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Accessibility
The phosphatase PTEN-mediated control of PI-3 kinase in T_{regs} cells maintains homeostasis and lineage stability

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Abstract

Foxp3⁺ regulatory T cells (T_{regs}) are required for immune homeostasis. One notable distinction between conventional T cells (T_{conv}) and T_{regs} is differential phosphatidylinositol 3-kinase (PI3K) activity: only T_{conv} downregulate PTEN, the primary negative regulator of PI3K, upon activation. Here, we show that control of PI3K in T_{regs} is essential for lineage homeostasis and stability. Mice lacking Pten in T_{regs} developed an autoimmune-lymphoproliferative disease characterized by excessive T_{H1} responses and B cell activation. Diminished control of PI3K activity in T_{regs} led to reduced CD25 expression, accumulation of Foxp3⁺CD25⁻ cells and ultimately, loss of Foxp3 expression in these cells. Collectively, these data demonstrate that control of PI3K signaling by PTEN in T_{regs} is critical to maintain their homeostasis, function and stability.

Regulatory T cells (T_{reg}) defined by expression of the transcription factor Foxp3 are required for normal immune homeostasis. Mutation of the Foxp3 gene in mouse and human leads to the scurvy phenotype and IPEX disease, respectively, both characterized by the lack of functional T_{reg}, autoimmunity, and systemic polyclonal lymphoproliferation. This central role of T_{reg} in immune tolerance has led to a focus on defining the signals that govern T_{reg} generation, function and stability. Critical among these signals are those...
delivered by the interleukin 2 receptor (IL-2R), as enhancement of IL-2R signaling dramatically expands Tregs in vivo⁴. Among the signals activated via the IL-2R, phosphorylation of STAT5 appears to be particular important for Treg maintenance and expansion⁵.

In addition to activation of STAT5, IL-2 also initiates signaling through phosphatidylinositol 3-kinase (PI3K), which can be activated as well via the TCR or CD28⁶,⁷. The primary negative regulator of PI3K is phosphatase and tensin homolog on chromosome 10 (PTEN), a lipid phosphatase, which catalyzes the reverse reaction of PI3K⁸. Demonstrating the importance of PI3K regulation in immune homeostasis, animals harboring one inactive PTEN allele, develop systemic autoimmunity, which has been attributed in part to defects in thymic negative selection, as well as in Fas-mediated and activation-induced cell death in potential effector T cells. While T cell-specific PTEN deficiency leads to a lethal CD4⁺ T cell lymphoma of thymic origin⁷,⁹,¹⁰, when lymphoma development is prevented by early thymectomy, systemic autoimmunity is observed, indicating that targeting PTEN in T cells is sufficient to disturb self-tolerance.

We and others, have previously shown that regulatory T cells, but not effector T cells, maintain high amounts of PTEN expression, thus preventing the downstream activation of PI3K targets following IL-2 stimulation, while allowing further JAK-STAT signaling¹¹,¹². Indeed, control of PTEN via the scaffold protein Disc large homolog 1 is critical for human Treg function in vitro¹³. This suggests that Tregs require a mechanism of PI3K control distinct from effector T cells.

Consistent with this notion, PI3K signaling inhibits in vitro Treg differentiation and homeostasis, while rapamycin promotes Treg proliferation and accumulation in the periphery¹⁴–¹⁸. Conversely, pharmacological inhibition of PI3K signaling enhances in vitro Treg differentiation¹⁴, ¹⁹ and expression of a constitutively active Akt allele in Tregs leads to an overall dampening of the Treg gene signature, including reduced expression of Foxp3, Il2ra (CD25), and Ctla4¹⁶.

To define the cell-intrinsic function of the PI3K pathway in Treg homeostasis, we generated mice lacking expression of PTEN specifically within the Foxp3⁺ population, termed Pten-ΔTreg mice. Disruption of PTEN did not result in catastrophic decreases in Foxp3⁺ Tregs; in fact, there was an increase in the CD25⁻ subset of Foxp3⁺ cells. However, despite the continued presence of the Foxp3⁺ Treg population, Pten-ΔTreg mice developed a systemic polyclonal lymphoproliferative disease and renal failure. Female mice heterozygous for Foxp3-Cre were initially healthy, but over time their wild-type Treg compartment was replaced by PTEN-deficient Tregs, and they ultimately succumbed to disease. Further studies revealed that loss of PTEN was associated with disruptions in cellular metabolism and energy utilization. Finally, we found that elevation of PI3K signaling in PTEN-deficient regulatory T cells led directly to the successive loss of CD25 followed by Foxp3, and the emergence of ‘ex’-Foxp3 cells (i.e., cells which were once Foxp3⁺ but appear to have permanently lost Foxp3 expression). Together, these data show that control of PI3K signaling via PTEN is required to maintain Treg homeostasis, function and Foxp3 expression.
Results

Generation of Pten-ΔT\textsubscript{reg} mice

Pten\textsuperscript{fl/fl} and Foxp3-Cre mice were crossed to generate Pten-ΔT\textsubscript{reg} mice, which lack PTEN expression specifically in the Foxp3\textsuperscript{+} population of cells (Fig. 1a). Unlike animals lacking PTEN in all T cells\textsuperscript{19}, Pten-ΔT\textsubscript{reg} mice developed an expanded T\textsubscript{reg} population in multiple tissues at young ages, while maintaining normal T cell development (Fig. 1b, Supplementary Fig. 1a). Though these PTEN-deficient T\textsubscript{regs} were generally phenotypically normal (Supplementary Fig. 1b), they expressed substantially lower amounts of the high-affinity IL-2 receptor subunit, CD25, than did control Pten\textsuperscript{+/+} Foxp3-Cre T\textsubscript{regs} (Fig. 1c). To exclude the possibility that downregulation of CD25 was a consequence of unappreciated developmental abnormalities, we used a tamoxifen-inducible system to delete PTEN \textit{in vitro} from pre-existing Foxp3\textsuperscript{+} cells. Deletion of PTEN led to a marked reduction in CD25 expression (Fig. 1d), thus demonstrating that PTEN deletion is sufficient to downregulate CD25 in otherwise normal T\textsubscript{regs}.

Development of disease in Pten-ΔT\textsubscript{reg} mice

Despite their enlarged T\textsubscript{reg} compartment, Pten-ΔT\textsubscript{reg} mice developed peripheral lymphoproliferative disease (defined as grossly visible lymphadenopathy) with age (Fig. 2a) that appeared as early as 12 weeks of age, and by 28 weeks of age affected approximately 80% of animals. While lymphoproliferation affected secondary lymphoid tissue, thymic cellularity was not increased, most likely due to the affects of autoimmunity (see below) with B cell infiltration of the thymus (data not shown). Interestingly, the lymphocyte compartments of diseased Pten-ΔT\textsubscript{reg} mice were notable for a further increase in the population of Foxp3\textsuperscript{+}CD25\textsuperscript{−} cells compared to healthy mice, and a large expansion of activated CD4\textsuperscript{+}CD44\textsuperscript{hi}CD62L\textsuperscript{lo} T cells (Fig. 2b). Expanded CD44\textsuperscript{hi}CD62L\textsuperscript{lo} cells were seen within the T\textsubscript{reg} compartment itself, in association with elevated ICOS expression (Fig. 2c).

