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*J Clin Invest.* 2006;116(9):2521-2531. <https://doi.org/10.1172/JCI28057>.

Research Article Immunology

One of the greatest barriers against harnessing the potential of CD4<sup>+</sup>CD25<sup>+</sup> Tregs as a cellular immunotherapy is their hypoproliferative phenotype. We have previously shown that the hypoproliferative response of Tregs to IL-2 is associated with defective downstream PI3K signaling. Here, we demonstrate that targeted deletion of the lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10) regulates the peripheral homeostasis of Tregs in vivo and allows their expansion ex vivo in response to IL-2 alone. PTEN deficiency does not adversely affect either the thymic development or the function of Tregs, which retain their ability to suppress responder T cells in vitro and prevent colitis in vivo. Conversely, reexpression of PTEN in PTEN-deficient Tregs as well as in activated CD4<sup>+</sup> T cells inhibits IL-2–dependent proliferation, confirming PTEN as a negative regulator of IL-2 receptor signaling. These data demonstrate that PTEN regulates the “anergic” response of Tregs to IL-2 in vitro and Treg homeostasis in vivo and indicate that inhibition of PTEN activity may facilitate the expansion of these cells for potential use in cellular immunotherapy.

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# PTEN inhibits IL-2 receptor–mediated expansion of CD4<sup>+</sup>CD25<sup>+</sup> Tregs

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**One of the greatest barriers against harnessing the potential of CD4<sup>+</sup>CD25<sup>+</sup> Tregs as a cellular immunotherapy is their hypoproliferative phenotype. We have previously shown that the hypoproliferative response of Tregs to IL-2 is associated with defective downstream PI3K signaling. Here, we demonstrate that targeted deletion of the lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10) regulates the peripheral homeostasis of Tregs in vivo and allows their expansion ex vivo in response to IL-2 alone. PTEN deficiency does not adversely affect either the thymic development or the function of Tregs, which retain their ability to suppress responder T cells in vitro and prevent colitis in vivo. Conversely, reexpression of PTEN in PTEN-deficient Tregs as well as in activated CD4<sup>+</sup> T cells inhibits IL-2–dependent proliferation, confirming PTEN as a negative regulator of IL-2 receptor signaling. These data demonstrate that PTEN regulates the “anergic” response of Tregs to IL-2 in vitro and Treg homeostasis in vivo and indicate that inhibition of PTEN activity may facilitate the expansion of these cells for potential use in cellular immunotherapy.**

## Introduction

CD4<sup>+</sup>CD25<sup>+</sup> Tregs are a naturally occurring population of T lymphocytes with a key role in suppressing the response of self-reactive T cells that escape negative selection in the thymus (1). In addition to regulating responses against self antigens, it is also well established that Tregs can exert potent suppressive effects against most other types of T cell–mediated immune responses (1). These observations highlight the potential of Treg subsets for use as cellular immunotherapy to inhibit the undesirable effects of certain classes of immune response, such as those observed in autoimmune disease and transplant rejection (2–4). However, efforts to harness the therapeutic potential of these cells are significantly hindered by the relatively low number of Tregs present in the periphery of normal healthy individuals as well as their anergic phenotype ex vivo (5, 6). Accordingly a number of studies have reported strategies to overcome these obstacles, largely by using costimulatory antibodies against CD3 and CD28 in conjunction with extremely high doses of IL-2 (3, 7).

Tregs constitutively express all 3 chains ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) of the high-affinity IL-2 receptor (IL-2R), and recent evidence indicates that a primary nonredundant function of IL-2 in vivo is regulation of self tolerance through an essential role in the development and homeostasis of CD4<sup>+</sup>CD25<sup>+</sup> Tregs (8, 9). Despite the widely recognized importance of IL-2 in Treg homeostasis, very little is known about the intracellular mechanisms that regulate IL-2R signaling in these cells. Indeed, a defining characteristic of Tregs is their inability to expand in vitro upon stimulation with IL-2 alone despite expression of all 3 chains of the high-affinity IL-2R (10). This observation

is in direct contrast with the established promitogenic effects of IL-2R signaling in activated IL-2R<sup>+</sup> non-Tregs (11).

