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 (Treg 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 Treg 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 Treg 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 repres- sors 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 Treg cells.

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 (Treg cells), another subset of T helper cells, which are essential for immune tolerance and prevention of autoimmunity (von Boehmer and Daniel, 2013). Treg cells are characterized by the expression of the transcription factor Forkhead box P3 (FoxP3) and are subdivided in two major categories: thymic-derived Treg cells (Treg cells) and peripherally derived and induced Treg cells (pTreg cells and iTreg cells), which arise from naive CD4+ T cells in response to signals in the periphery or culture and acquire FoxP3 expression.
and Treg cell function (Chaudhry and Rudensky, 2013). Interestingly, the development of Th17 and iTreg 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 Treg 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 Treg cells (Josefowicz et al., 2012). Consistent with the requirements for TGF-β1 and IL-2 in the generation of iTreg cells from naïve mouse CD4+ T cells, activation of IL-2–STAT5 and TGF-β1–SMAD pathways are important for iTreg 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 Treg 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 Treg 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+ Treg 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 Treg 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 Ca2+ 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 Treg 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 Treg cell differentiation. Surprisingly, we found that Itk−/− CD4+ T 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 iTreg 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 bona fide Treg cells both in vivo and in vitro. Our results suggest that Itk helps integrate signaling pathways that regulate the balance of Th17 and Treg cell differentiation, providing insight into the contribution of TCR signaling to iTreg cell development and suggesting Itk as a potential target to alter the balance between Th17 and Treg cells.

RESULTS
Itk-deficient cells exhibit increased Foxp3 induction
We have previously shown that Itk is a positive regulator of IL17A production and that naïve 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 naïve (CD4+CD44lowCD62LhighCD25−) 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 naïve Itk-deficient CD4+ T cells stimulated under Th17-polarizing conditions (18 ± 1.5%).
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 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+ Treg cells, also showed increased production of FoxP3+ cells under Th17 conditions, arguing that these findings were not 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 Th17 cells play a key role in regulating the immune response, a recent study has revealed that Th17 cells can also promote the differentiation of regulatory T cells (Treg) (1). This finding suggests that Th17 cells may have a dual role in immune regulation, balancing both inflammatory and regulatory functions. In order to better understand the mechanisms underlying this phenomenon, it is essential to further investigate the crosstalk between Th17 and Treg cells, as well as the factors that regulate their differentiation.

**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+ T cells from six mice in two experiments ± SEM. **, P < 0.05. (D) Sorted naive GFP−CD4+ T cells from WT and *Itk*−/− FoxP3GFP reporter mice differentiated as in A. Data are representative of more than five experiments.
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GFP+CD4+ T cells sorted from Itk−/− FoxP3GFP mice also gave rise to higher percentages of FoxP3+ iTreg 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−/− iTreg cells were functional, increasing numbers of sorted differentiated CD4+FoxP3+ cells (iTreg cells) from FoxP3GFP 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−/− iTreg cells were fully capable in suppressing proliferation of CD4+ T responder cells; notably, at low ratios, Itk−/− iTreg cells suppressed even better than WT. Evaluation of

**Figure 2.** Itk-deficient cells express more FoxP3 expression under iTreg cell conditions.

(A) Sorted naive CD4+ T cells from WT and Itk−/− mice were differentiated under iTreg 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 iTreg cell differentiation cultures determined by qRT-PCR; data are mean ± SEM of five separate experiments. *, P < 0.05. (C) Sorted naive CD4+ T cells from WT and Itk−/− 5CC7 transgenic mice were differentiated under Treg 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−/− FoxP3GFP reporter mice were differentiated under Treg cell conditions as in A. (E and F) The suppression capabilities of differentiated iTreg cells (E) or pTreg 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+FoxP3GFP+ cells (iTreg or pTreg cells) in the presence of 0.5 µg/ml anti-CD3 plus APCs (50,000). After 72 h, cultures were pulsed with [3H]thymidine. Data are the mean ± SEM from triplicate wells and are representative of three experiments.
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 iTreg 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.