We observed a large accumulation of germinal center B cells in both healthy and diseased Pten-ΔT\textsubscript{reg} mice (Supplementary Fig. 2a), accompanied by high concentrations of serum IgG1, IgG2a/c and IgG2b, and a decrease in IgG3 in diseased mice (Fig. 2d). Consistent with elevations in immunoglobulin concentrations, Pten-ΔT\textsubscript{reg} mice had marked increases in T\textsubscript{FH} cell numbers (defined as CD4\textsuperscript{+}ICOS\textsuperscript{+}CXCR5\textsuperscript{+}Foxp3\textsuperscript{−} cells) in the spleen (Supplementary Fig. 2b). Increases in T\textsubscript{FR} cell numbers were also observed (Supplementary Fig. 2b), consistent with the general increases in T\textsubscript{reg} cell numbers observed in Pten-ΔT\textsubscript{reg} mice. In addition, when conventional T cells from diseased Pten-ΔT\textsubscript{reg} mice were stimulated \textit{ex vivo}, a large population of IFN-γ producing cells was detected (Fig. 2e). There was no increase in IL-17 production from T cells isolated from either spleen, mesenteric lymph node or colonic lamina propria (Fig. 2e and data not shown). Consistent with this, both T\textsubscript{regs} and conventional T cells isolated from Pten-ΔT\textsubscript{reg} mice had elevated expression of the chemokine receptor CXCR3, which promotes cell homing to sites of T\textsubscript{H}1-type inflammation (Supplementary Fig. 2c)\textsuperscript{20}. These data collectively suggest that Pten-ΔT\textsubscript{reg} mice develop a T\textsubscript{H}1-type lymphoproliferative disease involving both T and B cells.
Most visceral organs from aged and visibly ill *Pten*-Δ*T*<sub>reg</sub> mice had normal gross and histologic appearance (data not shown), with the notable exception of the kidneys (Fig. 2f), which revealed a proliferative glomerulonephritis, interstitial infiltrates and cortical atrophy. This histologic picture was accompanied by elevations in serum concentrations of anti-double stranded DNA antibodies and creatinine (Fig. 2g,h). The rise in serum creatinine concentrations corresponded temporally with the onset of visible lymphadenopathy, indicating that kidney failure and autoimmunity occurred concomitantly. As with secondary lymphoid organs, we found a large population of Foxp3<sup>+</sup>CD25<sup>−</sup> cells within the diseased kidneys (Supplementary Fig. 2d).

As PTEN deletion in T cells and non-lymphoid cells can lead to malignancy in mice and humans<sup>9, 21</sup>, we addressed the possibility that *Pten*-Δ*T*<sub>reg</sub> mice had developed malignant disease rather than autoimmunity. One characteristic of autoimmunity in thymectomized *Pten*-Δ*T* mice is the maintenance of normal TCR V<sub>β</sub> diversity, while PTEN-null T cell malignancies are monoclonal<sup>10</sup>. We found comparable TCR V<sub>β</sub> diversity in both naïve CD4<sup>+</sup> cells and T<sub>reg</sub> isolated from diseased mice, strongly suggesting that the hyperplastic cells were not malignant (Supplementary Fig. 2e). Finally, the expanded population of T cells also predominately expressed low amounts of CD24 (HSA), in contrast with what is observed in PTEN-deficient T cell malignancies, which, like thymocytes, are CD24<sup>hi</sup> (Supplementary Fig. 2e)<sup>10, 22</sup>. Thus the expanded population of T cells in *Pten*-Δ*T*<sub>reg</sub> mice is a polyclonal non-malignant population.

**Defective regulation by PTEN-deficient T<sub>regs</sub>**

As we observed elevated numbers of T<sub>regs</sub> in *Pten*-Δ*T*<sub>reg</sub> mice, we next used BrdU labeling to assess their *in vivo* proliferative capacity. While PTEN-deficient Foxp3<sup>+</sup>CD25<sup>+</sup> T<sub>regs</sub> were more proliferative than wild-type controls, BrdU incorporation was highest in the Foxp3<sup>+</sup>CD25<sup>−</sup> T<sub>reg</sub> subset (Fig. 3a). Interestingly, these proliferative differences in Foxp3<sup>+</sup>CD25<sup>+</sup> and Foxp3<sup>+</sup>CD25<sup>−</sup> cells were seen in both *Pten*-Δ*T*<sub>reg</sub> mice as well as wild-type controls (Fig. 3a). To further assess the *in vivo* maintenance of PTEN-deficient T<sub>regs</sub>, we utilized the X-linked nature of the *Foxp3*-Cre knock-in transgene and bred female *Pten<sup>fl/fl</sup>* mice to be heterozygous for *Foxp3*-Cre<sup>23</sup>. Due to random X-inactivation, *Foxp3*-Cre heterozygous females should maintain a 50:50 ratio of Cre<sup>+</sup>YFP<sup>+</sup> PTEN-deficient T<sub>regs</sub>:Cre<sup>−</sup>YFP<sup>−</sup> PTEN-sufficient wild-type T<sub>regs</sub>. This was the case in young Cre heterozygous mice, which did not develop activated T cell accumulation at the same time as their *Pten*-Δ*T*<sub>reg</sub> counterparts. However, over time, we found that the PTEN-deficient T<sub>regs</sub> were increased in number relative to wild-type T<sub>regs</sub> in the blood and lymph nodes of *Foxp3*-Cre heterozygous females (Fig. 3b and data not shown), and this was accompanied by the eventual onset of disease (Fig. 3c,d), presumably due to the loss of wild-type T<sub>regs</sub>.

From these data, we hypothesized that the lymphoproliferative disease seen in *Pten*-Δ*T*<sub>reg</sub> mice could result from three distinct, but non-mutually exclusive processes: PTEN-deficient T<sub>regs</sub> were unable to perform normal regulatory functions and control effector T cell responses; PTEN deletion caused T<sub>reg</sub> lineage instability, therefore generating pathogenic PTEN-deficient effector cells; and transient expression of Foxp3 by non-T<sub>regs</sub> caused...
aberrant excision of PTEN, leading to the generation of pathogenic PTEN-deficient non-
T\textsubscript{regs}.

We first excluded the possibility of transient expression of Foxp3 in non-T\textsubscript{regs} leading to Cre-mediated Pten excision by assessing Pten recombination status at the genomic locus in sorted cell populations from young, healthy Pten-\Delta\textsubscript{T\textsubscript{reg}} mice (Supplementary Fig. 3)\textsuperscript{24}. Using primers to specifically detect the deleted Pten locus, we found that recombination of Pten was only seen in Foxp3\textsuperscript{+} populations and in neither naïve nor activated T cells, indicating that Cre-mediated Pten excision was faithful and confined to the Foxp3\textsuperscript{+} T\textsubscript{reg} population.