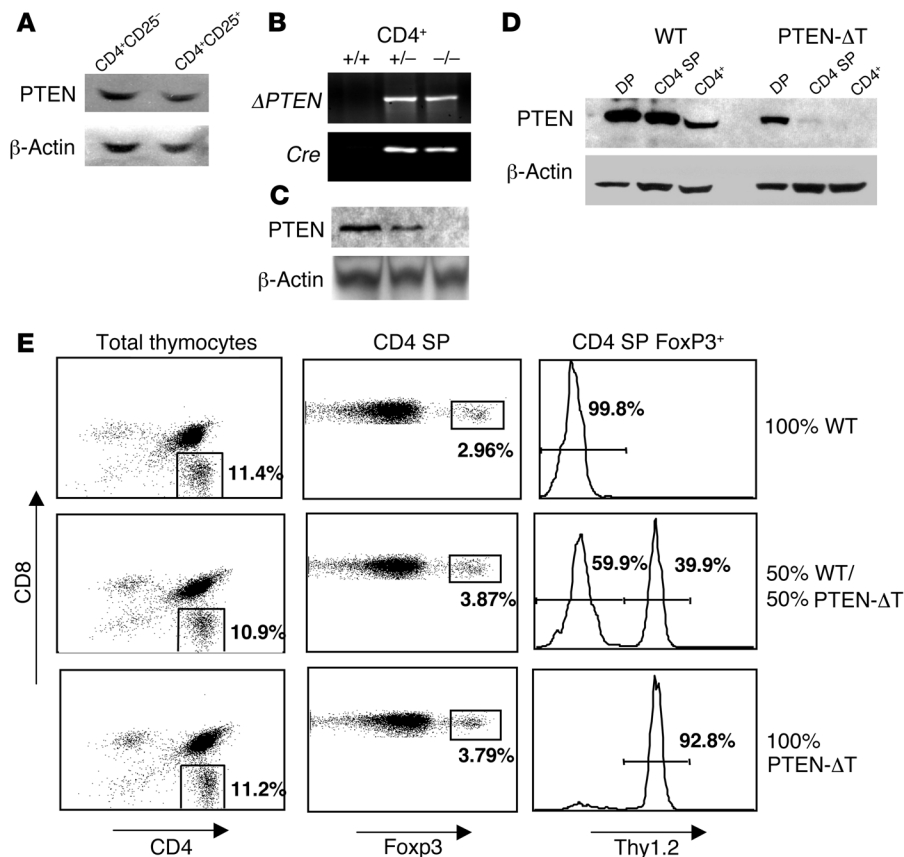
We have recently described a distinct IL-2R signaling pattern in Tregs, in which downstream mediators of PI3K are not activated while JAK/STAT-dependent signaling remains intact. This signaling pattern correlates with the hypoproliferative response of Tregs and is associated with expression of PTEN (phosphatase and tensin homolog deleted on chromosome 10) (12). PTEN, a phosphoinositol 3,4,5-triphosphatase, catalyzes the reverse reaction of PI3K, thereby negatively regulating the activation of downstream signaling pathways (13). We have demonstrated that the expression of PTEN is significantly downregulated after T cell activation yet remains relatively highly expressed in otherwise unmanipulated CD4<sup>+</sup>CD25<sup>+</sup> Tregs (12). While a substantial body of evidence exists indicating that PI3K-dependent signaling plays an essential role in driving IL-2–induced T cell proliferation (14, 15), a role for PTEN in regulating these events has not previously been defined.

In this report, we demonstrate that CD4<sup>+</sup>CD25<sup>+</sup> Tregs develop normally in mice with a specific deletion of PTEN in the T cell compartment (PTEN- $\Delta$ T). Furthermore, PTEN- $\Delta$ T Tregs proliferate readily upon stimulation with IL-2 alone in vitro and exhibit enhanced peripheral turnover in vivo. PTEN- $\Delta$ T Tregs retain their ability to suppress effector T cell responses both in vitro and in vivo. Furthermore, ex vivo deletion of PTEN in Tregs, using an inducible Cre system, also facilitates IL-2–mediated expansion of these cells, demonstrating that this phenotype is distinct from T cell developmental defects that might occur in the absence of PTEN expression. Finally, enforced expression of PTEN in both PTEN- $\Delta$ T Tregs and recently activated CD4<sup>+</sup> T cells inhibits their ability to expand in response to IL-2, confirming the ability of this lipid phosphatase to negatively regulate IL-2–dependent proliferation. These data identify PTEN as a negative regulator of IL-2R signaling in CD4<sup>+</sup> T cells while defining the mechanism that regulates CD4<sup>+</sup>CD25<sup>+</sup> Treg proliferation in response to IL-2R stimulation. These observations also indicate that targeting PTEN activity

**Nonstandard abbreviations used:** IL-2R, IL-2 receptor; MIGR1, MSCV-Ires-Gfp-EcoR1; NGFR, nerve growth factor receptor; 4-OHT, 4-OH tamoxifen; PTEN, phosphatase and tensin homolog deleted on chromosome 10; rIL-2, recombinant IL-2.

