Itk-mediated integration of T cell receptor and cytokine signaling regulates the balance between Th17 and regulatory T cells

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A proper balance between Th17 and T regulatory cells (T_{reg} cells) is critical for generating protective immune responses while minimizing autoimmunity. We show that the Tec family kinase Itk (IL2-inducible T cell kinase), a component of T cell receptor (TCR) signaling pathways, influences this balance by regulating cross talk between TCR and cytokine signaling. Under both Th17 and T_{reg} cell differentiation conditions, *Itk*$^{-/-}$ CD4$^+$ T cells develop higher percentages of functional FoxP3$^+$ cells, associated with increased sensitivity to IL-2. *Itk*$^{-/-}$ CD4$^+$ T cells also preferentially develop into T_{reg} cells in vivo. We find that Itk-deficient T cells exhibit reduced TCR-induced phosphorylation of mammalian target of rapamycin (mTOR) targets, accompanied by downstream metabolic alterations. Surprisingly, *Itk*$^{-/-}$ cells also exhibit reduced IL-2–induced mTOR activation, despite increased STAT5 phosphorylation. We demonstrate that in wild-type CD4$^+$ T cells, TCR stimulation leads to a dose-dependent repression of *Pten*. However, at low TCR stimulation or in the absence of Itk, *Pten* is not effectively repressed, thereby uncoupling STAT5 phosphorylation and phosphoinositide-3-kinase (PI3K) pathways. Moreover, Itk-deficient CD4$^+$ T cells show impaired TCR-mediated induction of *Myc* and *miR-19b*, known repressors of *Pten*. Our results demonstrate that Itk helps orchestrate positive feedback loops integrating multiple T cell signaling pathways, suggesting Itk as a potential target for altering the balance between Th17 and T_{reg} cells.

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Abbreviations used: Erk, extracellular signal-regulated kinase; Itk, IL2-inducible T cell kinase; iT_{reg} cell, induced T_{reg} cell; MLN, mesenteric LN; mTOR, mammalian target of rapamycin; PH, pleckstrin homology; PI3K, phosphoinositide-3-kinase; pT_{reg} cell, peripherally derived T_{reg} cell; qRT-PCR, quantitative RT-PCR.

One of the main functions of the adaptive immune system is to mount specific responses to pathogens while minimizing self-reactivity. To help orchestrate these responses, naive CD4$^+$ T cells differentiate into distinct types of effector T helper cells upon engagement of their TCR and co-stimulatory molecules in the context of cytokines and signals produced by innate immune cells (Zhu and Paul, 2010). Among effector CD4$^+$ T helper cell populations, Th17 cells play important roles in inflammatory responses against bacteria and fungi. Th17 cells are generated through the actions of IL-6 and TGF-β1, leading to the activation of STAT3 and expression of the transcription factors RORγt, and RORα. The activity of CD4$^+$ effector T cell populations is required for the eradication of infectious pathogens; however, excessive activation of Th17 responses can be harmful to the host, leading to immunopathology and autoimmunity.

The extent of the host's immune activation is controlled in large part by regulatory T cells (T_{reg} cells), another subset of T helper cells, which are essential for immune tolerance and prevention of autoimmunity (von Boehmer and Daniel, 2013). T_{reg} cells are characterized by the expression of the transcription factor Forkhead box P3 (FoxP3) and are subdivided in two major categories: thymic-derived T_{reg} cells (tT_{reg} cells) and peripherally derived and induced T_{reg} cells (pT_{reg} cells and iT_{reg} cells), which arise from naive CD4$^+$ T cells in response to signals in the periphery or culture and acquire FoxP3 expression

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and T_{reg} cell function (Chaudhry and Rudensky, 2013). Interestingly, the development of Th17 and iT_{reg} cells is closely related and reciprocally regulated; both share a requirement for the cytokine TGF-β1. In contrast, although IL-2 promotes expression of FoxP3 via activation of STAT5, IL-2 and STAT5 activation inhibit Th17 cell differentiation (Laurence et al., 2007). As these cell populations play opposing roles influencing the outcome of inflammatory and autoimmune diseases, understanding the balance between Th17 and T_{reg} cells and the factors that regulate them is of great importance, particularly for therapeutic approaches to autoimmunity (Barbi et al., 2013).

Over the last few years, several studies have focused on the molecular mechanisms regulating the induction of FoxP3, the master regulator of T_{reg} cells (Josefowicz et al., 2012). Consistent with the requirements for TGF-β1 and IL-2 in the generation of iT_{reg} cells from naive mouse $CD4^+$ T cells, activation of IL-2–STAT5 and TGF-β1–SMAD pathways are important for iT_{reg} cell differentiation. However, a growing body of data has revealed that other pathways contribute to the regulation of FoxP3 expression, including those downstream of the TCR. Notably, phosphoinositide-3-kinase (PI3K) and the downstream mammalian target of rapamycin (mTOR) and Akt pathways have been shown to play an instrumental role in regulating T_{reg} cell differentiation. mTOR is part of an evolutionary conserved pathway involved in regulation of cell growth, translation, migration, and metabolism (Powell et al., 2012). Inhibition of PI3K/Akt and mTOR pathways, including targeted deletion of mTOR, leads to FoxP3 expression upon TCR stimulation of CD4 cells (Battaglia et al., 2005; Kopf et al., 2007; Haxhinasto et al., 2008; Kang et al., 2008; Sauer et al., 2008; Delgoffe et al., 2009; Powell et al., 2012). Moreover, the hypoxia-inducible transcription factor α (HIF1α), a downstream target of mTOR pathways which contributes to the regulation of glucose metabolism, also helps regulate the balance between Th17 and T_{reg} cell differentiation (Dang et al., 2011; Shi et al., 2011). In the absence of HIF1α, $CD4^+$ T cells fail to up-regulate glycolytic pathways important for effector cell differentiation and instead develop into $FoxP3^+$ T_{reg} cells. Such data highlight the importance of mTOR and downstream metabolic pathways in cell fate decisions. Nonetheless, although much knowledge has been gained about these pathways, many questions remain regarding how the development and activity of T_{reg} cells and Th17 cells are controlled to permit protective immunity without pathological self-reactivity.

IL2-inducible T cell kinase (Itk) belongs to the Tec family of tyrosine kinases and is an important component of TCR-mediated signaling (Berg et al., 2005). In contrast to other more proximal molecules, loss of Itk does not prevent TCR signaling. Instead, the absence of Itk leads to impaired TCR signaling associated with decreased activation of PLC-γ and the downstream pathways involved in Ca^{2+} mobilization, Ras and extracellular signal-regulated kinase (Erk) cascades, and regulation of the actin cytoskeleton. Accordingly, mutation of *Itk* has been shown to both impair and alter T cell functional outcomes (Berg et al., 2005; Gomez-Rodriguez et al., 2011). We have previously shown that Itk is a positive modulator of IL17A production, with reduced percentages of IL17A-producing cells in Itk-deficient $CD4^+$ T cells generated under Th17 conditions (Gomez-Rodriguez et al., 2009). How Itk affects T_{reg} cell generation and its effects on the metabolic control of differentiation have not been explored.

Here, we have analyzed the influence of Itk on Th17 and T_{reg} cell differentiation. Surprisingly, we found that $Itk^{-/-}$ CD4 cells stimulated under Th17 conditions gave rise to a population of FoxP3-expressing cells. Itk-deficient $CD4^+$ also gave rise to higher percentages of FoxP3-expressing cells when differentiated under iT_{reg} cell conditions, even under conditions of limiting IL-2. Consistent with their TCR signaling defects, $Itk^{-/-}$ $CD4^+$ T cells exhibited reduced TCR-induced phosphorylation of mTOR downstream targets, including ribosomal S6 and Akt, accompanied by changes in metabolic signatures affected by mTOR, including reduced expression of *Hif1α*. Surprisingly, despite increased IL-2 responsiveness, including increased STAT5 phosphorylation, $Itk^{-/-}$ $CD4^+$ T cells exhibited decreased IL-2–induced phosphorylation of the mTOR target S6. We associate these phenotypes, in part, with defective repression of the gene encoding phosphatase and tensin homologue deleted on chromosome 10 (*Pten*), demonstrating that strong TCR stimulation leads to a dose-dependent repression of *Pten*. However, in $Itk^{-/-}$ $CD4^+$ T cells, repression of *Pten* is defective, thereby uncoupling IL-2–mediated activation of PI3K–mTOR pathways from STAT phosphorylation. We further show that Itk-deficient cells show decreased expression of *Myc* and its downstream target *miR-19b*, a known repressor of *Pten*, suggesting a potential mechanism by which Pten expression is altered by Itk deficiency. Importantly, we also observe increased conversion of naive $Itk^{-/-}$ CD4 cells to $FoxP3^+$ T cells in vivo and show that Itk-deficient $FoxP3^+$ cells function as bonafide T_{reg} cells both in vivo and in vitro. Our results suggest that Itk helps integrate signaling pathways that regulate the balance of Th17 and T_{reg} cell differentiation, providing insight into the contribution of TCR signaling to iT_{reg} cell development and suggesting Itk as a potential target to alter the balance between Th17 and T_{reg} cells.

RESULTS

Itk-deficient cells exhibit increased FoxP3 induction

We have previously shown that Itk is a positive regulator of IL17A production and that naive $CD4^+$ T cells from Itk-deficient cells express less IL17A than WT $CD4^+$ T cells under Th17 conditions (Gomez-Rodriguez et al., 2009). To further understand the defect in IL17A expression, we examined the expression of a variety of transcription factors in WT and $Itk^{-/-}$ cells differentiated under Th17 conditions. Surprisingly, one of the differentially expressed genes was *FoxP3*; sorted naive ($CD4^+CD44^{low}CD62L^{high}CD25^-$) $CD4^+$ T cells from Itk-deficient mice stimulated under Th17-polarizing conditions expressed significantly less *Il17a* and more *FoxP3* mRNA compared with WT cells (Fig. 1 A). Intracellular staining revealed that high percentages of FoxP3-expressing cells were generated from naive Itk-deficient $CD4^+$ T cells stimulated under Th17-polarizing conditions (18 ± 1.5%)