Next, we used the experimental autoimmune encephalomyelitis (EAE) model to analyze the \textit{in vivo} functional capacity of PTEN-deficient T\textsubscript{regs}. We found that while the initial onset of disease was similar in Pten-\Delta\textsubscript{T\textsubscript{reg}} and wild-type mice, Pten-\Delta\textsubscript{T\textsubscript{reg}} mice were unable to spontaneously resolve the disease, a process which has previously been shown to be T\textsubscript{reg} dependent\textsuperscript{25}. Notably, progression of symptoms in Pten-\Delta\textsubscript{T\textsubscript{reg}} mice occurred despite the mice maintaining high numbers of Foxp3\textsuperscript{+} cells in secondary lymphoid organs as well as in the brain itself (Fig. 4a,b), and as would be anticipated, disease severity was accompanied by increased production of IFN-\gamma and IL-17 by effector T cells (Fig. 4c). Interestingly, however, we found that Foxp3\textsuperscript{+} cells in Pten-\Delta\textsubscript{T\textsubscript{reg}} mice produced IL-17 to a similar degree as did non-T\textsubscript{regs} (Fig. 4c), suggesting that these PTEN-deficient T\textsubscript{regs} might be pathogenic \textit{in vivo}.

The apparent functional defects of PTEN-deficient Foxp3\textsuperscript{+} cells in our \textit{in vivo} models led to the question of whether these cells were bona fide T\textsubscript{regs}. We performed transcriptional analysis on PTEN-deficient Foxp3\textsuperscript{+}CD25\textsuperscript{+} and Foxp3\textsuperscript{+}CD25\textsuperscript{-} cells and found that both populations of PTEN-deficient T\textsubscript{regs} maintained normal expression of Treg signature genes\textsuperscript{26} including Foxp3, Cila4 and Nrp1 (Supplementary Fig. 4). Collectively, these data show that Pten-\Delta\textsubscript{T\textsubscript{reg}} mice were unable to resolve the inflammatory insult of EAE, despite the continued presence of high numbers of T\textsubscript{regs}.

Elevated glycolytic activity in PTEN-deficient T\textsubscript{regs}

Metabolic programming may dictate T cell fate decisions that occur during differentiation\textsuperscript{27}; PI3K activity promotes glycolytic metabolism in various cell types including conventional T cells\textsuperscript{28}, while in contrast T\textsubscript{regs} favor oxidative metabolism to meet their energy demands\textsuperscript{29}. Strikingly, we observed that T\textsubscript{regs} from Pten-\Delta\textsubscript{T\textsubscript{reg}} mice had a much higher glucose-stimulated increase in extracellular acidification rate (ECAR – an indirect measurement of glycolysis), compared to wild-type T\textsubscript{regs} (Fig. 5b). The mitochondrial ATP synthase inhibitor oligomycin did not further increase ECAR but decreased the oxygen consumption rate (OCR – an indicator of mitochondrial respiration), suggesting that maximal glycolytic capacity was reached by the initial addition of glucose in both populations (data not shown). The return of ECAR to basal levels in response to the addition of 2-deoxy-glucose indicates that glucose-stimulated changes in ECAR were due to glycolysis (Fig. 5b). In contrast to this, the ATP coupled respiration and the spare respiratory capacity (SRC) of PTEN-deficient T\textsubscript{regs} as measured by the OCR, after addition of oligomycin and FCCP respectively was similar to that of wild-type T\textsubscript{regs} (Fig. 5c). Further, addition of etomoxir, which blocks
mitochondrial fatty acid oxidation, followed by rotenone and antimycin (to block complex-I and complex-III of the electron transport chain respectively) reduced the SRC in both PTEN-deficient T\textsubscript{regs} and wild-type T\textsubscript{regs} to a similar degree (Fig. 5c). This overlapping mitochondrial profile between the two populations suggests that mitochondrial function was unimpaired in T\textsubscript{regs} in the absence of PTEN. Finally, to independently confirm the increase in glycolytic metabolism of Pten\textsuperscript{−ΔT_{reg}} cells, we measured their glycolytic rate via assessing the generation of \textsuperscript{3}H\textsubscript{2}O from [3-\textsuperscript{3}H]-glucose. Consistent with our Seahorse data, we found that Pten\textsuperscript{−ΔT_{reg}} cells possessed a higher glycolytic rate than their wild-type counterparts upon activation (Fig. 5d). Together, these findings indicate that PTEN deficiency in T\textsubscript{regs} renders them hyperglycolytic, a bioenergetic state that could disrupt their homeostasis and cause functional instability.

**PTEN-deficient T\textsubscript{regs} are unstable**

While the severity of EAE-related inflammation in Pten\textsuperscript{−ΔT_{reg}} mice as well as the ultimate development of autoimmune disease onset in Cre heterozygous females may be a result of an inherent inability of PTEN-deficient T\textsubscript{regs} to perform normal regulatory function, these data could also indicate compromised stability of PTEN-deficient T\textsubscript{regs}, particularly during inflammatory settings. One potential marker of T\textsubscript{reg} instability as a result of PTEN loss would be methylation of the T\textsubscript{reg}-specific demethylated region (TSDR)\textsuperscript{30}. Methylation of the Foxp3 locus has been associated previously with maintenance of Foxp3 expression and resultant T\textsubscript{reg} stability\textsuperscript{30}. Therefore, we analyzed TSDR methylation in Foxp3\textsuperscript{+}CD25\textsuperscript{+} and Foxp3\textsuperscript{+}CD25\textsuperscript{−} cells purified by sorting from wild-type and Pten-\textsuperscript{ΔT_{reg}} mice. We observed a moderate reduction of TSDR demethylation in CD25\textsuperscript{+} PTEN-deficient T\textsubscript{regs} compared to wild-type CD25\textsuperscript{+} T\textsubscript{regs} (Fig. 6). Interestingly, a similar “loss” of demethylation was observed in both populations of Foxp3\textsuperscript{+}CD25\textsuperscript{−} T\textsubscript{regs}, which is consistent with lower expression of Foxp3 protein in both wild-type and PTEN-deficient CD25\textsuperscript{−} T\textsubscript{regs} (\textsuperscript{31–33} and Supplementary Fig. 5).