**Conflict of interest:** P.T. Walsh, S.J. Bensinger, and L.A. Turka own intellectual property related to the PTEN and regulatory T cells.

**Citation for this article:** *J. Clin. Invest.* 116:2521–2531 (2006). doi:10.1172/JCI28057.



**Figure 1**

Thymic development of CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs in the absence of PTEN. (A) PTEN is expressed at equivalent levels in CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T cell subsets from normal mice. (B) Specific recombination at the *Pten* locus in the presence of Cre was shown by PCR amplification of an 849-bp product in genomic DNA isolated from CD4<sup>+</sup> T cells from *Cre*-ve (+/+), *Pten*<sup>fllox/+</sup>*Cre*<sup>+</sup> (+/-), and *Pten*<sup>fllox/fllox</sup>*Cre*<sup>+</sup> (-/-) littermates. (C) Expression of PTEN protein in CD4<sup>+</sup> T cells isolated as above. β-Actin was used as a loading control. (D) CD4<sup>+</sup>CD8<sup>+</sup> double-positive (DP), CD4<sup>+</sup> single-positive (SP), and peripheral CD4<sup>+</sup> T cells were isolated by FACS from 3-week-old wild-type and PTEN-ΔT mice. Cells were subsequently lysed and analyzed for expression of PTEN by immunoblotting. (E) T cell-depleted bone marrow cells from wild-type (Thy1.1<sup>+</sup>) and PTEN-ΔT (Thy1.2<sup>+</sup>) mice were used to reconstitute lethally irradiated Thy1.1<sup>+</sup> hosts. Mice were reconstituted with either 100% wild-type, 100% PTEN-ΔT, or a mixture of 50% each. Thymic regulatory subsets (CD4<sup>+</sup>SP Foxp3<sup>+</sup>) were analyzed 10 weeks after reconstitution. Data shown are representative of results from 3 chimeric mice per condition.

may facilitate expansion of CD4<sup>+</sup>CD25<sup>+</sup> Tregs ex vivo or in vivo for potential therapeutic use.

**Results**

*CD4<sup>+</sup>CD25<sup>+</sup> Tregs develop normally in the absence of PTEN.* In normal mice, PTEN is expressed at equivalent levels in Treg and CD4<sup>+</sup>CD25<sup>-</sup> T cell subsets (Figure 1A). However, as PTEN deficiency in mice results in embryonic lethality, to examine the role of PTEN in CD4<sup>+</sup>CD25<sup>+</sup> Tregs, we used mice with targeted deletion of PTEN specific to the T cell compartment. Mice homozygous for expression of the *Pten*<sup>fllox</sup> allele were crossed with CD4<sup>+</sup>*Cre* transgenic mice. From the resulting litters (with animals termed *PTEN-ΔT* mice), genomic DNA from purified CD4<sup>+</sup> T cells was screened by PCR for expression of the *Cre* transgene. Specific recombination at the *Pten*<sup>fllox</sup> locus was detected using primers flanking the 5' and 3' loxP sites, which amplify an 849-bp product only after Cre-mediated deletion of