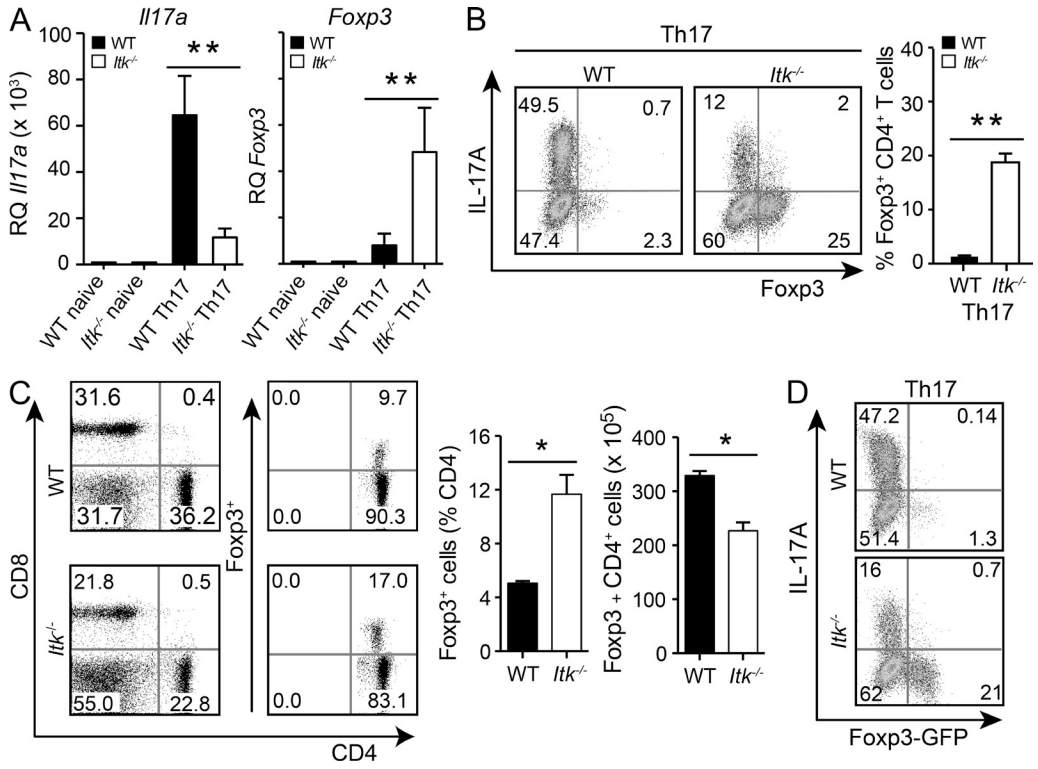

Figure 1. Itk-deficient cells express FoxP3 under Th17 cell differentiation conditions. (A and B) Sorted naive CD4 T cells were differentiated under Th17 conditions (1 µg/ml anti-CD3, 3 µg/ml anti-CD28, 20 ng/ml IL6, and 5 ng TGF-β1 plus APCs) for 2 d. (A) *Il17a* and *FoxP3* mRNA was determined by qRT-PCR. Mean ± SEM from five different experiments is shown. **, $P < 0.0001$. RQ, relative quantification. (B) Alternatively, cells were restimulated with PMA and ionomycin, and IL17A and FoxP3 were analyzed by intracellular staining. (right) Mean FoxP3+ cells from >10 experiments ± SEM. Similar results were observed after 86 h of culture. (C) FoxP3 expression in CD4+ cells in splenocytes from WT and *Itk*$^{-/-}$ mice. (right) Mean percentages and absolute numbers of FoxP3+CD4+ cells from six mice in two experiments ± SEM. *, $P < 0.05$. (D) Sorted naive GFP−CD4+ T cells from WT and *Itk*$^{-/-}$ FoxP3^{GFP} reporter mice differentiated as in A. Data are representative of more than five experiments.

compared with WT cells (1 ± 0.3%; Fig. 1 B). This observation did not appear to be secondary to a relative lack of expansion of effector cells, as the *Itk*$^{-/-}$ CD4+ T cells exhibited only a mild impairment in cell expansion under these conditions (Gomez-Rodriguez et al., 2009).

Although Itk-deficient mice have slightly reduced numbers of FoxP3+CD4+ T cells compared with WT mice, the percentage of CD4+ T cells that express FoxP3 is higher because of the overall low numbers of CD4+ T cells in these mice (Fig. 1 C). To rule out the possibility that the increase in FoxP3+ cells in culture was the result of an enrichment of FoxP3 producers that might remain even after sorting naive *Itk*$^{-/-}$ CD25− CD4+ T cells, we crossed Itk-deficient mice with FoxP3^{GFP} mice, which express GFP regulated by the FoxP3 control elements (Bettelli et al., 2006). Again, we obtained high percentages of FoxP3^{GFP+}-expressing cells from sorted naive CD4+CD25−FoxP3^{GFP-} *Itk*$^{-/-}$ T cells cultured under Th17 conditions (21 versus 1.3% in WT cells; Fig. 1 D and Fig. S1), supporting the conclusion that the increased percentages of FoxP3+ cells obtained after differentiation were not derived from FoxP3+ cells present before culturing. Moreover, stimulation of CD4+ T cells from Itk-deficient 5CC7 TCR transgenic mice on a *Rag2*$^{-/-}$ background, which should lack FoxP3+ T_{reg} cells, also showed increased production of FoxP3-producing cells under Th17 polarizing conditions, arguing that these findings were not the result of altered development (not depicted). Thus, the increased FoxP3 expression in *Itk*$^{-/-}$ CD4+ T cells under Th17 conditions appeared to result from an intrinsic alteration in differentiation.

Itk-deficient CD4+ T cells differentiate more efficiently into T_{reg} cells

To determine whether the increased differentiation into FoxP3+-expressing cells was a more global property of *Itk*$^{-/-}$ CD4+ T cells, we evaluated the differentiation of naive cells under T_{reg} cell conditions. When naive CD4+ cells were stimulated with standard concentrations of anti-CD3 (1 µg/ml) in the presence of WT APCs plus IL-2 and TGF-β1, a significantly higher percentage of Itk-deficient cells became FoxP3+ than cells from WT mice (65.4 ± 2.9% vs. 40.1 ± 1.6% in WT cells; Fig. 2 A). Accordingly, naive *Itk*$^{-/-}$ CD4+ T cells differentiated under these iT_{reg} cell–inducing conditions exhibited higher amounts of *FoxP3* mRNA than WT iT_{reg} cells (Fig. 2 B). Similar observations were obtained when naive CD4+ T cells from 5CC7 transgenic mice were differentiated in the presence of T_{reg} cell–inducing cytokines (Fig. 2 C). Naive

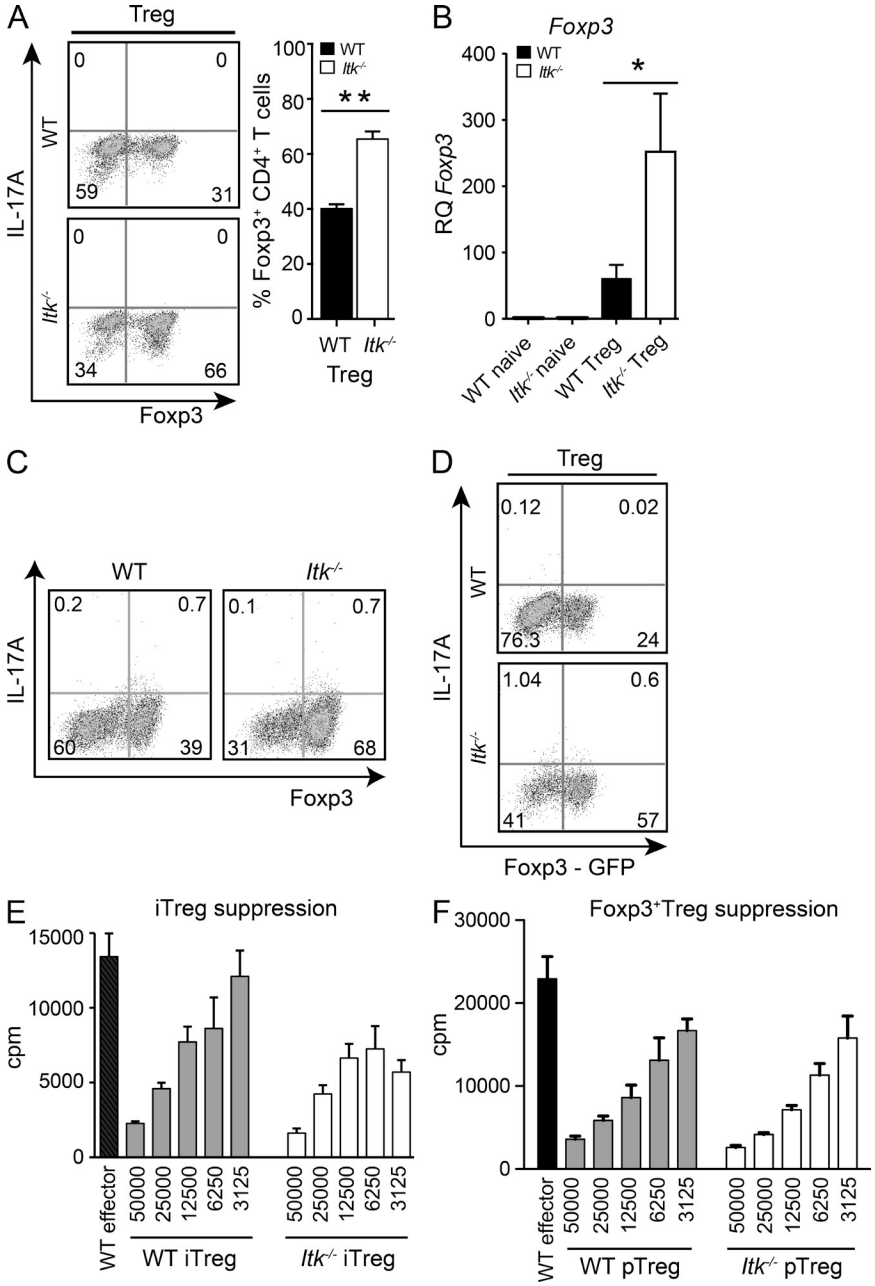

Figure 2. Itk-deficient cells express more FoxP3 expression under iT_{reg} cell conditions. (A) Sorted naive CD4+ T cells from WT and *Itk*$^{-/-}$ mice were differentiated under iT_{reg} cell conditions with APCs (using 1 µg/ml anti-CD3) for 2 d and then restimulated with PMA and ionomycin, and IL17A and FoxP3 were analyzed by intracellular staining. (right) Mean ± SEM of FoxP3+ cells quantitated from more than five experiments. **, $P < 0.0001$. (B) *FoxP3* mRNA from iT_{reg} cell differentiation cultures determined by qRT-PCR; data are mean ± SEM of five separate experiments. *, $P < 0.05$. RQ, relative quantification. (C) Sorted naive CD4+ T cells from WT and *Itk*$^{-/-}$ 5CC7 transgenic mice were differentiated under T_{reg} cell conditions in the presence of PCC peptide. Data are representative of two independent experiments. (D) Sorted naive CD4 T cells from WT and *Itk*$^{-/-}$ FoxP3^{GFP} reporter mice were differentiated under T_{reg} cell conditions as in A. (E and F) The suppression capabilities of differentiated iT_{reg} cells (E) or pT_{reg} cells (F) from WT and *Itk*$^{-/-}$ mice were evaluated by co-culturing WT CD4+CD25− effector cells (50,000) with the indicated number of sorted CD4+FoxP3^{GFP+} cells (iT_{reg} or pT_{reg} cells) in the presence of 0.5 µg/ml anti-CD3 plus APCs (50,000). After 72 h, cultures were pulsed with [^{3}H]thymidine. Data are the mean ± SEM from triplicate wells and are representative of three experiments.