To more directly evaluate PTEN-deficient T\textsubscript{reg} stability, sort purified in vitro-induced Foxp3\textsuperscript{+}CD25\textsuperscript{+} T\textsubscript{regs} were cultured in the presence of IL-2. We observed that PTEN-deficient T\textsubscript{regs} began to downregulate CD25 expression by 48h, and subsequently lost expression of Foxp3 (Fig. 7a). Blockade of PI3K signals prevented both CD25 and Foxp3 loss, but had no effect on wild-type T\textsubscript{regs} (Fig. 7a). As loss of CD25 expression preceded loss of Foxp3, the Foxp3\textsuperscript{+}CD25\textsuperscript{−} phenotype may be an initial, and perhaps inciting, step in T\textsubscript{reg} destabilization. The fact that neither supplemental IL-2 nor PI3K inhibition alone was able to fully stabilize Foxp3 and CD25 expression (Supplementary Fig. 6a), provides further evidence for the importance of the proper integration and modulation of PI3K and CD25 signals in T\textsubscript{reg} stability.

As PI3K activation is upstream of both mTORC1 and mTORC2, we assessed the activation of these two arms of the mTOR pathway via the phosphorylation of their downstream targets S6 (mTORC1) and Akt (Serine 473, mTORC2). While PTEN-deficient T\textsubscript{regs} exhibited enhanced phosphorylation of Akt both at baseline and following TCR/CD28 stimulation, pS6 levels were not increased over control T\textsubscript{regs} either at rest or after stimulation (Supplementary Fig. 6b), indicating a preferential over-activation of mTORC2.
Similarly, inhibition of Akt (which is activated by PI3K and mTORC2) was more effective than rapamycin, which primarily affects mTORC1, at restoring CD25 expression on PTEN-deficient T\textsubscript{regs} \textit{in vitro} (Supplementary Fig. 6c).

STAT5 binds to the Foxp3 promoter, thus regulating its expression and possibly stability.\textsuperscript{5} As CD25 downregulation preceded loss of Foxp3 expression \textit{in vitro}, we assessed the downstream phosphorylation of STAT5 within PTEN-deficient Foxp3\textsuperscript{+}CD25\textsuperscript{+} T\textsubscript{regs}. Importantly, we found brief stimulation with IL-2 induced similar amounts of pSTAT5 in Foxp3\textsuperscript{+}CD25\textsuperscript{+} cells from both Pten\textsuperscript{−}\textsubscript{iΔT\textsubscript{reg}} mice and wild-type mice, demonstrating that elevated PI3K signaling does not alter the ability of T\textsubscript{regs} to respond to IL-2 stimulation (Fig. 7b).\textsuperscript{11}

As noted above, a deleted Pten allele was only detected in Foxp3\textsuperscript{+} cells in young, healthy Pten\textsuperscript{−}\textsubscript{iΔT\textsubscript{reg}} mice (Supplementary Fig. 3). Thus, we speculated that appearance of the genomic deletion in the Foxp3\textsuperscript{−} population would be suggestive of PTEN-deficient T\textsubscript{reg} instability. In fact, we found evidence of Pten deletion at the genomic level in the activated CD4\textsuperscript{+} CD44\textsuperscript{hi}CD62L\textsuperscript{lo}Foxp3\textsuperscript{−} population of cells in diseased mice (Supplementary Fig. 7), consistent with this hypothesis. To further examine if these PTEN-deleted effector cells did indeed derive from \textit{bona fide} T\textsubscript{regs}, we took advantage of fate-mapping to assess and quantify the loss of Foxp3 in T\textsubscript{regs} following deletion of PTEN. Pten\textsuperscript{fl/fl} mice were crossed to Foxp3\textsuperscript{eGFP-Cre-ERT2} Rosa26-YFP mice\textsuperscript{34} to generate Pten\textsuperscript{−}\textsubscript{iΔT\textsubscript{reg}} mice. In these animals, treatment with tamoxifen leads to deletion of PTEN in Foxp3\textsuperscript{+} cells, which are simultaneously “marked” by excision of a stop codon in the ubiquitously expressed Rosa26 locus, allowing the transcription and expression of YFP. While in theory, eGFP can be used as a surrogate for Foxp3 expression, in practice we found that the eGFP signal in the Foxp3 locus was relatively weak compared with the much stronger YFP signal in the Rosa26 locus (data not shown). This, coupled with the known high degree of spectral emission overlap between GFP and YFP, made it simpler to assess Foxp3 expression directly by antibody staining while preserving Rosa26-YFP expression (Supplementary Fig. 8). A population of Foxp3\textsuperscript{−} cells within the YFP\textsuperscript{+} population could be distinguished in the blood of both Pten\textsuperscript{−}\textsubscript{iΔT\textsubscript{reg}} mice and wild-type Foxp3\textsuperscript{−}\textsubscript{Cre} Rosa26-YFP mice (iFoxp3-Cre) as early as 1 week after initiation of a 5 day course of tamoxifen (Fig. 8a). Notably, by 10 weeks post-tamoxifen treatment, the percentage of ‘ex’-Foxp3 cells in Pten\textsuperscript{−}\textsubscript{iΔT\textsubscript{reg}} mice was ~30%, i.e., three times higher than that seen in control animals (Fig. 8a). This increase in ‘ex’-Foxp3 cells persisted for as long as 18 weeks post-tamoxifen (the last time point at which animals were bled). Confirming data from female Pten\textsuperscript{−}\textsubscript{T\textsubscript{reg}} mice heterozygous for Foxp3\textsuperscript{−}\textsubscript{Cre}, reduction of CD25 expression was specific for T\textsubscript{regs} in which PTEN was deleted (Fig. 8b). As ‘ex’-Foxp3 cells were observed in both control and Pten\textsuperscript{−}\textsubscript{iΔT\textsubscript{reg}} mice, we also examined their expression of CD25, and found similar reductions in CD25 expression following loss of Foxp3 in both animals (Fig. 8c). Collectively, these data indicate that PTEN is important for the maintenance of T\textsubscript{reg} CD25 expression and T\textsubscript{reg} stability \textit{in vivo}.

**Discussion**

The data presented in this manuscript, together with another group\textsuperscript{35}, demonstrate that PTEN-mediated control of PI3K activity is critical for the function and stability of murine
Foxp3+ regulatory T cells. Disruption of PTEN results in reduced CD25 expression, the accumulation of Foxp3+CD25− cells, and ultimately, the loss of Foxp3 expression. Prior to the discovery and characterization of the Foxp3 transcription factor, high expression of CD25 was used to demarcate Tregs from effector T cells. As such, little relevance has been attributed to the small portion of Foxp3+CD25− cells, and they have only been assessed in minimal detail compared to canonical Foxp3+CD25+ cells. Here, we have identified a population of Foxp3+CD25− cells in Pten-ΔTreg mice, which appear to be intermediates during the complete destabilization of canonical Foxp3+CD25+ Tregs. Accordingly, these Foxp3+CD25− “Treg” cells displayed a transitional phenotype, where their Treg transcriptional profile was maintained despite a partially methylated Foxp3 TSDR, consistent with their lower Foxp3 protein expression.