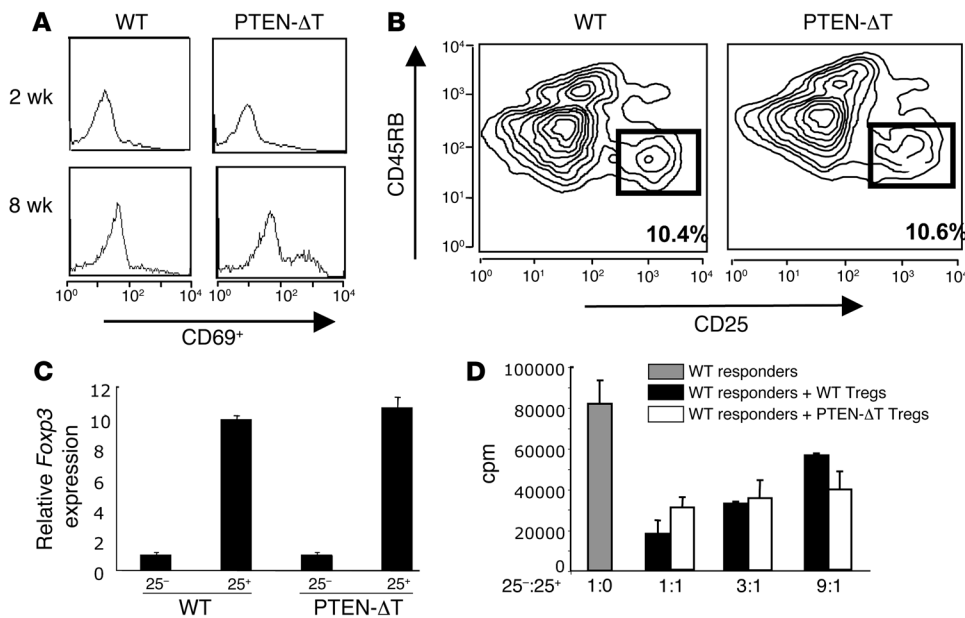
exons 4 and 5 (Figure 1B) (16). T cell-specific deletion of PTEN was confirmed by Western blotting for total PTEN expression in purified CD4<sup>+</sup> T cells from 3-week-old homozygous mutant *Pten*<sup>fllox/fllox</sup>*Cre*<sup>+</sup>, heterozygous mutant *Pten*<sup>fllox/+</sup>*Cre*<sup>+</sup>, or wild-type *Cre*<sup>-</sup> mice (Figure 1C).

The precise phase of thymic development at which CD4<sup>+</sup> T cells commit to the Treg lineage is controversial, and although Cre recombinase becomes active during the double-positive phase of T cell development in the CD4<sup>+</sup>*Cre* mouse (17), the in vivo half-life of PTEN protein expression may be sufficient that PTEN-ΔT Tregs develop in the presence of PTEN. Therefore, we examined levels of PTEN expression during different phases of T cell development in the thymus of 3-week-old PTEN-ΔT and wild-type mice. As shown in Figure 1D, PTEN protein levels were notably lower (compared with littermate controls) in double-positive thymocytes from PTEN-ΔT mice and were almost undetectable in CD4<sup>+</sup> single-positive thymocytes.

To further determine whether PTEN deficiency affects Treg development we made bone marrow chimeric mice by reconstituting lethally irradiated congenic hosts with wild-type (Thy1.1) and/or PTEN-ΔT (Thy1.2) bone marrow. Mice were reconstituted with either 100% wild-type, 100% PTEN-ΔT, or a 50% wild-type/50% PTEN-ΔT mixture of bone marrow. Ten weeks after reconstitution, thymic development appeared normal in all chimeras, with similar percentages of double-negative, double-positive, and single-positive subsets (Figure 1E and data not shown). Furthermore, examination of Foxp3 expression among CD4 single-positive cells from all groups demonstrated equivalent levels of Treg

development (Figure 1E). To analyze the relative proportion of wild-type and PTEN-ΔT-derived cells within the CD4<sup>+</sup>Foxp3<sup>+</sup> Treg subsets of the mixed chimeras, we measured expression of congenic Thy1 markers. As shown in Figure 1E, although the proportion of wild-type-derived Tregs was slightly higher in the 50/50 chimeric mice (59.9% versus 39.9%), this was most likely due to residual host-derived T cell development, similar to what was seen in the 100% PTEN-ΔT chimeric mice, in which 92.8% of Tregs were PTEN deficient. Together, these data indicate that CD4<sup>+</sup>CD25<sup>+</sup> Tregs develop normally in the absence of PTEN.