GFP−CD4+ T cells sorted from *Itk*$^{-/-}$ FoxP3^{GFP} mice also gave rise to higher percentages of FoxP3+ iT_{reg} cells compared with WT mice (Fig. 2 D), arguing that these observations were not the result of outgrowth of FoxP3+ cells present before culturing. Thus, naive CD4+ T cells deficient in Itk give rise to increased FoxP3+ cells in vitro.

To evaluate whether *Itk*$^{-/-}$ iT_{reg} cells were functional, increasing numbers of sorted differentiated CD4+FoxP3+ cells (iT_{reg} cells) from FoxP3^{GFP} mice were co-cultured with naive WT CD4+CD25− effector T cells in the presence of anti-CD3 and APCs, and cell proliferation was evaluated (Fig. 2 E). In vitro differentiated *Itk*$^{-/-}$ iT_{reg} cells were fully capable in suppressing proliferation of CD4+ T responder cells; notably, at low ratios, *Itk*$^{-/-}$ iT_{reg} cells suppressed even better than WT. Evaluation of sorted CD4+CD25+ FoxP3^{GFP+} T_{reg} (pT_{reg}) cells from WT FoxP3^{GFP} and *Itk*$^{-/-}$ FoxP3^{GFP} mice co-cultured with freshly isolated WT CD4+CD25− responder cells and anti-CD3 also demonstrated that WT and *Itk*$^{-/-}$ pT_{reg} cells were equally capable of suppressing proliferation of responder T cells (Fig. 2 F). Thus, *Itk*$^{-/-}$ T_{reg} cells were functional, suggesting that the suppressive capability of both pT_{reg} and iT_{reg} cells is independent of Itk.

Itk$^{-/-}$ CD4+ T cells give rise to increased FoxP3 expression across a range of TCR doses

Itk is an important contributor to signaling downstream from the TCR through its roles in activating PLC-γ– and actin-mediated pathways (Berg et al., 2005). To evaluate whether altered TCR signaling contributes to the increased induction of FoxP3

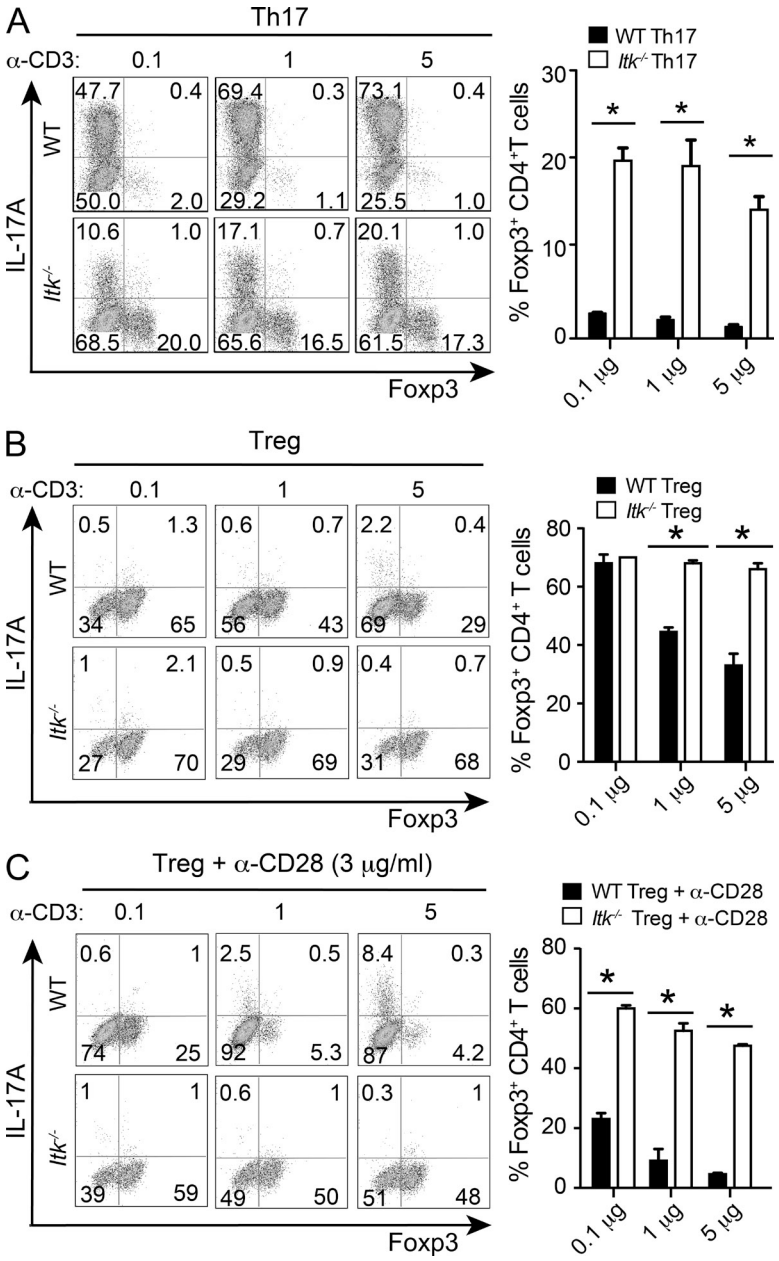

Figure 3. *Itk*$^{-/-}$ CD4$^+$ T cells give rise to increased FoxP3 expression across TCR doses. (A–C) Sorted naive CD4$^+$ T cells were differentiated under Th17 (A), T_{reg} (B), and T_{reg} + anti-CD28 (C) conditions, with 0.1, 1, and 5 μg/ml of anti-CD3 alone (T_{reg}) or with anti-CD28 (Th17 and T_{reg} + anti-CD28), and then restimulated with PMA and ionomycin, and IL17A and FoxP3 were analyzed by intracellular staining. (right) Mean ± SEM of five experiments. *, $P < 0.005$.

expression in Itk-deficient cells, we stimulated cells across a range of anti-CD3 concentrations. Although WT CD4$^+$ T cells do not express much FoxP3 under Th17 conditions, we consistently observed a small increase in the percentage of FoxP3-positive cells as cells were stimulated with decreasing amounts of anti-CD3 (increasing from 0.8 ± 0.2% to 2.1 ± 0.1%, $P < 0.005$; Fig. 3 A). In contrast, Itk-deficient CD4$^+$ T cells differentiated in the presence of Th17 cytokines showed high percentages of FoxP3-expressing cells at all concentrations of CD3 stimulation.

Previous data have suggested low or interrupted TCR stimulation favors FoxP3 expression both in vivo and in vitro (Kretschmer et al., 2005; Kim and Rudensky, 2006; Sauer et al., 2008; Turner et al., 2009; Gottschalk et al., 2010). Under iT_{reg} cell differentiation conditions, we also observed an increase in the generation of WT FoxP3$^+$ cells at lower concentrations of anti-CD3 (increasing from 29.7 ± 2.9% to 65.3 ± 3.1%, $P < 0.005$; Fig. 3 B). Indeed, under conditions of low TCR stimulation (0.1 μg/ml anti-CD3), similar percentages of FoxP3 producers could be generated from both WT and *Itk*$^{-/-}$ cells. However, *Itk*$^{-/-}$ cells developed high percentages of FoxP3$^+$ cells at all concentrations of anti-CD3 tested (Fig. 3 B).

Co-stimulation with anti-CD28 can potentiate signaling pathways downstream of the TCR (Boomer and Green, 2010). Accordingly, TCR stimulation in the presence of anti-CD28 further decreased the percentage of FoxP3-expressing cells seen with WT cells stimulated under T_{reg} cell conditions (Fig. 3, compare B with C; Kim and Rudensky, 2006; Benson et al., 2007). Although the requirement for Itk in CD28 signaling has

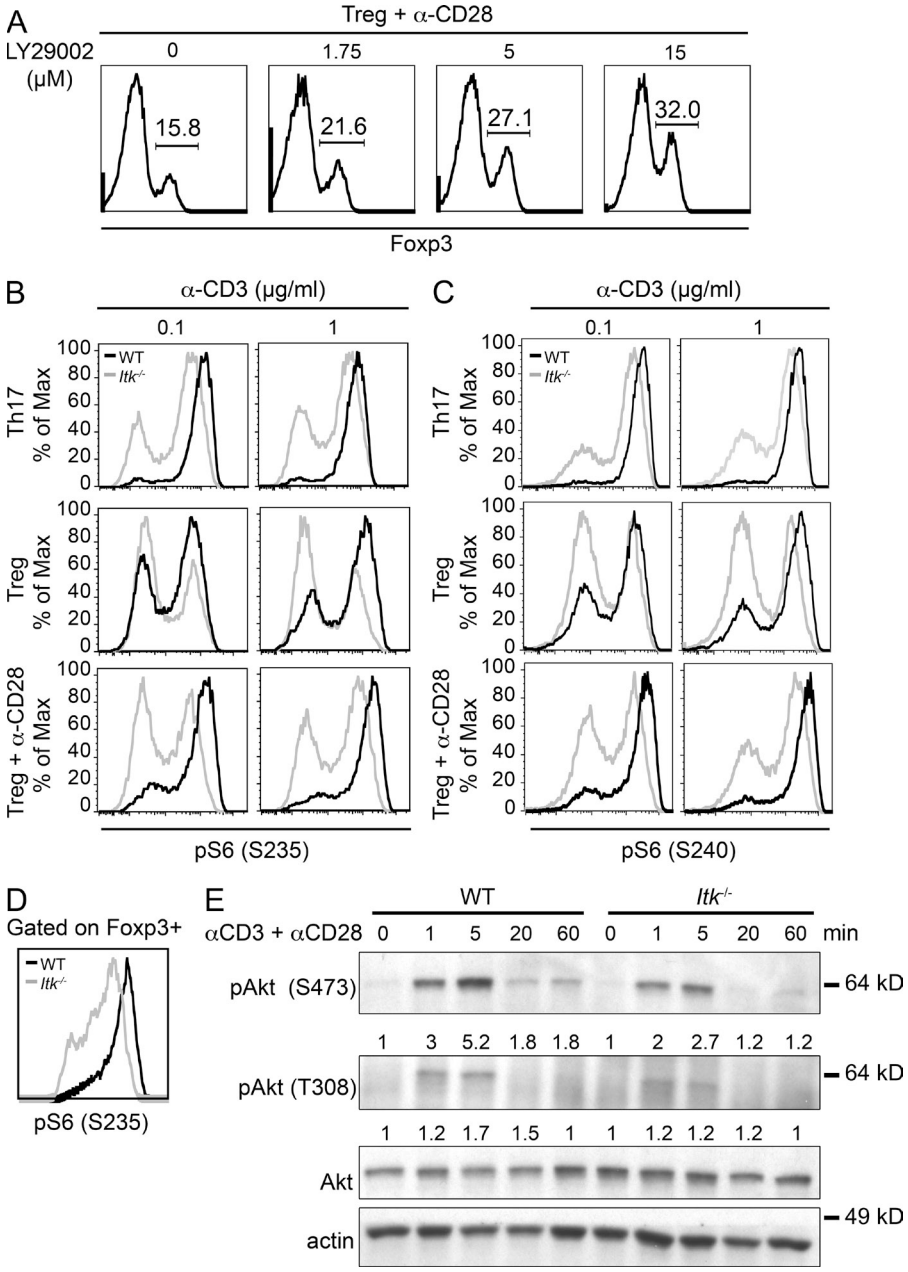

Figure 4. Defective activation of the mTOR–Akt pathway in *Itk*$^{-/-}$ CD4$^+$ T cells. (A) Sorted naive WT CD4$^+$ T cells were differentiated under T_{reg} cell (1 μg/ml anti-CD3) conditions plus anti-CD28 and varying concentrations of the PI3K inhibitor Ly294002 and evaluated for FoxP3. (B and C) Sorted naive CD4$^+$ T cells were differentiated under Th17, T_{reg}, and T_{reg} + anti-CD28 conditions with different concentrations of anti-CD3, in the presence of APCs for 24 h, and evaluated for intracellular levels of pS6 (S235) (B) or pS6 (S240) (C). (D) Sorted naive CD4$^+$ T cells were differentiated under T_{reg} cell (0.1 μg/ml anti-CD3) conditions, and levels of pS235 S6 in FoxP3$^+$ cells were determined. (E) Naive WT and *Itk*$^{-/-}$ CD4$^+$ T cells were stimulated with anti-CD3 and anti-CD28 for the indicated times, and samples were immunoblotted for Akt pSer473, pThr308, total Akt, and actin. The intensities relative to time 0 are indicated. Data are representative of three independent experiments.

