicate. Analysis was done in Bioconductor, using robust multi array normalization (www.bioconductor.org). Individual *P* values were corrected according to Benjamini and Hochberg (46). Only transcripts with both an adjusted *P* value <0.05 and a fold-change of >1.5 were considered differentially expressed. Functional annotation used DAVID (http://david.abcc.ncifcrf.gov).

ACKNOWLEDGMENTS. We thank Eugene Ng for cell sorting and Drs. James Elliott, Rose Zamoyska, Chris Rudd, and Patrick Maxwell for discussions and the CSC Microarray Facility for array hybridizations. This work was supported by the Medical Research Council (U.K.).

1. Fisher AG (2002) Cellular identity and lineage choice. *Nat Rev Immunol* 2:977–982.
2. Fontenot JD, Rudensky AY (2005) A well adapted regulatory contrivance: Regulatory T cell development and the forkhead family transcription factor Foxp3. *Nat Immunol* 6:331–337.
3. Sakaguchi S (2005) Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* 6:345–352.
4. Marie JC, Letterio JJ, Gavin M, Rudensky AY (2005) TGF-β1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells. *J Exp Med* 201:1061–1067.
5. Brunkow ME, *et al.* (2001) Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet* 27:68–73.
6. Hori S, Nomura T, Sakaguchi S (2003) Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299:1057–1061.
7. Fontenot JD, Gavin MA, Rudensky AY (2003) FoxP3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 3:330–336.
8. Fontenot JD, *et al.* (2005) Regulatory T cell lineage specification by the forkhead transcription factor FoxP3. *Immunity* 22:329–341.