*Intact suppression by PTEN-deficient CD4<sup>+</sup>CD25<sup>+</sup> Tregs.* Previous reports have demonstrated that loss of PTEN in the T cell compartment, achieved by crossing *Pten*<sup>fllox/-</sup> mice with *lck-Cre* transgenic mice, results in lethality by about 15 weeks of age due to the development of CD4<sup>+</sup> T cell lymphomas (18). Our mice had a very similar phenotype, developing CD4<sup>+</sup> T cell lymphomas by

**Figure 2**

Isolation and analysis of CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup> Tregs from PTEN-ΔT mice. (A) Expression of the T cell-activation marker CD69 on CD4<sup>+</sup> T cells from PTEN-ΔT mice and wild-type littermates at age 2 weeks, before the onset of disease, and at age 8 weeks. (B) Frequency of CD4<sup>+</sup> T cells that are CD25<sup>hi</sup>CD45RB<sup>lo</sup> from 2-week-old PTEN-ΔT and wild-type littermate mice. (C) Real-time PCR analysis of *Foxp3* expression on FACS-purified CD4<sup>+</sup>CD25<sup>-</sup>CD45RB<sup>hi</sup> and CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup> cells from 2-week-old PTEN-ΔT and wild-type littermate mice. (D) Purified CD4<sup>+</sup>CD25<sup>-</sup>CD45RB<sup>hi</sup> cells ( $1 \times 10^5$ ) from littermate control mice were stimulated with anti-CD3 (0.5 μg/ml) plus irradiated APCs ( $3 \times 10^6$ ) for 72 hours in the presence of the indicated ratios of CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup> Tregs purified from either PTEN-ΔT mice or wild-type mice. Tritiated thymidine was added to cultures for the final 16 hours before harvesting. All data are representative of at least 2 independent experiments. Data shown represent mean  $\pm$  SD of triplicate samples.

10–12 weeks (data not shown). In agreement with these reports, lymphoma was preceded by an increased total number of CD4<sup>+</sup> lymphocytes and an accumulation of activated CD4<sup>+</sup> T cells in the periphery, which became apparent after 6 weeks. This accumulation of activated CD4<sup>+</sup> T cells in the periphery was detected as a significant increase in the numbers of CD4<sup>+</sup> cells expressing the activation marker CD69 (Figure 2A). As previously reported, the expression levels of CD25 on CD4<sup>+</sup> T cells did not vary significantly between PTEN-ΔT mice and wild-type littermate controls (data not shown).

We next sought to determine whether loss of PTEN in the T cell compartment affected the function of CD4<sup>+</sup>CD25<sup>+</sup> Tregs. To avoid contamination of putative Tregs with recently activated T cells, mice were sacrificed at 2–3 weeks of age, a time at which no increase in the number of CD69<sup>+</sup>CD4<sup>+</sup> T cells was detectable (Figure 2A). Phenotypic analysis of PTEN-ΔT mice demonstrated similar numbers of CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup> cells when compared with littermate controls (Figure 2B). After purification by FACS, real-time PCR demonstrated that cells from both PTEN-ΔT and control mice expressed comparable levels of *Foxp3* mRNA (Figure 2C). Furthermore, PTEN-ΔT Tregs were able to suppress the proliferation of wild-type responder cells in vitro to the same extent as Tregs isolated from control mice (Figure 2D). Taken together, these data demonstrate that CD4<sup>+</sup>CD25<sup>+</sup> Tregs develop normally in the absence of PTEN.