been controversial (Michel et al., 2001; Li and Berg, 2005), we found only a small reduction in the percentage of Itk-deficient FoxP3-expressing cells generated with anti-CD28 compared with anti-CD3 alone; this was most notable under conditions of highest TCR stimulation. Similar results were observed at the level of mRNA expression (not depicted). Thus, strong TCR and CD28 co-stimulation negatively influenced the induction of FoxP3 expression in WT CD4$^+$ T cells, but *Itk*$^{-/-}$ cells were relatively resistant to these effects.

Reduced PI3K–Akt–mTOR signaling in *Itk*$^{-/-}$ CD4$^+$ T cells

TCR plus CD28 co-stimulation engagement stimulates a variety of downstream signaling molecules and transcription factors; one prominent pathway is the activation of PI3K, which has been implicated upstream of both Akt and mTOR pathways. Intriguingly, both Akt and mTOR have been shown to restrain the generation of iT_{reg} cells (Powell et al., 2012). To evaluate whether PI3K activation contributes to the negative effects of TCR/CD28 engagement on FoxP3 expression in WT cells, we evaluated the effects of PI3K inhibition on cells stimulated with anti-CD3 and anti-CD28. The presence of the PI3K inhibitor LY2940002 in T_{reg} cell cultures enhanced the production of FoxP3 by WT CD4$^+$ T cells in the presence of anti-CD3 plus anti-CD28, increasing the expression from 16.4 to 32.2% (Fig. 4 A); higher concentrations of the PI3K inhibitor were toxic for the cells (not depicted). Thus, both CD28 and its downstream effector, PI3K, exert detrimental effects on FoxP3 expression.

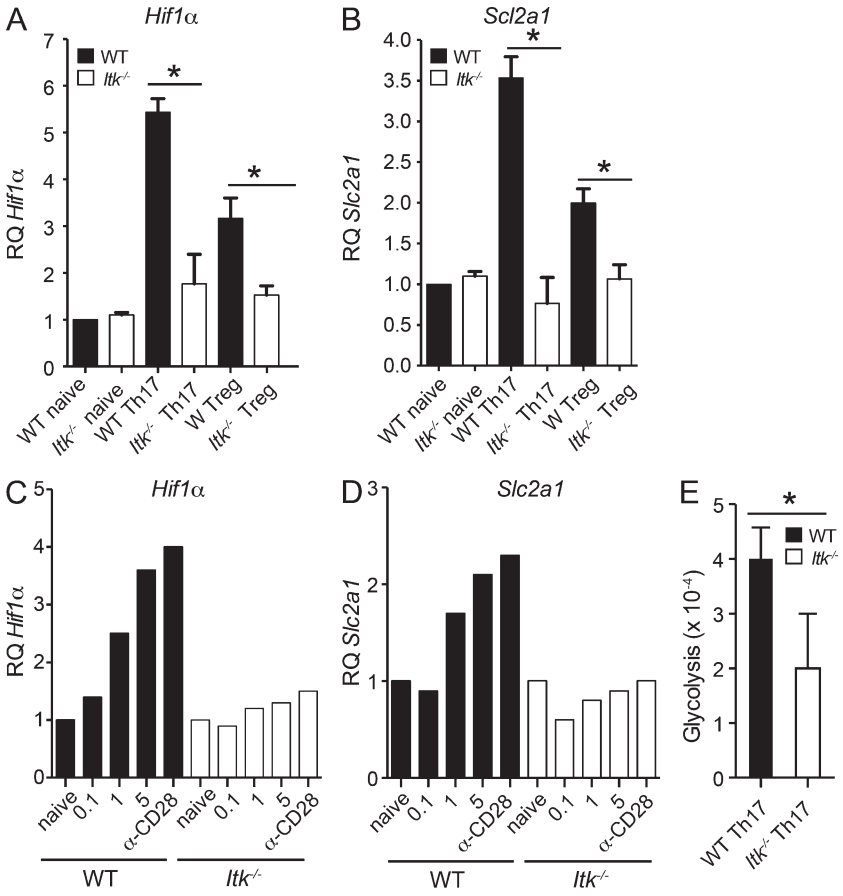

Figure 5. *Itk*$^{-/-}$ CD4$^+$ T cells express decreased downstream metabolic effectors of mTOR. (A and B) Naive and Th17- and T_{reg} cell–differentiated WT and *Itk*$^{-/-}$ CD4$^+$ T cells were examined for expression of *Hif1α* (A) and *Slc2a1* (encoding Glut1; B) by qRT-PCR after 48 h. Data represent mean ± SEM from greater than five experiments. *, P < 0.05. (C and D) WT and *Itk*$^{-/-}$ naive CD4$^+$ cells were differentiated for 48 h under T_{reg} cell conditions using the indicated amounts of anti-CD3 ± anti-CD28, and expression of *Hif1α* (C) and *Slc2a1* (D) was evaluated by qRT-PCR. Data are representative of three independent experiments. (E) Glycolysis of cells differentiated for 2 d under Th17 conditions was analyzed by Seahorse Bioscience. Mean ± SEM from triplicate samples. *, P < 0.05. RQ, relative quantification.

Given the TCR signaling defects in Itk-deficient T cells, we investigated whether mTOR and Akt activation were altered in these cells. mTOR exists in two complexes, mTORC1 and mTORC2 (Powell et al., 2012). In mTORC1, mTOR is complexed with Raptor, resulting in activation of translation and phosphorylation of the downstream targets involved in cell growth, translation, migration, and metabolism. As a downstream readout of mTOR1 complex activation, we evaluated the intracellular levels of phosphorylated S6 ribosomal protein (pS6) by flow cytometry. In WT cells, a clear population of pS6 (S235)$^+$ cells could be observed, which was decreased under low TCR conditions, as well as under T_{reg} cell conditions (Fig. 4 B). Consistent with defects in TCR signaling, we observed a reduction in pS6 (S235) levels in *Itk*$^{-/-}$ cells differentiated under both Th17 and T_{reg} cell conditions; defects were observed across a range of anti-CD3 concentrations at all times examined between 6 and 48 h (Fig. 4 B and not depicted). Reduced pS6 (S235) was also observed in *Itk*$^{-/-}$ FoxP3$^+$ cells relative to WT FoxP3$^+$ cells, suggesting that the reduced pS6 was not secondary to altered cell populations in *Itk*$^{-/-}$ cell cultures (Fig. 4 D). Because S235 can be a target of both mTORC1 and ribosomal S6 kinase, a downstream effector of Erk, we also evaluated phosphorylation of pS6 (S240), which is a more specific target for mTORC1 in T cells, phosphorylation of which is completely abolished by Rapamycin treatment (not depicted; Salmond et al., 2009). Itk-deficient cells also showed reduced phosphorylation of pS6 (S240) (Fig. 4 C), again providing evidence for defective activation of mTORC1.

In mTORC2, mTOR is complexed with Rictor and activates Akt by phosphorylating Akt S473 in CD4$^+$ T cells (Powell et al., 2012). We also observed reduced Akt phosphorylation on both S473 and T308 in stimulated Itk-deficient compared with WT CD4$^+$ T cells, although these defects were less pronounced (Fig. 4 E). Together, these data indicate that Itk is required for full TCR-induced activation of mTOR and Akt pathways in CD4$^+$ T cells and suggest that alterations in these pathways may contribute to the increased expression of FoxP3 in Itk-deficient cells.

Itk-deficient CD4 cells show altered metabolic profiles

mTOR has been shown to play a major role in the regulation of metabolism and growth control, including activation of genes regulating glycolysis (Chi, 2012). Intriguingly, T cell activation leads to changes in metabolic profiles, including the induction of glycolytic pathways in effector CD4$^+$ T cells (van der Windt and Pearce, 2012); although effector CD4 cells activate glycolytic pathways, T_{reg} cells exhibit depressed glycolysis and reduced expression of glucose transporters and glycolytic enzymes compared with effector CD4$^+$ T cell populations (Michalek et al., 2011; Xu et al., 2012; MacIver et al., 2013). Notably, CD4$^+$ T cells deficient in HIF1α, an mTOR-induced

