

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

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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 inhibition of phosphatidylinositol 3-kinase (PI3K) p110 α , p110 δ , 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 antagonized 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 TGF β 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). TGF β 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 TGF β and is instead controlled by phosphatidylinositol 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, G66976, Ro-32-0432, Ro-31-8220), glycogen synthase kinase-3 (SB21673), PPAR δ (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 TGF β , resulting in $>$ 90% Foxp3 induction in the absence of exogenous cytokines (Fig. 1*a–c*, TGF β +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*].

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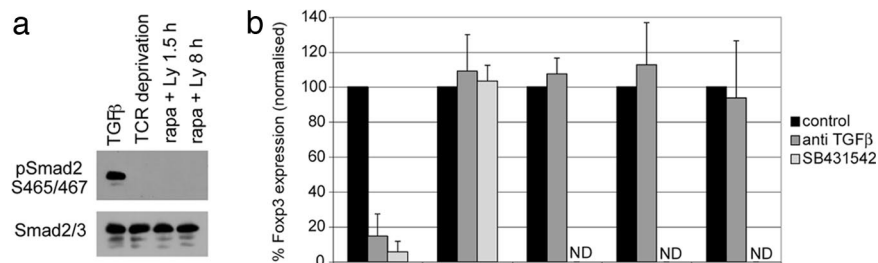


Fig. 4. No apparent TGFβ involvement in Foxp3 induction by PI3K and mTOR inhibitors. (a) Naive LN CD4 T cells were activated for 18 h in serum-free AIM-V medium and then exposed to TGFβ (1 ng/ml, 90 min, lane 1); TCR signal deprivation (90 min, lane 2); or TCR signal deprivation, rapamycin, and LY294002 (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. 3c). Taken together, our analysis suggests that PI3K/mTOR signaling controls not only Foxp3 and its direct targets, but a wider Treg-like transcriptional program (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. 4a lane 1) but not in cells subjected to TCR signal deprivation (Fig. 4a, lane 2) or PI3K and mTOR inhibition (Fig. 4a, 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. 4b). 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 methylated 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). *Oct4*, 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

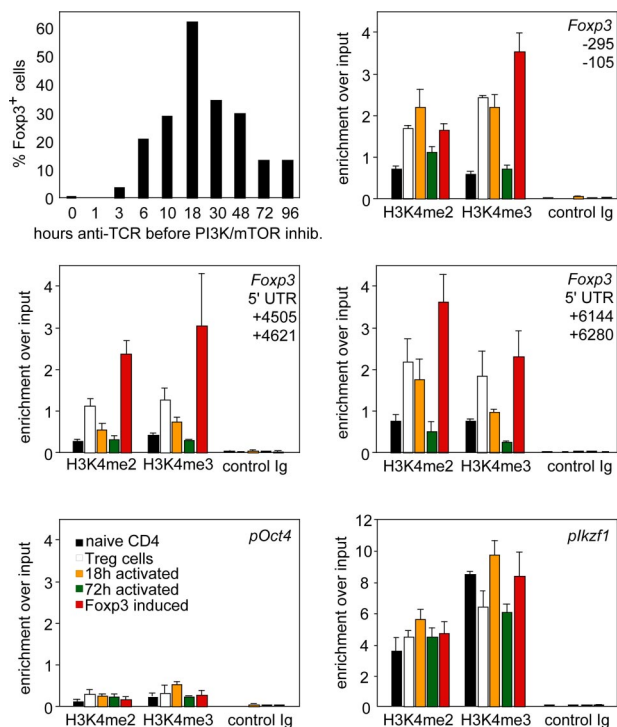


Fig. 5. Inducibility of Foxp3 by PI3K and mTOR inhibition is transient, and H3K4 methylation at the Foxp3 TSS and 5' UTR marks the inducible state. Naive CD4 T cells were activated for the indicated time with anti-TCR and anti-CD28 and then cultured for 36 h with LY294002 and rapamycin (Upper Left). 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 *plkzf1* are shown as controls.

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