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 Treg 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
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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<sub>reg</sub> 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 plus anti-CD28. The presence of the PI3K inhibitor LY294002 in T<sub>reg</sub> cell cultures enhanced the production of FoxP3 by WT CD4<sup>+</sup> T cells in the presence of anti-CD3 plus anti-CD28, increasing the expression from 16.4 to 32.2% (Fig. 4A); 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.

Reduced PI3K–Akt–mTOR signaling in Itk<sup>−/−</sup> CD4<sup>+</sup> 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<sub>reg</sub> 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 plus anti-CD28. The presence of the PI3K inhibitor LY294002 in T<sub>reg</sub> cell cultures enhanced the production of FoxP3 by WT CD4<sup>+</sup> T cells in the presence of anti-CD3 plus anti-CD28, increasing the expression from 16.4 to 32.2% (Fig. 4A); 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 4. Defective activation of the mTOR–Akt pathway in Itk<sup>−/−</sup> CD4<sup>+</sup> T cells. (A) Sorted naive WT CD4<sup>+</sup> T cells were differentiated under T<sub>reg</sub> 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<sup>+</sup> T cells were differentiated under Th17, T<sub>reg</sub>, and T<sub>reg</sub> + 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<sup>+</sup> T cells were differentiated under T<sub>reg</sub> cell (0.1 µg/ml anti-CD3) conditions, and levels of pS235 S6 in FoxP3<sup>+</sup> cells were determined. (E) Naive WT and Itk<sup>−/−</sup> CD4<sup>+</sup> 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.
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+ T cells express decreased downstream metabolic effectors of mTOR. (A and B) Naive and Th17- and Treg 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 Treg 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 Treg 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 Treg 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).
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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 iTreg 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)P3, 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 iTreg cells (not depicted). Nonetheless, CD25 levels were lower on Itk<sup>−/−</sup> activated CD4<sup>+</sup> 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 iTreg 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<sup>−/−</sup> T cells, we examined the effects of Itk deficiency on activation of IL-2–induced signaling. To up-regulate CD25, naive CD4<sup>+</sup> T cells were differentiated under neutral conditions without TGF-β, conditions under which we did not observe increased development of FoxP3<sup>+</sup> iTreg cells, and then washed and treated with hIL-2. Consistent with the increased FoxP3 induction in Itk<sup>−/−</sup> 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 iTreg cell conditions with low concentrations of anti-CD3, so that WT and Itk-deficient cells had equivalent percentages of FoxP3<sup>+</sup> 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.