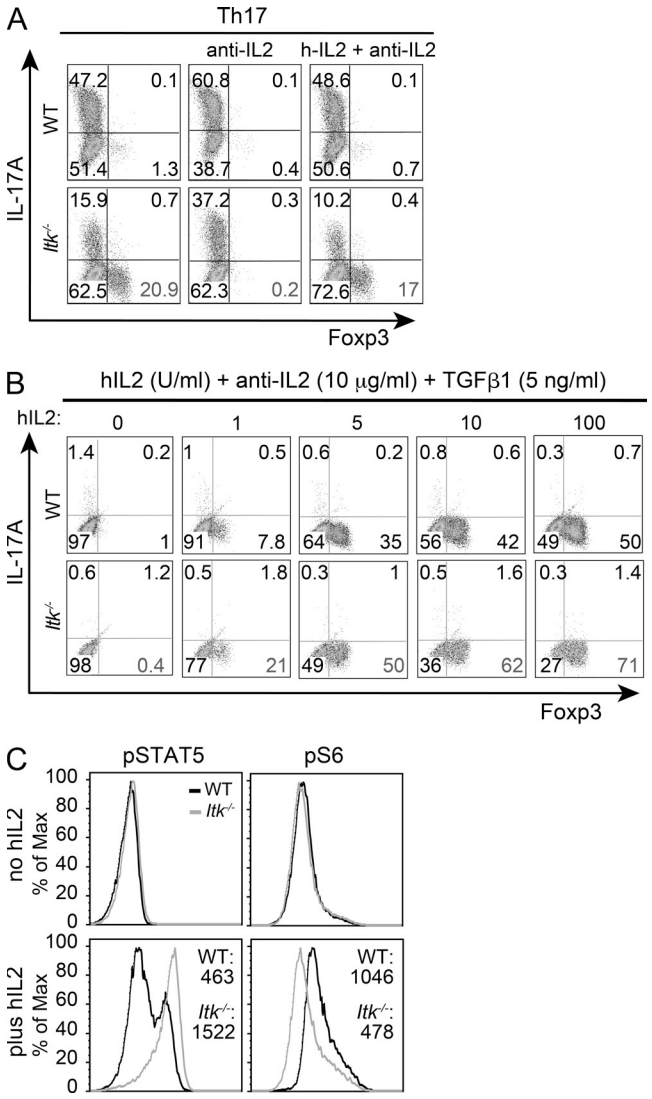

Figure 6. *Itk*$^{-/-}$ CD4$^+$ T cells have altered responses to IL2. (A) Sorted naive CD4$^+$ T cells from WT and *Itk*$^{-/-}$ mice were differentiated for 2 d under Th17 conditions in the presence of APCs in the absence or presence of blocking anti-IL2 or anti-IL2 plus hIL2, cells were restimulated with PMA and ionomycin, and IL17A and FoxP3 were analyzed by intracellular staining. (B) Sorted naive CD4$^+$ T cells in the presence of APCs were differentiated for 2 d under T_{reg} cell conditions with anti-IL2 antibody in the presence of different concentrations of hIL2 (U/ml) and 5 ng/ml TGF-β1. (C) Naive CD4$^+$ T cells from WT and *Itk*$^{-/-}$ mice were activated for 48 h under Th-null conditions, and then the cells were starved and restimulated with 100 U/ml hIL2. Phospho-STAT5 (pSTAT5) and pS6 were evaluated by intracellular staining. Mean fluorescent intensities are indicated. Data are representative of at least three experiments.

transcription factor that helps induce the expression of genes encoding glycolytic enzymes, preferentially differentiate into T_{reg} cells rather than Th17 cells (Dang et al., 2011; Shi et al., 2011), similar to our findings in Itk-deficient cells.

Consistent with defects in the induction of mTOR pathways, we found that Itk-deficient CD4$^+$ T cells exhibited reduced mRNA for *Hif1α* and the glucose transporter 1 (*Slc2a1*), two key regulators of glycolytic metabolism. This decrease was observed under both Th17 and T_{reg} cell conditions (Fig. 5, A and B). Moreover, consistent with a role for TCR signaling in this regulation, we found that titrating down the level of TCR stimulation in WT cells also led to decreased expression of *Hif1α* and *Slc2a1* (Fig. 5, C and D). However, *Itk*$^{-/-}$ cells showed reduced *Hif1α* and *Scl2a1* mRNA levels across a wide range of TCR stimulation. Thus, Itk-deficient cells failed to induce these metabolic regulators in response to TCR signals.

To further evaluate the functional consequences of these changes, we analyzed the glycolytic activity of WT and Itk-deficient cells by measuring the extracellular acidification rate, an indicator of glycolysis. Consistent with the observed changes in mTOR pathways and expression of downstream metabolic effectors, *Itk*$^{-/-}$ cells exhibited reduced glycolytic rates compared with WT cells (Fig. 5 E and not depicted). Thus, Itk-deficient T cells exhibit altered metabolic profiles upon activation.

Itk$^{-/-}$ cells exhibit increased responses to IL-2

Although these data indicate an important role for TCR signaling in regulating cellular metabolism and the differentiation of T_{reg} cells, cytokines, particularly IL-2, are also important contributors to T_{reg} cell differentiation. IL-2 plays critical roles in the development, maintenance, survival, expansion, and suppressive activity of both FoxP3$^+$ pT_{reg} and iT_{reg} cells (Boyman and Sprent, 2012). Furthermore, although IL-2 and the downstream activation of STAT5 are required to induce the expression of FoxP3 by T_{reg} cells, IL-2 can interfere with the differentiation of Th17 cells through the activation of STAT5 (Boyman and Sprent, 2012). However, IL-2 also activates PI3K-mediated pathways and thus would be predicted to increase T cell activation and metabolism.

To further dissect the altered differentiation of Itk-deficient CD4$^+$ T cells, we examined the influence of IL-2 on the generation of FoxP3 producers in *Itk*$^{-/-}$ cells under Th17 cell differentiation conditions. Addition of neutralizing IL-2 antibodies to the Th17 cultures enhanced IL17A production from WT cells as previously reported (Laurence et al., 2007). However, this increase was more apparent in the absence of Itk, with 2.4–6× increases in the percentages of IL17A$^+$ cells in *Itk*$^{-/-}$ CD4$^+$ T cells differentiated in the presence of blocking IL-2 antibodies (Fig. 6 A and not depicted). Notably, FoxP3-expressing cells were virtually abolished in *Itk*$^{-/-}$ cell cultures in the presence of anti-IL2. Thus, the aberrant expression of FoxP3 in Itk-deficient cells required IL-2. Conversely, addition of exogenous human IL-2 (hIL-2) reduced the production of IL17A in both WT and *Itk*$^{-/-}$ Th17 cultures (Fig. 6 A). However, in the *Itk*$^{-/-}$ cultures the percentages of FoxP3 producers also dramatically increased.

To further evaluate the responses of *Itk*$^{-/-}$ cells to IL-2, naive CD4$^+$ T cells were differentiated under T_{reg} cell conditions with different concentrations of hIL2 in the presence of anti–mouse IL-2 antibodies to eliminate the contribution of autocrine IL-2 production. Increased percentages of FoxP3 producer cells were observed at all concentrations of IL-2 in *Itk*$^{-/-}$ cultures, even under limiting IL-2 conditions (Fig. 6 B).

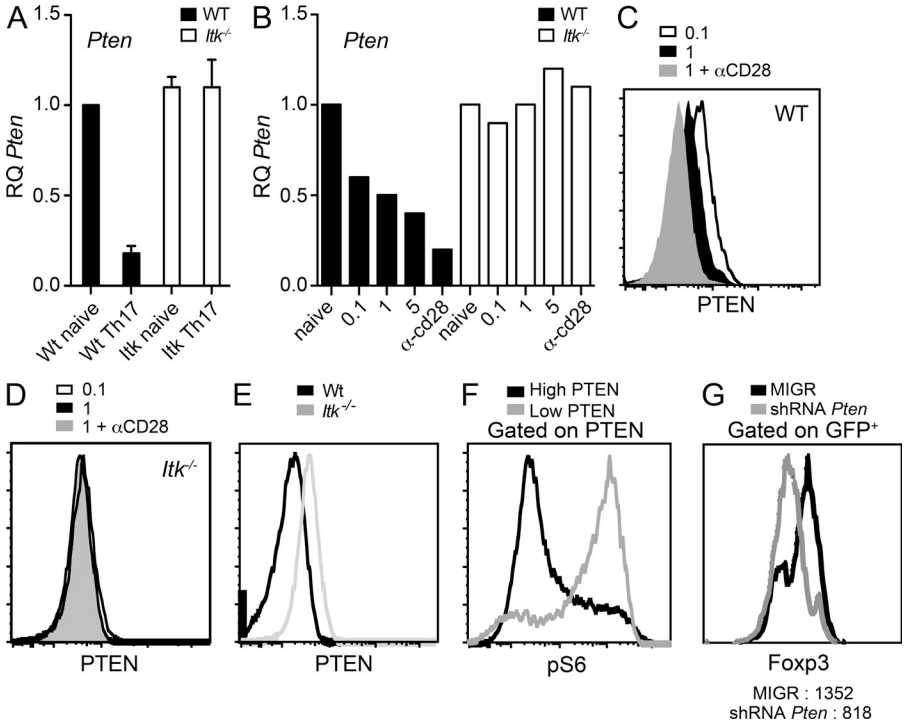

Figure 7. TCR engagement down-regulates *Pten*. (A) Sorted naive CD4+ T cells from WT and *Itk*$^{-/-}$ mice were differentiated under Th17 conditions for 2 d, and *Pten* mRNA was evaluated by qRT-PCR (data represent the mean ± SEM of three different experiments). (B) Sorted naive CD4+ T cells were differentiated under T_{reg} cell conditions with different concentrations of anti-CD3 or 1 µg/ml anti-CD3 + anti-CD28, and *Pten* mRNA was evaluated by qRT-PCR. Data are representative of three experiments. RQ, relative quantification. (C–F) Cells were differentiated as in B, and Pten was determined by intracellular staining. 0.1 µg/ml anti-CD3, 1 µg/ml anti-CD3, and 1 µg/ml anti-CD3 + anti-CD28 are shown. (C and D) WT (C) and *Itk*$^{-/-}$ (D) are shown. Data are representative of two independent experiments. (E) Comparison of Pten in CD4+ T cells from WT and *Itk*$^{-/-}$ differentiated with 1 µg/ml anti-CD3 + anti-CD28. (F) WT CD4 T cells were differentiated with 0.1 µg/ml anti-CD3 or 1 µg/ml anti-CD3 + anti-CD28, gated for high or low Pten, and levels of pS6 (S240) were determined. (G) Sorted naive Itk-deficient CD4 T cells were stimulated for 48 h and then infected with a retrovirus containing shRNA3 against *Pten*. 1 d later, cells were differentiated under T_{reg} + anti-CD28 conditions. Cells were stained for FoxP3 expression: GFP+-gated cells are shown. MIGR and shRNA Pten are shown. Mean fluorescent intensities are indicated. Data are representative of two independent repeats.

Nonetheless, CD25 levels were lower on *Itk*$^{-/-}$ activated CD4+ T cells than on WT cells (not depicted). Itk-deficient CD4 cells also secreted less IL-2 24 h after stimulation, although comparable levels were expressed and secreted 48 h after stimulation (not depicted). Thus, the increased generation of iT_{reg} cells in the absence of Itk was associated with increased responsiveness to IL-2.

Altered IL-2 signaling in the absence of Itk

Previous studies have demonstrated that strong TCR signaling can impair IL-2–induced phosphorylation of STAT5 (Lee et al., 1999; Yamane and Paul, 2013), an important transcription factor for FoxP3 induction, although the mechanism for these observations is not fully understood. Given the defective TCR signaling in *Itk*$^{-/-}$ T cells, we examined the effects of Itk deficiency on activation of IL-2–induced signaling. To up-regulate CD25, naive CD4+ T cells were differentiated under neutral conditions without TGF-β, conditions under which we did not observe increased development of FoxP3+ iT_{reg} cells, and then washed and treated with hIL-2. Consistent with the increased FoxP3 induction in *Itk*$^{-/-}$ cells exposed to IL-2, Itk-deficient cells showed increased IL-2–induced pSTAT5 compared with WT cells (Fig. 6 C, left).