We observed nearly normal CD25 expression in newly-generated PTEN-deficient Tregs in the thymus, suggesting that the destabilization process occurred after the generation of canonical Tregs expressing high levels of CD25. Strikingly, the small population of Foxp3+CD25− “Treg” present in wild-type mice was seen to have much the same transcriptional profile as their PTEN-deficient counterparts. It is possible that loss of PTEN expression causes proliferation and/or confers a survival advantage upon a normal population of unstable Foxp3+CD25− cells, which are non-pathogenic in wild-type mice either due to proper regulation by Foxp3+CD25+ Tregs or because they are short-lived.

Alternatively, the large population of Foxp3+CD25−ICOS+ cells in Pten-ΔTreg mice could be an expanded population of effector Tregs (eTreg), which have been reported to express low CD25 at the steady state and require ICOS rather than IL-2 for maintenance. Interestingly, inflammation has been shown to drive the proliferation of eTreg and skew cells away from a cTreg phenotype. This finding, along with our data above, suggest that PTEN loss may be crucial in either eTreg differentiation or maintenance.

The functional stability of Tregs remains a controversial issue, with several conflicting reports regarding the existence, origin and physiological/pathological role of unstable Tregs. Recently, non-Treg cells have been demonstrated to transiently express Foxp3 upon activation, thus generating a small population of ‘ex’-Foxp3 cells, which are not ‘ex’-Treg. These data indicated that the Treg lineage itself was stable, but “unfaithful” expression of Foxp3 may occur, leading to a population of cells with unstable Foxp3 expression. However, the loss of Foxp3 in bona fide Tregs occurs in multiple autoimmune settings. Activated by self-antigen, these destabilized Tregs acquired effector function and pathogenicity in models of autoimmunity. Consistent with these findings, we find no evidence that PTEN deletion is occurring in non-Tregs. We show that ‘ex’-Foxp3 cells may also be generated from bona fide Foxp3+CD25+ Tregs that lack PTEN in a stepwise manner in vitro, with loss of CD25 preceding loss of Foxp3. Finally, using lineage reporter mice, we observe that the basal rate of generation of ‘ex’-Foxp3 cells from Tregs in vivo increases approximately three-fold when PTEN is absent.

Despite a normal transcriptional profile compared to PTEN-sufficient Tregs, Foxp3+CD25− cells expressed lower Foxp3 protein than canonical Foxp3+CD25+ Tregs, suggesting that the Foxp3+CD25− Treg population may be enriched for cells with unstable Foxp3 expression. Interestingly, this difference in Foxp3 expression was observed in wild-type Tregs as well as in PTEN-deficient Tregs.
in PTEN-deficient T\(_{\text{regs}}\); this association of Foxp3 and CD25 expression has been seen by other groups previously\(^{31-33}\).

The etiology of lymphoproliferation and autoimmunity in \(\text{Pten-}\Delta \text{T}_{\text{reg}}\) mice may be multifactorial. We provide evidence that PTEN-deficient T\(_{\text{regs}}\) exhibit lineage instability and can convert into Foxp3\(^{-}\) cells. As the TCR repertoire of T\(_{\text{regs}}\) may be biased to self-antigen recognition\(^{39}\), this suggests the possibility that disease may be caused by conversion of PTEN-deficient T\(_{\text{regs}}\) into pathogenic effectors. Although this process of Foxp3 loss in T\(_{\text{regs}}\) occurs in wild-type mice as well, the absence of disease might be due to the comparative lesser degree of T\(_{\text{reg}}\) instability and/or the fact that loss of PTEN confers an important survival advantage\(^{10}\) upon ‘ex’-Foxp3 cells. However if ‘ex’-Foxp3 cells are pathogenic, they must be “restrainable” by wild-type T\(_{\text{regs}}\), as female mice heterozygous for \(\text{Foxp3-Cre}\) do not become ill until their wild-type T\(_{\text{regs}}\) are largely lost, and \(\text{Pten-i}\Delta \text{T}_{\text{reg}}\) remain healthy after tamoxifen injection and resultant loss of Foxp3 in a substantial percentage of T\(_{\text{regs}}\). Alternatively, ‘ex’-Foxp3 cells that accumulate as a result of PTEN loss may not be pathogenic \(\text{per se}\), with disease in \(\text{Pten-}\Delta \text{T}_{\text{reg}}\) mice being a consequence of an intrinsic defect in regulation by PTEN-deficient T\(_{\text{regs}}\). The inability of \(\text{Pten-}\Delta \text{T}_{\text{reg}}\) mice to resolve EAE in consistent with this possibility.

Activation of signaling through PI3K and mTOR inhibits peripheral T\(_{\text{reg}}\) induction\(^{14}\). One possible mechanism for PI3K control of T\(_{\text{regs}}\) is via the Foxo1 and Foxo3a (Foxos) transcription factors, which bind to and promote Foxp3 transcription, but are inactivated by PI3K\(^{-}\). Indeed, deletion of Foxos severely inhibits both T\(_{\text{reg}}\) generation and function \(\text{in vivo}\), while constitutive nuclear localization of Foxos prevents these defects\(^{18}\). Additionally, the maintenance of T\(_{\text{reg}}\) stability and function by the Neuropilin-1 (Nrp-1)/Semaphorin 4a (Sema4a) signaling axis is due in part to upregulation of PTEN and subsequent nuclear localization of Foxos upon Nrp-1 ligation\(^{40}\). Interestingly however, \(\text{Pten-}\Delta \text{T}_{\text{reg}}\) mice develop a different phenotype than animals in which T\(_{\text{regs}}\) lack Foxo1 expression (as the latter does not affect CD25\(^{-}\) expression, nor promote inflammation induced T\(_{\text{reg}}\) instability) and thus have heightened mTORC2 activation\(^{18}\). These disparate phenotypes suggest that multiple factors downstream of PTEN are critical in instructing the T\(_{\text{reg}}\) lineage.

Modulation of PI3K signaling can alter cellular metabolism\(^{27}\). For example, overexpression of PTEN inhibits glycolysis and skews energy generation toward oxidative metabolism\(^{41}\). Interestingly, T\(_{\text{regs}}\) preferentially utilize lipid oxidation for energy, a process characterized by high AMP-activated protein kinase (AMPK) activity\(^{29}\). This contrasts with effector T cells, which primarily utilize glycolysis as a means of energy generation\(^{29,42}\). Our data demonstrate that loss of PTEN enhances glycolysis in T\(_{\text{regs}}\). The importance of metabolic programs in cellular activation and cell fate decisions is well recognized\(^{43,44}\), including a role in determining if cells become \text{in vitro} induced T\(_{\text{regs}}\) or induced effectors\(^{29}\). Whether alterations in metabolism can affect a pre-existing lineage decision is not known, however the finding that T\(_{\text{reg}}\) loss of PTEN shifts their metabolic profile suggests that this may be a contributing mechanism by which PTEN deletion destabilizes the T\(_{\text{reg}}\) lineage, perhaps via an effect on CD25 expression, which precedes loss of Foxp3 during \text{in vitro} culture.
T cells may express other lipid phosphatases in addition to PTEN that control PI3K signals. One such family, the PH-domain leucine-rich repeat protein phosphatase (PHLPP), is expressed at high levels in murine and human T_{regs}, and is required for optimal iT_{reg} development and T_{reg} function. Our data indicate that despite the expression of additional lipid phosphatases in T_{regs}, PTEN has a non-redundant function in PI3K control and T_{reg} maintenance.