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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 Treg 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 iTreg 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 iTreg 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+CD45RBhighCD25− 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 iTreg 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+CD45RBhighCD25− T cells from WT mice, and a second group received sorted naive CD4+CD45RBhighCD25− T cells from Itk−/− mice. Similar to our in vitro results, naive Itk−/− cells showed a higher tendency to convert to FoxP3+ iTreg 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 iTreg cells in vivo, we transferred congeneric sorted naive WT CD4+CD45RBhigh CD25−CD45.1 T cells along with WT or Itk−/− sorted differentiated CD4+FoxP3GFPhigh iTreg cells and monitored weight loss in the mice over the course of 2 mo. Although at high numbers of iTreg cells both WT and Itk−/− iTreg cells could control colitis (Fig. 9 B), at suboptimal doses of iTreg cells Itk−/− iTreg cells helped control colitis better than WT iTreg 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 iTreg cells did not require Itk. Together, these data implicate Itk as a functionally important regulator of the balance between Treg and Th17 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.
A careful balance between inflammatory and T\textsubscript{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\textsubscript{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\textsubscript{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-\gamma, Ca\textsuperscript{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\textsubscript{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\textsuperscript{+} 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\textsubscript{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+ iTreg 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 PI3K inhibited through 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 PI3K (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 Treg cell development. Recent data have demonstrated that decreased ERK activation also promotes Treg 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 Treg cell differentiation. As that loss of Itk does not appear to impair Treg 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−/− Foxp3GFP mice were generated by crossing Itk−/− mice with Foxp3GFP mice (Betelli 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 Biotech). Naïve (CD4+CD44+CD62L+CD25−) T cells or Foxp3+ Treg (CD4+Foxp3+CD25+) 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 naïve 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 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-B1, and 10 µg/ml of each anti-IL-4, anti-IFN-γ, and anti-IL-12 antibodies. Treg cells: 100 U/ml hIL-2, 5 ng/ml TGF-B1 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. Treg cells + anti-CD28 condition: 100 U/ml hIL-2, 5 ng/ml TGF-B1 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 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 naïve CD4+ T WT cells and APCs were incubated with the inhibitor LY294002 (Sigma-Aldrich) for 40 min before stimulation with the indicated differentiated conditions for 48 h. Naïve 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 IL-2, sorted naïve CD4+ T cells from WT and Itk−/− mice were stimulated under Th17, Treg, or Th-null conditions, in the presence of APCs for 2 d in the absence or presence of 10 µg/ml of blocking anti-IL-2 or anti-IL-2 plus the indicated amount of hIL-2 (100 U/ml). Alternatively, sorted naïve 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-IL-12, anti-IL-4, and anti-IFN-γ. Under these conditions, Foxp3+ cells were not generated. Cells were starved for 3 h and then stimulated with hIL-2 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′−GATCCCCGCGACCAGCTAGTATACACTAAA-CTCAGGTTCATTGCTGAGGTTTGG-3′; Pten shRNA1 R, 5′−ATTCCAAACACACAGCTAGAAGTATACACATCGAGTTT-GATAATGTCCTGTTGCGCCGG-3′; Pten shRNA2 F, 5′−GATCCCGACACTGATCGTCAGAAGAACCCTGCGAGTTTC-ATTCTCTGATCAGTCTGGATTGTTCCTT-3′; Pten shRNA2 R, 5′−ATTCCAACACACAGCTAGTATACACTAAA-CTCAGGTTCATTGCTGAGGTTTGG-3′; Pten shRNA3 F, 5′−GATCCCGGAAAAAGATTAACCTCCCAATCTCGAGGCTCCCTGCGAAGCTATACAGTCTGGATTGTTCCTT-3′; and Pten shRNA3 R, 5′−ATTCCAACACACAGCTAGTATACACTAAA-CTCAGGTTCATTGCTGAGGTTTGG-3′ were annealed and subcloned in the vector 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 naïve 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 iTreg + 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 naïve CD4+ T cells to Treg cells in vivo. naïve mice from WT and Itk−/− were obtained by sorting for CD4+ CD25+ CD45Ro T cells (Powrie et al., 1994). Sorted CD45R0+ T cells were transferred to congenic Rag1−/− recipient C57BL/6 mice by retroviral injection (1.0 × 10^8 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 Treg cells. Treg cells were obtained by differentiation of sorted naïve CD4+ T cells from WT-Foxp3GFP and Itk−/− Foxp3GFP reporter mice in the presence of APCs, with 0.1 µg/ml anti-CD3 mAb, 5 ng/ml TGF-B1, and 100 U/ml hIL-2. After stimulation for 3 d, cells were harvested and sorted for CD4+ Foxp3GFP+ cells. To assess the suppressive function of Treg cells, Itk−/− or WT Treg cells were cotransfected at 1:3 and 1:6 ratio with 218,000 CD4+ CD25+ CD45R0+ T cells from WT (B6.SL mice) to Rag1−/− mice. Weight loss 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+ Treg and iTreg cells in vitro. Foxp3+ Treg cells were obtained by sorting for CD4+ CD25+ Foxp3GFP+ and Itk−/− Foxp3GFP reporter mice. Treg cells were obtained by differentiation of sorted naïve CD4+ T cells from WT-Foxp3GFP and Itk−/− Foxp3GFP reporter mice in the presence of APCs, with 0.1 µg/ml anti-CD3 mAb, 5 ng/ml TGF-B1, and 100 U/ml hIL-2. After stimulation for 3 d, cells were harvested and sorted for the CD4+ Foxp3GFP+ population. To test the suppressive function of Foxp3+ Treg and iTreg cells, sorted naïve 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+ Treg or iTreg cells from Itk-deficient WT cells were added into the culture (as indicated). Proliferation was measured by [3H]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 Golgisto (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
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^−ΔΔCT 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^−ΔΔCT method and ABI 7500 SDS 1.3.1 software.

Immunoblot. 10^5 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).

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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