However, IL-2 activates multiple intracellular pathways in addition to STAT5-mediated signaling, including mitogen-activated protein kinase (MAPK) and PI3K–Akt–mTOR pathways (Boyman and Sprent, 2012; Liao et al., 2013). Surprisingly, despite their increased phosphorylation of STAT5, Itk-deficient cells showed less IL-2–induced phosphorylation of S6, suggesting reduced mTOR activation compared with WT cells (Fig. 6 C). Similar results were observed when cells were initially stimulated under iT_{reg} cell conditions with low concentrations of anti-CD3, so that WT and Itk-deficient cells had equivalent percentages of FoxP3+ cells (not depicted). These results suggest that Itk deficiency did not simply increase IL-2 responses, but rather altered signaling in response to IL-2, such that STAT5 activation was uncoupled from mTOR activation.

Elevated *Pten* expression in the absence of Itk

The reduction in S6 phosphorylation in response to both TCR and IL-2 in Itk-deficient cells suggested that Itk deficiency more globally prevented effective activation of PI3K- and mTOR-mediated pathways. One of the major molecules that antagonizes pathways downstream of PI3K is the lipid phosphatase, Pten, which removes the D3 phosphate from $PI_{(3,4,5)}P_3$, the major product of PI3K (Song et al., 2012). Intriguingly, previous work has demonstrated that TCR signaling can down-regulate Pten (Bensinger et al., 2004). Furthermore, higher levels of Pten were observed in T_{reg} cells

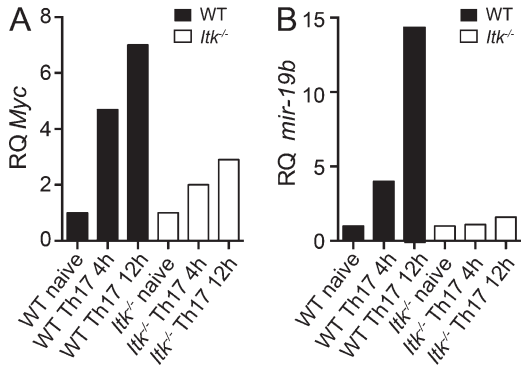

Figure 8. Itk-deficient cells have impaired induction of *Myc* and *miR-19b*. (A and B) Sorted naive CD4+ T cells from WT and *Itk*$^{-/-}$ mice were differentiated under Th17 conditions, and *Myc* mRNA (A) and *mir-19b* (B) were evaluated by qRT-PCR. Data shown are representative of three different experiments. RQ, relative quantification.

compared with conventional T cells, leading to altered IL2 signaling (Bensinger et al., 2004). To determine whether alterations in Pten may contribute to the phenotypes of Itk-deficient cells, we evaluated *Pten* message after CD4$^+$ T cell differentiation. Under Th17 conditions, we observed a marked reduction in *Pten* mRNA in WT cells: *Pten* mRNA decreased ~10-fold compared with naive WT cells (Fig. 7 A). However, strikingly, in *Itk*$^{-/-}$ cells *Pten* mRNA did not decrease upon differentiation. Similar results were observed under T_{reg} cell–inducing conditions (Fig. 7 B). To further examine whether this was related to TCR signaling, we examined *Pten* mRNA in cells stimulated across a range of anti-CD3 doses. Stimulation of WT cells with increasing amounts of anti-CD3 led to a dose-dependent decrease in *Pten* mRNA (Fig. 7 B); the presence of anti-CD28 plus anti-CD3 in WT iT_{reg} cell cultures led to an even more profound reduction in *Pten* mRNA. Similar results were observed at the level of Pten protein (Fig. 7 C). Notably, expression of *Hif1α*, a downstream readout of mTOR, was reciprocally related to the expression of *Pten* and increased under these same conditions (Fig. 5 C). However, neither *Pten* mRNA nor protein changed significantly in Itk-deficient cells at any concentration of anti-CD3 (Fig. 7, B, D, and E). Even under conditions of low anti-CD3 stimulation, in which Itk and WT cells showed similar frequencies of FoxP3$^+$ cells, Itk-deficient cells expressed more *Pten*. Thus, TCR- and Itk-mediated pathways play an important role in controlling the expression of *Pten*, a major regulator of the PI3K–mTOR axis.

To further examine the effects of Pten on downstream readouts, we examined mTOR activation in stimulated WT cells that expressed different levels of Pten. Pten protein levels were examined by flow cytometry, and marker gates were used to examine cells that expressed either the highest or lowest levels of Pten. Notably, cells expressing higher levels of Pten protein showed decreased pS6 (Fig. 7 F). To evaluate whether higher *Pten* levels in Itk-deficient cells contributed to the increase in FoxP3 expression, we treated Itk-deficient cells with *Pten*-specific shRNA. Notably, Itk-deficient cells transduced with retroviral vectors expressing shRNA for *Pten* showed reduced FoxP3 expression as compared with cells transduced with a control retrovirus (Fig. 7 G). Thus, altered *Pten* repression in Itk-deficient cells appears to contribute to their altered differentiation.

Itk-deficient cells have impaired induction of *Myc* and *miR-19b*

To understand potential mechanisms for altered Pten repression in Itk-deficient cells, we considered known repressors of *Pten* expression. Among these, Myc has been shown to lead to *Pten* repression through the induction of miR19b (Olive et al., 2009). Interestingly, Myc is also important for the induction of genes important for glycolysis. Recent data have demonstrated that strong TCR signals are required for the efficient induction of Myc (Guy et al., 2013). Similarly, we find that *Myc* mRNA induction was markedly impaired in Itk-deficient CD4 cells at early time points and this correlated with decreased induction of *miR-19b* (Fig. 8, A and B). Thus, Itk is required for transduction of signals leading to expression of *Myc* and *miR-19b*, two known repressors of *Pten*, upon TCR engagement.

Itk-deficient CD4 T cells show increased conversion into iT_{reg} cells that can function in vivo

Finally, to determine whether these observations were relevant in vivo, we used an adoptive transfer colitis model in which sorted naive CD4$^+$CD45RB^{high}CD25$^-$ T cells were transferred into C57BL/6 *Rag1*$^{-/-}$ CD45.1 congenic mice (Powrie et al., 1994). In this model, colitis can be partially controlled by conversion of naive CD4$^+$ T cells into iT_{reg} cells, which can be followed by evaluating T cell populations in the mesenteric LNs (MLNs), spleen, and colon over several weeks. Two experimental groups were examined: one group received sorted naive CD4$^+$CD45RB^{high}CD25$^-$ T cells from WT mice, and a second group received sorted naive CD4$^+$CD45RB^{high}CD25$^-$ T cells from *Itk*$^{-/-}$ mice. Similar to our in vitro results, naive *Itk*$^{-/-}$ cells showed a higher tendency to convert to FoxP3$^+$ iT_{reg} cells than naive WT cells, a finding which was observed both 4 and 6 wk after transfer, most notably in the MLNs (Fig. 9 A and not depicted).

To evaluate the function of Itk-deficient T_{reg} cells in vivo, we transferred congenic sorted naive WT CD4$^+$CD45RB^{high} CD25$^-$CD45.1 T cells along with WT or *Itk*$^{-/-}$ sorted differentiated CD4$^+$FoxP3^{GFPhigh} iT_{reg} cells and monitored weight loss in the mice over the course of 2 mo. Although at high numbers of iT_{reg} cells both WT and *Itk*$^{-/-}$ iT_{reg} cells could control colitis (Fig. 9 B), at suboptimal doses of iT_{reg} cells *Itk*$^{-/-}$ iT_{reg} cells helped control colitis better than WT iT_{reg} cells, resulting in increased animal weight (Fig. 9 C) and reduced percentages of CD45.1 IFN-γ– and IL17-producing cells in the gut (Fig. 9 D and not depicted). Thus, both in vitro and vivo, the function of T_{reg} cells did not require Itk. Together, these data implicate Itk as a functionally important regulator of the balance between T_{reg} and Th17 cells.

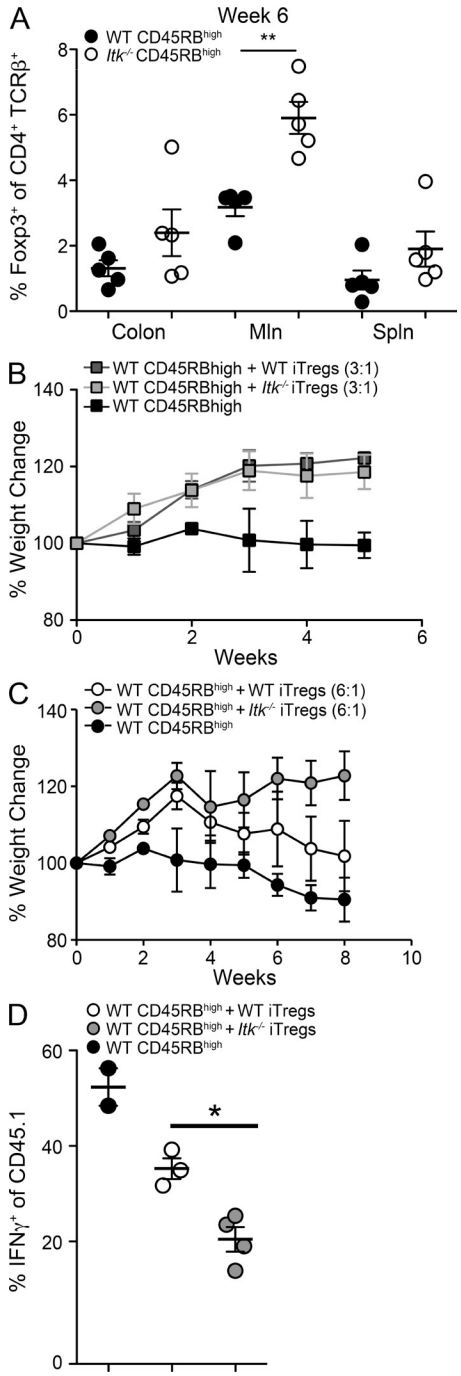

Figure 9. Functional experiments of Itk-deficient CD4+ T cells in vivo. (A) Sorted naive $CD4^+CD25^-CD45RB^{high}$ T cells from WT and $Itk^{-/-}$ mice were transferred by retroorbital injection (1.5×10^5 cells) to $Rag1^{-/-}$ recipient C57BL/6 mice, which were followed over 6 wk. MLNs, colons, and spleens were harvested, and lymphocytes were analyzed for conversion to $CD4^+FoxP3^+$ T cells (data from 6 wk are shown). Data are representative of three independent experiments examining five mice each. Mean ± SEM. **, $P < 0.001$. (B and C) iT_{reg} cells were differentiated in culture (using 0.1 µg/ml anti-CD3 mAb) from sorted naive $FoxP3^-$ cells from WT and $Itk^{-/-}$ $FoxP3^{GFP}$ reporter mice and sorted for $CD4^+FoxP3^{GFPhigh}$ cells after differentiation. $Itk^{-/-}$ and WT iT_{reg} cells were cotransferred retroorbitally at 1:3 (B) or 1:6 (C) ratios with 218,000 sorted naive congenic $CD4^+CD25^-CD45RB^{high}$ T WT cells (from B6.SL mice) into $Rag1^{-/-}$ hosts. Weight loss was followed over 8 wk. Data are representative of three experiments examining five mice each. (D) Colons from C were harvested after 8 wk, and $CD4^+$ T lymphocytes were stained for IFN-γ. Mean ± SEM. *, $P < 0.05$.