The identification of PI3K as a nodal control point for T_{reg} homeostasis is particularly relevant given the clinical development of PI3K inhibitors for the treatment of autoimmunity or cancer. In the latter arena, the target of PI3K inhibition is the tumor cell itself, although interestingly it was reported recently that inactivation of PI3Kδ in T_{regs} disabled tumor-induced immune suppression and enabled immune-mediated tumor regression in murine models. Whether PI3K blockade has a dominant role in inhibiting regulatory or effector T cell responses is likely to be context and dose-dependent. Our own results showing that high PI3K activity may cripple regulatory responses underscore the complexity of immune system control via this pathway.

**Online methods**

**Mice**

\( Pten^{\text{fl/fl}} \) mice were bred with \( \text{Foxp3-YFP-Cre} \) knock-in mice provided by A. Rudensky (Memorial Sloan Kettering Cancer Center, New York, NY), to generate \( Pten-\Delta T_{\text{reg}} \) mice or with \( \text{Foxp3-GFP-Cre-ERT2 Rosa26-YFP} \) mice provided by A. Rudensky) to generate mice with tamoxifen inducible loss of PTEN in T_{regs} \( \text{(Pten-i\Delta T_{reg}} \) mice). Control animals were either littermates or appropriate age/sex-matched \( Pten^{+/+} \text{Foxp3-YFP-Cre or Foxp3-GFP-Cre-ERT2 Rosa26-YFP} \) mice. \( \text{Foxp3-GFP-hCre} \) mice and \( \text{Rosa26 cre-ER} \) mice have been previously described. Unless otherwise stated, all figures are representative of experiments performed with healthy 6 – 8 week old mice. All mice were housed in specific pathogen-free conditions at Massachusetts General Hospital and the University of California, San Francisco, and all procedures were performed in accordance with protocols approved by each institution’s Institutional Animal Care and Use Committee.

**Detection of PTEN protein and assessment of the \( Pten \) genomic locus**

Specific deletion of PTEN in Foxp3^{+} cells at the protein level was assessed via western blotting as described previously. PTEN recombination at the genomic DNA level was performed on highly pure sorted cell populations using previously described primers to specifically detect \( Pten^{\text{flox}} \) and deleted \( Pten \) alleles.

**Flow cytometry and cell sorting**

Fluorescent anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD24 (30-F1), anti-CD25 (PC-61), anti-CD39 (Duha59), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-CD73 (TY/11.8), anti-CD122 (5H4), anti-CD132 (TUGm2), anti-CXCR3 (CXCR3-173), anti-CXCR5 (L138D7), anti-ICOS (7E.17G9), anti-IFN-\( \gamma \) (XMG1.2), anti-IL-17 (TC11-18H10.1), anti-TCRV\( \beta \)2 (B20.6) and anti-TCRV\( \beta \)8 (MR5-2) antibodies were purchased from Biolegend, while anti-PTEN (A2B1), anti-CTLA-4 (UC10-4F10-11), anti-PD-1 (29F.1A12) and anti-CD127 (SB/
199) fluorescent antibodies were purchased from BD Biosciences. Anti-Foxp3 intracellular staining kit and anti-Foxp3 (FJK-16s) antibodies were purchased from eBioscience. For intracellular cytokine staining, cells were restimulated with Leukocyte Activation Cocktail (BD Biosciences) for 5 h at 37°C. For phosoho-flow cytometry, cells were serum starved at 37°C for 1 h and activated for 15 minutes with either IL-2 (pSTAT5) or αCD3 and αCD28 (pAkt and pS6). Cells were fixed with paraformaldehyde and permeabilized with methanol. Unconjugated anti-pSTAT5, anti-Akt Ser 473 and anti-S6 antibodies were purchased from Cell Signaling and secondary detection antibody was purchased from Jackson ImmunoResearch. For flow cytometric determination of PTEN, cells were stained with anti-CD25 and anti-CD4 antibodies and then fixed and permeabilized with BD cytofix/cytoperm reagent to preserve endogenous YFP fluorescence. Cells were then stained with an anti-PTEN antibody and run on LSR II flow cytometer. Cells were analyzed on a BD FACSCalibur, BD LSRII or Beckman Coulter Navios and sorted on a BD FACS Aria II to over 95% purity; data were analyzed using FlowJo (Tree Star).

**T_reg culture conditions**

Tregs were cultured in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, non-essential amino acids, sodium pyruvate, L-glutamine, HEPES, β-ME with 200 (for *in vitro* induced Tregs) or 2,000 IU/ml (nTregs) recombinant human IL-2 (Chiron). *In vitro* induction of Foxp3 from naïve CD4+ T cells was done by stimulation of T cells with plate-bound anti-CD3 (0.1 μg/ml clone 145-2C11 overnight) and anti-CD28 (1 μg/ml clone PV-1 overnight) in the presence of 5 ng/ml TGF-β. PI3K inhibitors were used at the following concentrations: 10 μM LY294002, 100 nM Rapamycin, 100 nM Wortmannin, 1 μM Akt Inhibitor VIII.

**Acute deletion of PTEN *in vitro***

Tregs from *Pten*+/+ or *Pten*fl/fl mice that also contained Rosa26-CreER were sorted by expression of CD4+CD25+CD62L+ and stimulated *in vitro* with anti-CD3 and anti-CD28 coated beads (Dynabeads Mouse T-Activator CD3/CD28, Invitrogen). On day 4 of culture, cells were either treated with 500 nM 4-hydroxytamoxifen (4-OHT, Sigma) or vehicle (ethanol) for the duration of the experiment. CD25 expression was assessed on CD4+Foxp3+ cells 3 and 10 days after start of treatment.

**ELISA**

Serum was collected from mice via cardiac puncture for serum Igs and anti-double stranded DNA (anti-dsDNA) antibodies or by serial bleeding for serum creatinine. Immunoglobulin concentrations were detected using the eBioscience Ready-SET-Go! Mouse Ig Isotyping ELISA kit, creatinine concentrations were detected using an ELISA kit from US Biological following the manufacturer’s instructions and anti-dsDNA antibodies were detected with an ELISA kit from Alpha Diagnostic as per the manufacturer’s instructions.
**In vivo BrdU labeling**

Mice were injected with 1 mg BrdU (BD Biosciences) i.p. every 12 h for 3 consecutive days. Mice were sacrificed 12 hours after the final injection and cells were stained using a BrdU labeling kit (BD Biosciences) following the manufacturer’s instructions.