9. Khattri R, Cox T, Yasayko SA, Ramsdell F (2003) An essential role for scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol* 3:337–342.
10. Tran DQ, Ramsey H, Shevach EM (2007) Induction of FOXP3 expression in naive human CD4+FOXP3 T cells by T-cell receptor stimulation is TGFβ dependent but does not confer a regulatory phenotype. *Blood* 110:2983–2990.
11. Hsieh CS, Zheng Y, Liang Y, Fontenot JD, Rudensky AY (2006) An intersection between the self-reactive regulatory and nonregulatory T cell receptor repertoires. *Nat Immunol* 7:401–410.
12. Pacholczyk R, *et al.* (2007) Nonself-antigens are the cognate specificities of Foxp3+ regulatory T cells. *Immunity* 27:493–504.
13. Kretschmer K, *et al.* (2005) Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol* 6:1219–1227.
14. Chen W, *et al.* (2003) Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-β induction of transcription factor Foxp3. *J Exp Med* 198:1875–1886.
15. Coombes JL, *et al.* (2007) A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGFβ and retinoic acid dependent mechanism. *J Exp Med* 204:1757–1764.
16. Sun CM, *et al.* (2007) Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med* 204:1775–1785.
17. Benson MJ, Pino-Lagos K, Rosemblatt M, Noelle RJ (2007) All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. *J Exp Med* 204:1765–1774.
18. Gao W, *et al.* (2007) Contrasting effects of cyclosporine and rapamycin in de novo generation of alloantigen-specific regulatory T cells. *Am J Transplant* 7:1722–1732.
19. Battaglia M, Stabilini A, Roncarolo MG (2005) Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood* 105:4743–4748.
20. Mantel PY, *et al.* (2006) Molecular mechanisms underlying FOXP3 induction in human T cells. *J Immunol* 176:3593–3602.
21. Kane LP, Weiss A (2003) The PI-3 kinase/Akt pathway and T cell activation: Pleiotropic pathways downstream of PIP3. *Immunol Rev* 192:7–20.
22. Camps M, *et al.* (2005) Blockade of PI3Kgamma suppresses joint inflammation and damage in mouse models of rheumatoid arthritis. *Nat Med* 11:936–943.
23. Knight ZA, *et al.* (2006) A pharmacological map of the PI3K family defines a role for p110alpha in insulin signaling. *Cell* 125:733–747.
24. Foukas LC, *et al.* (2006) Critical role for the p110alpha phosphoinositide-3-OH kinase in growth and metabolic regulation. *Nature* 441:366–370.
25. Bilancio A, *et al.* (2006) Key role of the p110delta isoform of PI3K in B-cell antigen and IL-4 receptor signaling: Comparative analysis of genetic and pharmacologic interference with p110delta function in B cells. *Blood* 107:642–650.
26. Okkenhaug K, *et al.* (2002) Impaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice. *Science* 297:1031–1034.
27. Sabatini DM (2006) mTOR and cancer: Insights into a complex relationship. *Nat Rev Cancer* 6:729–734.
28. Barnett SF, *et al.* (2005) Identification and characterization of pleckstrin-homology-domain-dependent and isoenzyme-specific Akt inhibitors. *Biochem J* 385:399–408.
29. Hagenbeek TJ, *et al.* (2004) The loss of PTEN allows TCR alphabeta lineage thymocytes to bypass IL-7 and Pre-TCR-mediated signaling. *J Exp Med* 200:883–894.
30. Hill JA, *et al.* (2007) Foxp3 transcription-factor-dependent and -independent regulation of the regulatory T cell transcriptional signature. *Immunity* 27:786–800.
31. Cobb BS, *et al.* (2006) A role for Dicer in immune regulation. *J Exp Med* 203:2519–2527.
32. Inman GJ, *et al.* (2002) SB-431542 is a potent and specific inhibitor of TGF-β superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Mol Pharmacol* 62:65–74.
33. Floess S, *et al.* (2007). Epigenetic control of the Foxp3 locus in regulatory T cells. *PLoS Biol.* e38.
34. Kim HP, Leonard WJ (2007) CREB/ATF-dependent T cell receptor-induced FoxP3 gene expression: A role for DNA methylation. *J Exp Med* 204:1543–1551.
35. Tone Y, *et al.* (2008) Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. *Nat Immunol* 9:194–202.
36. Spivakov M, Fisher AG (2007) Epigenetic signatures of stem-cell identity. *Nat Rev Genet* 8:263–271.
37. Patton DT, *et al.* (2006) The phosphoinositide 3-kinase p110delta is critical for the function of CD4+CD25+Foxp3+ regulatory T cells. *J Immunol* 177:6598–6602.
38. Haxhinasto S, Mathis D, Benoist C (2008). The AKT-mTOR axis regulates de novo differentiation of CD4+Foxp3+ cells. *J Exp Med* 205:565–574.
39. Walsh PT, *et al.* (2006) PTEN inhibits IL-2 receptor-mediated expansion of CD4+ CD25+ Tregs. *J Clin Invest* 116:2521–2531.
40. So T, Croft M (2007) Cutting edge: OX40 inhibits TGF-β- and antigen-driven conversion of naive CD4 T cells into CD25+Foxp3+ T cells. *J Immunol* 179:1427–1430.
41. Lio CW, Hsieh CS (2008) A two-step process for thymic regulatory T cell development. *Immunity* 28:100–111.
42. Aschenbrenner K, *et al.* (2007). Selection of Foxp3+ regulatory T cells specific for self antigen expressed and presented by Aire+ medullary thymic epithelial cells. *Nat Immunol* 8:351 358.
43. Anderson MS, *et al.* (2005) The cellular mechanism of Aire control of T cell tolerance. *Immunity* 23:227–239.
44. Kaye J, *et al.* (1989) Selective development of CD4+ T cells in transgenic mice expressing a class II MHC-restricted antigen receptor. *Nature* 341:746–749.
45. Baxter J, *et al.* (2004) Histone hypomethylation is an indicator of epigenetic plasticity in quiescent lymphocytes. *EMBO J* 23:4462–4472.
46. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J Roy Stat Soc B* 57:289–300.