DISCUSSION

A careful balance between inflammatory and T_{reg} cell responses is required to avoid deleterious damage to the host while mounting successful immune responses. Understanding the molecular mechanisms and factors that regulate the balance of T_{reg} and Th17 cell differentiation is therefore of great importance. We demonstrate here that inhibition of the Itk protein tyrosine kinase impairs Th17 differentiation while positively regulating T_{reg} cell differentiation, both in culture and in vivo. Our results indicate that Itk participates in the cross talk between TCR and cytokine signaling that differentially affects the activation of distinct signaling pathways involving mTOR and STAT5 activation downstream of IL-2. We further link these findings, in part, to impaired induction of Myc and repression of *Pten*, associated with decreased mTOR activation and altered metabolic control. The confirmation of these observations in vivo suggests that Itk may be a potential therapeutic target for Th17-mediated pathology.

Itk is a Tec family tyrosine kinase that is activated upon TCR signaling and is required for full TCR-induced activation of PLC-γ, Ca^{2+} mobilization, and ERK activation. Loss of Itk also affects actin cytoskeletal reorganization and T cell adhesion through effects on multiple guanine-nucleotide exchange factors, including Vav and SLAT (Gomez-Rodriguez et al., 2011; Singleton et al., 2011). Intriguingly, data have shown that interrupted or shorter duration of TCR signaling, as might occur with decreased adhesion, leads to increased FoxP3 expression (Sauer et al., 2008; Miskov-Zivanov et al., 2013), consistent with previously recognized defects associated with Itk deficiency (Finkelstein et al., 2005). However, our results suggest that Itk also strongly influences pathways activated by PI3K and mTOR.

PI3K is activated by TCR and CD28 signaling and is involved in cell cycle progression, cell survival, proliferation, and regulation of cell trafficking, in part through the activation of $PI_{(3,4,5)}P_3$-binding pleckstrin homology (PH) domain–containing proteins. Important downstream components of these processes in many cell types are the Akt and mTOR pathways, which play critical roles in regulating cellular metabolism and differentiation downstream of multiple receptors. Notably, it is now well recognized that inhibition of mTOR or Akt in $CD4^+$ T cells leads to expression of FoxP3 (Powell et al., 2012). This regulation is likely to occur via multiple mechanisms, including effects of Akt on the FoxO transcription factors (Hedrick et al., 2012), which are required for FoxP3 expression, as well as effects on distinct mTOR-mediated pathways. Thus, the mTOR and Akt axes play major roles as a gatekeeper of effector versus T_{reg} cell differentiation.

Among the critical pathways controlled by mTOR is the regulation of cellular metabolism. Indeed, in recent years

there has been an increasing recognition of how metabolism contributes to/helps control T cell fate and the role of mTOR in this process. A critical element of this process is the expression of the transcription factor HIF1α; $CD4^+$ T cells deficient in HIF1α exhibit increased FoxP3 and reduced IL17A under Th17-inducing conditions (Dang et al., 2011; Shi et al., 2011). HIF1α-deficient $CD4^+$ T cells also show increased responsiveness to FoxP3-inducing cytokines, similar to our observations in Itk-deficient cells (Dang et al., 2011). Nonetheless, how TCR signaling contributes to this regulatory axis is not well appreciated. Our results suggest that Itk and TCR signaling play a critical role in regulating the expression of HIF1α by functioning as a rheostat that determines the extent of activation of PI3K- and mTOR-activated pathways. At least part of this occurs through effects on the expression of *Pten*. We further show that *Pten* expression is tightly regulated by TCR signaling, with strong TCR signals leading to a marked repression of *Pten* message, supporting previous work from Bensinger et al. (2004). This repression is defective in Itk-deficient cells, even under conditions in which WT and $Itk^{-/-}$ $CD4^+$ T cells develop equivalent percentages of $FoxP3^+$ iT_{reg} cells, suggesting that these observations are not secondary to altered differentiation. Furthermore, treatment of Itk-deficient $CD4^+$ cells with *Pten*-specific shRNA reduced the generation of $FoxP3^+$ cells, supporting the idea that altered *Pten* expression contributes to rather than results from the increased generation of $FoxP3^+$ cells. Indeed, under conditions of strong TCR signals, where we see decreased expression of *Pten*, we see reciprocal regulation and increased pS6 and elevated expression of the mTOR target gene, *Hif1α*. Our results suggest that by altering the expression of *Pten*, impaired TCR signaling can affect the activation of signaling pathways downstream of multiple receptors, supporting the idea that TCR signaling controls the ability of T cells to integrate diverse inputs. This regulation provides a distinct positive feedback mechanism by which PI3K-mediated pathways can be exquisitely controlled to affect effector cell differentiation.

Indeed, although TCR and IL-2 signaling are often evaluated independently, both activate several similar downstream readouts. Moreover, several lines of data indicate that TCR signaling can influence IL-2 signaling. Although TCR signaling up-regulates CD25, which is required for IL-2 responses in mouse T cells, high TCR engagement paradoxically decreases STAT5 phosphorylation so that more efficient STAT5 phosphorylation occurs with low TCR engagement (Lee et al., 1999; Yamane and Paul, 2013). Nonetheless, although STAT5 is often used as a surrogate for IL2 responses, STAT5 activation is only one of multiple pathways activated by IL-2 (Liao et al., 2013). Our results suggest that TCR signaling can differentially affect the activation of pathways downstream of IL-2 and demonstrate that at least one way this is accomplished is through modulating expression of *Pten*, thereby uncoupling STAT5 and PI3K activation. Whether altered *Pten* regulation contributes to the influence of Itk and TCR signaling on responses to other cytokines and pathways of differentiation remains to be evaluated. In $CD8^+$ T cells, activation of Akt and mTOR pathways has been closely linked to regulation of cell trafficking, in part through effects on expression of homing receptors (Finlay and Cantrell, 2011; Finlay et al., 2012). Although we have not directly explored these pathways, decreased $CD4^+$ T cell numbers have been observed in the lungs of Itk-deficient mice in models of allergic asthma (Ferrara et al., 2006). It is intriguing to speculate that migration of effector cells may also be influenced in Itk-deficient mice by virtue of alterations in phospholipid metabolism. Furthermore, these effects are not limited to those influenced by Akt and mTOR, as $PI_{(3,4,5)}P_3$ is involved in the activation of multiple PH domain–containing proteins, including Itk itself and other T cell signaling molecules.

Our results therefore suggest a model in which decreased or impaired TCR signaling, such as may occur under conditions of limited antigen or altered peptide ligands, leads to decreased activation of mTOR and altered cell metabolism through multiple mechanisms. First, it is likely that decreased or impaired TCR signaling prevents full activation of PI3K and downstream effectors including mTOR. However, we show here that impaired TCR signaling via loss of Itk also leads to a negative feedback loop in which defective repression of *Pten* prevents downstream activation of PI3K- and mTOR-mediated pathways not just from the TCR, but from multiple cellular inputs. This, in turn, results in impaired induction of *Hif1α* and downstream activation of glucose metabolism. Interestingly, we have also observed that Itk deficiency prevents full induction of Myc and miRNA 19b, which are known repressors of *Pten* expression. However, Myc itself is known to increase expression of multiple genes involved in glucose metabolism and nutrient transport including *Hif1α*, perhaps secondary to its role as a global amplifier of gene expression in lymphocytes (Wang et al., 2011; Nie et al., 2012). It is also of note that nutrient uptake is a major regulator of mTOR that may be more important than PI3K in T cells (Sinclair et al., 2013). Similarly, Myc expression is also repressed by increased *Pten*, providing another level of systems amplification (Bonnet et al., 2011). Thus, Itk deficiency likely affects multiple aspects of T cell metabolic control via altered regulation of both *Myc* and *Pten*. It should be noted that altered inositol phosphate regulation is also likely to affect the activation of multiple PH domain–containing proteins involved in TCR and other signaling pathways, including Itk, adding another level to these feedback mechanisms. Our results suggest that together, these circuits may conspire to dampen glycolytic activation and other downstream readouts of mTOR and Akt in response to multiple receptor signaling pathways, causing a reprogramming of T cell differentiation. It is notable that previous data has implicated Btk, a related Tec family tyrosine kinase, in amplifying inositol phosphate signaling in B cells via enhancing the recruitment of PIP5K (Saito et al., 2003; Schwartzberg, 2003). These data suggest that Tec kinases help modulate multiple lymphocyte-signaling cascades in part through participating in amplification loops involving inositol phosphate and metabolic-mediated pathways.

However, altered *Myc* and *Pten* expression are likely not to be the only mechanisms by which Itk deficiency and decreased TCR signaling influence T_{reg} cell development. Recent data have demonstrated that decreased ERK activation also promotes T_{reg} cell differentiation (Chang et al., 2012; Liu et al., 2013; unpublished data), although studies have showed varying effects on Th17 development. Itk-deficient cells also have defective TCR-induced ERK activation. Erk itself is also known to be part of a positive TCR feedback loop that acts very proximally, at the level of Lck (Štefanová et al., 2003), and has been implicated in regulating STAT5 phosphorylation (Lee et al., 1999). Given the effects of Itk on TCR signaling, including the activation of ERK, Itk may be a critical pivot in multiple positive-feedback loops that both amplify TCR signaling and alter responses to cytokines, thereby contributing to the balance of Th17 and T_{reg} cell differentiation. As that loss of Itk does not appear to impair T_{reg} cell function, our results raise the possibility of Itk as a therapeutic candidate for the treatment of diseases involving Th17-mediated inflammation.

MATERIALS AND METHODS

Mice. *Itk*$^{-/-}$ (Liao and Littman, 1995) and WT mice were backcrossed 12 generations onto the C57BL/6 background. *Itk*$^{-/-}$ FoxP3^{GFP} mice were generated by crossing *Itk*$^{-/-}$ mice with FoxP3^{GFP} mice (Bettelli et al., 2006). Transgenic 5CC7 *Rag2*$^{-/-}$ *Itk*$^{-/-}$ mice were generated by interbreeding *Itk*$^{-/-}$ and 5CC7 *Rag2*$^{-/-}$ mice (Seder et al., 1992). Congenic B6.SL female mice were purchased from Taconic. All mice used were between 7 and 10 wk old. Animal husbandry and experiments were performed in accordance with approved protocols by the National Human Genome Research Institute's Animal Use and Care Committee, National Institutes of Health.