**Induction of EAE**

Mice were immunized subcutaneously with MOG 35–55 peptide (UCLA Biopolymers Core) emulsified in Complete Freund’s Adjuvant (CFA, Difco) with M. tuberculosis (Difco). Additionally, mice were injected with Pertussis toxin (PT, List Biological Laboratories) i.p. on days 0 and 2 following immunization. Animals were evaluated daily in a blinded manner for signs of disease by the following criteria: 0: no disease; 1: flaccid tail paralysis; 2: hind limb paresis; 3: bilateral hind limb paralysis; 4: fore and hind limb paralysis.

**Tamoxifen treatment**

Foxp3^eGFP-Cre-ERT2^ Rosa26-YFP (iFoxp3^ Cre) and Pten^fl/fl^ Foxp3^eGFP-Cre-ERT2^ Rosa26-YFP (Pten-ΔT_reg) mice were injected with 2 mg of tamoxifen (Sigma) i.p. for 5 consecutive days to induce Foxp3^eGFP-Cre-ERT2^ activity. To simultaneously detect Foxp3 by antibody staining while preserving Rosa-YFP fluorescence, cells were pre-fixed with 2% paraformaldehyde (Electron Microscopy Sciences), fixed and permeabilized using an eBioscience kit and stained intracellularly with an anti-Foxp3 antibody as above.

**Histology**

Histologic analysis was performed as previously described^{23}.

**Metabolic studies**

Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured by the glycolysis-stress and mito-stress tests, respectively using the XF24 extracellular flux analyzer (Seahorse Bioscience). Briefly, sorted cells were rested for 20–22 hrs in the presence of 1ng/ml IL-2 and 5ng/ml IL-7 followed by the glycolysis-stress test that consisted of seeding 0.5–1 × 10^6 purified cells in glucose free XF Assay media onto a 24 well XF plate coated with cell tak (BD biosciences, CN 354240) followed by sequential addition of glucose (20mM), oligomycin (1μM) and 2-deoxy glucose (20mM). The mito-stress test was performed using glucose (25mM) containing XF assay media and consisted of sequential addition of oligomycin (1μM), FCCP (1.5μM), etomoxir (200μM) followed finally by rotenone (100nM) and antimycin (1μM).

The glycolytic rate was determined by measuring the conversion of 5-^3^H glucose to tritiated ^3^H2O as described previously^{50}. Briefly, 0.5–1.5 × 10^6 purified cells were either unstimulated or stimulated in the presence of plate-bound anti-CD3 (5ug/ml) and anti-CD28 (5ug/ml) and IL-2: 200U/ml. Cells were harvested between 22–24hrs, washed in 1X PBS, resuspended and incubated in Krebs buffer (pH 7.4, 115 mM NaCl, 2 mM KCl, 25mM NaHCO3, 1mM MgCl2, 0.25% BSA) for 30 min at 37°C. Following incubation, 10μCi of radio-labeled 5-^3^H glucose along with 5 μl 1M cold glucose was added to the cells and...
incubated for another additional 1 h at 37°C. Reactions were quenched by addition of 0.5ml of 0.2M HCl. The diffusion chamber setup, the scintillation counting and the calculation of the glycolytic rate were performed as described previously. A cell free sample with 10μCi of radio-labeled 5-3H glucose was included as a negative control and 0.5 μCi of radio-labeled 3H2O was included as a positive control.

**Methylation analysis of Foxp3 locus**

Genomic DNA from sorted cells was bisulfite converted using the EZ DNA Methylation-Direct kit (Zymo Research) according to the manufacturer’s protocol. Methylation-specific PCR primer sequences 5′-TATT TTTTTGGGTTTTGGGATATTA-3′ (forward) and 5′-AACCAACCACTTCTT ACACATATCTAT-3′ (reverse) were used to amplify intron 1 of Foxp3 (corresponding to Foxp3 CNS2). PCR products were subcloned into pGEM-T Easy vectors (Promega) and sequenced (9–21 sequences per mouse).

**Transcriptional profiling**

Foxp3+CD25+ and Foxp3+CD25− cells were double-sorted to high purity, and RNA was extracted with TRizol (Life Technologies) according to the manufacturer’s instructions. Samples were run in triplicate using a GeneChip Mouse Gene ST 1.0 Array (Affymetrix) and analyzed using GENE-E, GenePattern and Gene Set Enrichment Analysis (Broad Institute).

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Characterization of Pten-ΔT<sub>reg</sub> mice. (a) Foxp3<sup>+</sup> and Foxp3<sup>−</sup> cells were sort purified from the indicated mice and cell populations were analyzed by immunoblotting for PTEN and total Akt. (b) Analysis of CD4 and Foxp3 in cells isolated from the indicated tissues (pLN, peripheral lymph node; mLN, mesenteric lymph node). n = 10, representative of 10 experiments; *p < 0.001, **p < 0.0001 by two-way ANOVA. (c) Expression of CD25 on Foxp3<sup>+</sup> cells isolated from thymus, spleen, pLN and mLN from Pten-ΔT<sub>reg</sub> and wild-type mice. n = 10, representative of 10 experiments; *p < 0.05, **p < 0.01, ***p < 0.001 by two-way ANOVA. (d) CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>+</sup> sorted T<sub>regs</sub> from Pten<sup>+/+</sup> or Pten<sup>-/-</sup> Rosa26-CreER mice were cultured for the indicated times in vitro in the presence of 4-OH tamoxifen (4-OHT) to induce Cre-mediated excision of Pten and assessed for CD25 expression. Representative of 2 experiments. All error bars shown are mean ± SD.
Figure 2.

Pten-ΔT<sub>reg</sub> mice develop spontaneous systemic lymphoproliferative disease with age. (a) (Left) Cell numbers from the indicated tissues of Foxp3-Cre control mice, healthy appearing Pten-ΔT<sub>reg</sub> mice, and diseased Pten-ΔT<sub>reg</sub> mice (as assessed by gross lymphadenopathy, typical age 15 – 18 weeks). n = 3 for diseased Pten-ΔT<sub>reg</sub> or 5 for other groups; representative of 5 experiments; *p < 0.0001, **p < 0.01 by two-way ANOVA. (b) Flow cytometric analysis of CD4<sup>+</sup> cells (top) and CD4<sup>+</sup>Foxp3<sup>−</sup> cells (bottom) from spleen of wild-type mice, Pten-ΔT<sub>reg</sub> mice, and diseased Pten-ΔT<sub>reg</sub> mice. Representative of 10 experiments. (c) Foxp3<sup>+</sup>T<sub>reg</sub>s were isolated from wild-type, Pten-ΔT<sub>reg</sub> and diseased Pten-ΔT<sub>reg</sub> mice and analyzed for expression of CD44, CD62L and ICOS. Representative of 10 experiments. (d) Serum IgG1, IgG3a/c, IgG2b, IgG3, IgA and IgM concentrations from the three groups of mice as defined above. n = 10 for diseased Pten-ΔT<sub>reg</sub> and 5 for other groups, representative of 3 experiments; *p < 0.0001, **p < 0.001, ***p = 0.0001 by one-way ANOVA. (e) CD4<sup>+</sup> cells from lymph nodes of control mice and diseased Pten-ΔT<sub>reg</sub> mice were isolated, restimulated ex vivo and stained for IFN-γ and IL-17, representative of 5 experiments. (f) Hematoxylin and eosin staining of renal tissue from a representative wild-type (top) and Pten-ΔT<sub>reg</sub> mouse (bottom); 10x magnification. (g) Serum creatinine concentrations in Pten-ΔT<sub>reg</sub> mice with age. n = 8, representative of 2 experiments. Red symbols indicate age at which visible lymphadenopathy occurred; red line indicates the level at which kidney failure is diagnosed in mice. (h) Anti-double stranded DNA antibody
concentrations in $Pten$-$\Delta T_{reg}$ mice. $n = 10$, representative of 2 experiments; *$p < 0.0001$ by one-way ANOVA. All error bars shown are mean ± SD.
Figure 3.
Expansion of PTEN-deficient T\(_{\text{reg}}\)s in vivo. (a) Wild-type and Pten-ΔT\(_{\text{reg}}\) mice were injected i.p. with BrdU every 12 h for 3 consecutive days. Cells were isolated from thymus, spleen, pLN and mLN and assessed for BrdU incorporation. \(n = 9\), representative of 4 experiments; *\(p < 0.001\), **\(p < 0.002\), ***\(p < 0.0001\) by t-test. (b) Female Pten\(^{fl/fl}\) mice were bred to express one allele of Foxp3-Cre (Cre heterozygous mice) and followed over time along with female Pten-ΔT\(_{\text{reg}}\) mice (homozygous for Foxp3-Cre). \(n = 20\). (c) Flow cytometric analysis of peripheral blood CD4\(^{+}\)Foxp3\(^{-}\) T cells from mice as in panel b plus a wild-type Foxp3-Cre control mouse. (d) Aged Pten\(^{fl/fl}\) Cre heterozygous female mice develop disease similar to Pten-ΔT\(_{\text{reg}}\) mice with delayed onset, \(n = 20\) (Cre het) or 35 (Pten-ΔT\(_{\text{reg}}\)). All error bars shown are mean ± SD.
Figure 4.
Loss of PTEN prevents the ability of T<sub>reg</sub> to resolve autoimmune inflammation. (a) Wild-type and Pten-ΔT<sub>reg</sub> mice were immunized s.c. with myelin oligodendrocyte peptide emulsified in complete Freund’s adjuvant (CFA) to induce experimental autoimmune encephalomyelitis (EAE), and clinical disease severity was monitored for 35 days post-immunization. n = 10, representative of 3 experiments; p < 0.0001 by two-way ANOVA. (b) Representative flow cytometric analysis for CD4 and Foxp3 in the spleen, draining lymph nodes and brain of mice in panel a at day 35. (c) T cells as in panel b were restimulated <i>ex vivo</i> and assessed for production of IFN-γ and IL-17. Left set of panels are gated on CD4<sup>+</sup>Foxp3<sup>−</sup> cells and the right set of panels are gated on CD4<sup>+</sup>Foxp3<sup>+</sup> cells. n = 5, representative of 3 experiments. (d) Compilation of data from the experiments in c. N.D. = not detected; *p < 0.0001, **p < 0.001, ***p < 0.01 by two-way ANOVA. All error bars shown are mean ± SD.
Figure 5.
PTEN deficiency skews T<sub>reg</sub> metabolism toward glycolysis. (a, b) T<sub>reg</sub>s from Pten-ΔT<sub>reg</sub> and wild-type mice were purified and subjected to a glycolysis stress (a) or mito-stress test (b) to assess the bioenergetic profile of these cells. \( n = 3 \) samples per group, representative of 2 experiments. (c) The indicated populations of cells were isolated from Pten-ΔT<sub>reg</sub> or wild-type mice, stimulated and treated with \([3-3^H]\) glucose to measure the generation of \(^3\text{H}_2\text{O}\) as an indicator of the glycolytic rate of cells. \( n = 4 \), representative of 3 experiments; \(*p < 0.01\) by two-way ANOVA. All error bars shown are mean ± SD.
Figure 6.
TSDR methylation analysis. Methylation status of CpG motifs of the Foxp3 locus was assessed by bisulfite sequencing of the indicated populations of sorted cells (data were averaged from 4 – 5 mice per group and > 76 total sequences per group).
Figure 7.
PTEN deletion leads to Treg instability in vitro. (a) CD4+Foxp3+CD25+ iTregs from Pten\textsuperscript{fl/fl} Foxp3\textsuperscript{GFP-hCre} mice with indicated genotypes were sorted and cultured in the presence of IL-2 with or without the PI3K inhibitor (10 μM LY294002) for 6 d and expression of Foxp3 and CD25 was monitored at indicated times. Representative of 3 experiments. (b) Foxp3\textsuperscript{+} cells were isolated from wild-type and Pten-ΔT\textsubscript{reg} mice, serum starved for 1 h and stimulated for 30 min in the presence of 200 IU/ml hIL-2. Cells were then immediately fixed, permeabilized and assessed for pSTAT5 levels by phospho-flow cytometry. Grey = isotype control. Representative of 3 experiments.
Figure 8.
PTEN deletion leads to T\textsubscript{reg} instability \textit{in vivo}. Control and Pten-i\Delta T\textsubscript{reg} mice were treated with a 5 d course of tamoxifen as per Materials and Methods. Peripheral blood was assessed at the indicated time points. (a) Detection and quantification of ‘ex’-Foxp3 cells. All plots are gated on CD4\textsuperscript{+}YFP\textsuperscript{+} cells, except Week -1, which is gated on total CD4\textsuperscript{+} cells. \(n=5\), representative of 3 experiments; *\(p<0.05\), **\(p<0.0001\) by two-way ANOVA. (b) Expression of CD25 on Foxp3\textsuperscript{+} cells. CD25 staining of Foxp3\textsuperscript{+}YFP\textsuperscript{+} T\textsubscript{regs} (unshaded) and Foxp3\textsuperscript{+}YFP\textsuperscript{-} (shaded) unlabeled T\textsubscript{regs} from a representative animal at week 20 as per panel a. \(n=5\), representative of 3 experiments, *\(p<0.0001\) by two-way ANOVA. (c) Expression of CD25 on ‘ex’-Foxp3 cells. CD25 staining of Foxp3\textsuperscript{-}YFP\textsuperscript{+} (dashed) and Foxp3\textsuperscript{+}YFP\textsuperscript{+} (solid) cells for the animal shown in panel b. \(n=5\), representative of 3 experiments; *\(p<0.0001\) by two-way ANOVA. All error bars are mean ± SD.