T cell purification and culture. CD4$^+$ T cells were purified by negative selection using a magnetic cell separation system according to the manufacturer's protocol (Miltenyi Biotec). Naive (CD4$^+$CD44^{low}CD62L^{high}CD25$^-$ T cells) or FoxP3$^+$ T_{reg} (CD4$^+$ FoxP3$^{GFP\text{-}high}$) cells were purified by cell sorting at a purity >99%. Cells were cultured in complete RPMI 1640 media as indicated in Gomez-Rodriguez et al. (2009). In brief, sorted naive CD4$^+$ T cells (2×10^5) were co-cultured at a ratio of 1:5 with mitomycin-treated T-depleted splenocytes as APCs for the indicated amount of time in 48-well plates containing 1 µg/ml anti-CD3 (2C11) plus 3 µm/ml anti-CD28 (Bio X Cell) or as indicated in the figure legends under different conditions. Th17 cells: 20 ng/ml IL-6, 5 ng/ml TGF-β1, and 10 µg/ml of each anti–IL-4, anti–IFN-γ, and anti–IL-12 antibodies. T_{reg} cells: 100 U/ml hIL2, 5 ng/ml TGF-β1 with 0.1, 1, or 5 µg anti-CD3, and 10 µg/ml of each anti–IL-4, anti–IFN-γ, and anti–IL-12 antibodies. T_{reg} cells + anti-CD28 condition: 100 U/ml hIL2, 5 ng/ml TGF-β1 with 1 µg/ml anti-CD3 plus 3 µg/ml anti-CD28, and 10 µg/ml of each anti–IL-4, anti–IFN-γ, and anti–IL-12 antibodies. Th-null conditions: 1 µg/ml anti-CD3 plus 3 µm/ml anti-CD28 and 10 µg/ml of each anti–IL-4, anti–IFN-γ, and anti–IL-12 antibodies. Cytokines were purchased from PeproTech. Cytokine antibodies were purchased from Bio X Cell. For inhibition experiments, sorted naive CD4$^+$ T WT cells and APCs were incubated with the inhibitor LY29400 (Sigma-Aldrich) for 40 min before stimulation with the indicated differentiation conditions for 48 h. Naive CD4 T cells from 5CC7 transgenic mice were cultured as indicated before except that PCC peptide was used instead of anti-CD3. For evaluating responses to IL2, sorted naive CD4$^+$ T cells from WT and *Itk*$^{-/-}$ mice were stimulated under Th17, T_{reg}, or Th-null conditions, in the presence of APCs for 2 d in the absence or presence of 10 µg/ml of blocking anti-IL2 or anti-IL2 plus the indicated amount of hIL2 (100 U/ml). Alternatively, sorted naive CD4$^+$ T cells from WT and *Itk*$^{-/-}$ mice were activated for 2 d in the presence of APCs plus 1 µg/ml anti-CD3, 3 µg/ml anti-CD28, and 10 µg/ml of each anti-IL12, anti-IL4, and anti–IFN-γ. Under these conditions, FoxP3$^+$ cells were not generated. Cells were starved for 3 h and then stimulated with hIL2 for different time points to evaluate pS6 and pSTAT5.

Retrovirus production and infection. Oligonucleotides for shRNA against *Pten* were annealed and subcloned in the vector MIGR (IRES-GFP): *Pten* shRNA1 F, 5′-GATCCCGGCCACAGCTAGAACTTATCAAACTCGAGTTTGATAAGTTCTAGCTGTGGTTTTTG-3′; *Pten* shRNA1 R, 5′-AATTCAAAAACCACAGCTAGAACTTATCAAACTCGAGTTTGATAAGTTCTAGCTGTGGCCGG-3′; *Pten* shRNA2 F, 5′-GATCCCGGACCACTGACTCTGATCCAGAGAATGAACCCTCGAGGGTTCATTCTCTGGATCAGAGTCAGTGGTTTTTTG-3′; *Pten* shRNA2 R, 5′-AATTCAAAAAACCACTGACTCTGATCCAGAGAATGAACCCTCGAGGGTTCATTCTCTGGATCAGAGTCAGTGGTCCGG-3′; *Pten* shRNA3 F, 5′-GATCCCGGGAAGATATATTCCTCCAATTCAGGACCCCTCGAGGGGTCCTGAATTGGAGGAATATATCTTCTTTTTG-3′; and *Pten* shRNA3 R, 5′-AATTCAAAAAGAAGATATATTCCTCCAATTCAGGACCCCTCGAGGGGTCCTGAATTGGAGGAATATATCTTCCCGG-3′. MIGR and shRNA *Pten*-MIGR plasmids (12.5 µg) were cotransfected with helper plasmid (2.5 µg) and were used to transfect 293 T cells with FuGENE (Roche). After 48 h, retroviral supernatants were collected. Sorted naive *Itk*$^{-/-}$ CD4$^+$ T cells from *Itk*$^{-/-}$ mice in the presence of APCs were activated with anti-CD3 and anti-CD28 for 36 h. Retrovirus supernatants were added to the cells and spun at 2,500 rpm for 1.5 h at room temperature with 8 µg/ml polybrene (Sigma-Aldrich). Data are shown for shRNA3. After 24 h, infected cells were differentiated under T_{reg} + anti-CD28 conditions for 48 h, restimulated with PMA + ionomycin, and stained for intracellular cytokines and FoxP3.

In vivo colitis model to evaluate conversion on naive CD4$^+$ T cells to T_{reg} cells in vivo. Naive cells from WT and *Itk*$^{-/-}$ were obtained by sorting for CD4$^+$CD25$^-$CD45RB^{high} T cells (Powrie et al., 1994). Sorted CD45RB^{high} cells were transferred to congenic *Rag1*$^{-/-}$ recipient C57BL/6 mice by retro-orbital injection (1.5×10^5 cells). Weight loss was followed over 6–8 wk. Blood was collected weekly, and MLNs, colons, and spleens were harvested after 4 and 6 wk and analyzed for conversion to CD4$^+$FoxP3$^+$ T cells.

Inhibition of colitis by iT$_{reg}$ cells. iT$_{reg}$ cells were obtained by differentiation of sorted naive CD4$^+$ T cells from WT-FoxP3^{GFP} and *Itk*$^{-/-}$FoxP3^{GFP} reporter mice in the presence of APCs, with 0.1 µg/ml anti-CD3 mAb, 5 ng/ml TGF-β1, and 100 U/ml hIL2. After stimulation for 3 d, cells were harvested and sorted for CD4$^+$FoxP3^{GFPhigh}. To assess the suppressive function of iT$_{reg}$ cells, *Itk*$^{-/-}$ or WT iT$_{reg}$ cells were cotransferred at 1:3 and 1:6 ratio with 218,000 CD4$^+$CD25$^-$CD45RB^{high} T WT cells (B6.SL mice) to *Rag1*$^{-/-}$ mice. Weight lost was followed over 8 wk. Blood was collected weekly, and MLNs, guts, and spleens were harvested after 8 wk and stained for IL17A and IFN-γ cytokines and transcription factor FoxP3.

Suppression assay of FoxP3$^+$ T_{reg} and iT$_{reg}$ cells in vitro. FoxP3$^+$ T_{reg} cells were obtained by sorting for CD4$^+$CD25^{high}FoxP3^{GFPhigh} from WT-FoxP3^{GFP} and *Itk*$^{-/-}$ FoxP3^{GFP} reporter mice. iT$_{reg}$ cells were obtained by differentiation of sorted naive CD4$^+$ T cells from Wt-FoxP3^{GFP} and *Itk*$^{-/-}$ FoxP3^{GFP} reporter mice in the presence of APCs, with 0.1 µg/ml anti-CD3 mAb, 5 ng/ml TGF-β1, and 100 U/ml hIL2. After stimulation for 3 d, cells were harvested and sorted for the CD4$^+$FoxP3^{GFPhigh} population. To test the suppressive function of FoxP3$^+$ T_{reg} and iT$_{reg}$ cells, sorted naive CD4$^+$ effector WT T cells at 50×10^3 /well were stimulated with 0.5 µg/ml anti-CD3 in the presence of mitomycin-treated APCs at 50×10^3 /well. Graded numbers of FoxP3$^+$ T_{reg} or iT$_{reg}$ cells from Itk-deficient and WT cells were added into the culture (as indicated). Proliferation was measured by [^{3}H]thymidine incorporation after 3 d of culture.

Flow cytometric analyses. Differentiated cells were stimulated for 4 h with 2 ng/ml PMA (Sigma-Aldrich) and 1 µg/ml ionomycin (Sigma-Aldrich) in the presence of Golgi stop (BD), and intracellular staining for FoxP3 and IL17A was performed according to the manufacturer's instructions (eBioscience). For intracellular levels of phospho-S6 (Ser 235 and Ser 240; Cell

Signaling Technology), phospho-STAT5 (BD), and PTEN (BD), $CD4^+$ T cells were fixed with 4% PFA and permeabilized with methanol at −20 C. Cells were stained with the indicated phospho-antibodies for 60 min in the dark at 4°C. Analysis was performed by using FlowJo software (Tree Star).

RNA isolation, reverse transcription, and quantitative PCR. Total RNA was isolated from the differentiated cells at different time points with the RNeasy Mini kit (QIAGEN) and was reverse transcribed with random hexamer primers and M-MLV reverse transcription (Life Technologies). Quantitative RT-PCR (qRT-PCR) was performed on a 7500 Fast Real-Time PCR instrument (Life Technologies) using TaqMan assay for the mRNAs indicated in the figures.The samples were normalized to 18S RNA, and data are expressed as relative to WT naive levels using the $2^{-\Delta\Delta CT}$ method and ABI 7500 SDS 1.3.1 software (Applied Biosystems).

microRNA isolation and TaqMan. MicroRNA was isolated using the mirVana isolation kit (Life Technologies) according to the manufacturer's recommendations. RNA was converted into cDNA using specific microRNA primers with the TaqMan MicroRNA Reverse Transcription kit (Life Technologies). qRT-PCR was performed using the TaqMan assay with specific microRNA primers (Life Technologies). To determine relative expression levels samples were normalized to U6 microRNA using the $2^{-\Delta\Delta CT}$ method and ABI 7500 SDS 1.3.1 software.

Immunoblot. 10^6 $CD4^+$ T cells from WT and $Itk^{-/-}$ were stimulated with 5 µg/ml anti-CD3 and 5 µg/ml anti-CD28 for different times and immediately lysed in Laemmli buffer by boiling. Proteins were separated in 8% SDS-PAGE gel and transferred to nitrocellulose membranes, which were blocked and incubated with anti–phospho-Akt (Ser 473, Thr 308), anti-Akt (Cell Signaling Technology), or anti-actin (Sigma-Aldrich) as indicated by the manufacturer, washed, incubated with HPR-labeled goat anti–rabbit, and developed with the enhanced chemiluminescence detection system (GE Healthcare). The intensities were quantified using ImageJ software (National Institutes of Health).

Glycolysis. WT and $Itk^{-/-}$ $CD4^+$ T cells differentiated under Th17 and T_{reg} cell conditions for 2 d were collected and washed with PBS. The glycolytic activity of differentiated cells was measured by the extracellular acidification rate, an indicator of glycolysis, using an Extracellular Flux Analyzer (Seahorse Bioscience).

Statistical analyses. Results are expressed as mean ± SEM. Statistical differences between the analyzed groups were calculated with the paired Student's *t* test.Values of $P < 0.05$ are considered significant. Graphs were created in Excel (Microsoft) and Prism (GraphPad Software).

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