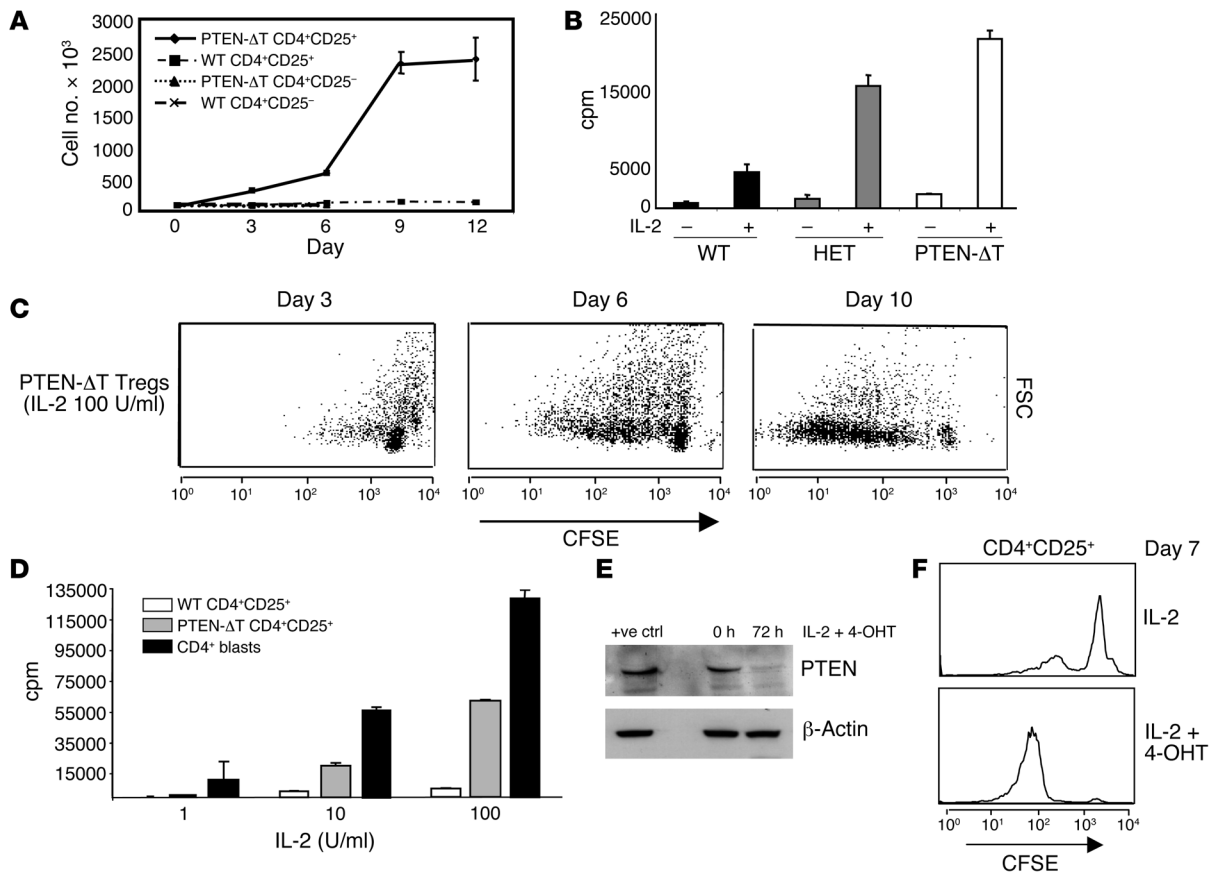
*PTEN-deficient CD4<sup>+</sup>CD25<sup>+</sup> Tregs proliferate in response to IL-2.* We have previously demonstrated that the hypoproliferative response

of CD4<sup>+</sup>CD25<sup>+</sup> Tregs to IL-2R stimulation is associated with a distinct signaling pattern characterized by intact STAT5 phosphorylation but an inability to activate pathways downstream of PI3K (12). In contrast, IL-2R stimulation of activated CD4<sup>+</sup> T cells readily activates PI3K-dependent signaling pathways and leads to a robust proliferative response. We further demonstrated that while CD4<sup>+</sup>CD25<sup>+</sup> Tregs express high levels of PTEN, a negative regulator of PI3K-dependent signaling, little or no PTEN is expressed in recently activated CD4<sup>+</sup> T cells. Therefore, we hypothesized that PTEN activity negatively regulates IL-2-induced expansion of Tregs. In order to test this hypothesis, we cultured CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup> cells from PTEN-ΔT mice in the presence of recombinant IL-2 (rIL-2) (100 U/ml) and assessed total cell numbers at various time points. As shown in Figure 3A, viable PTEN-ΔT Treg cell numbers increased an average of 15–25 fold over a 2-week period in culture while no accumulation of wild-type Tregs or CD4<sup>+</sup>CD25<sup>-</sup> T cells from either wild-type or

PTEN-ΔT mice was observed. CFSE dilution clearly illustrated the kinetics of cell division over the first 10 days in culture, and cell proliferation was also assessed by incorporation of tritiated thymidine after 48 hours in culture with rIL-2 (Figure 3, B and C). This expansion of PTEN-ΔT Tregs was due to the proliferative effects of IL-2R signaling, as no difference in the levels of cell survival between wild-type and PTEN-ΔT Tregs was observed (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI28057DS1).

The proliferative response of PTEN-ΔT Tregs to IL-2 is dose dependent, with cell division evident at as low as 10 U/ml of rIL-2 (Figure 3D). This level of proliferation observed in PTEN-ΔT Tregs is less than that seen in activated CD4<sup>+</sup> T cell blasts. In part, this may be due to endogenous IL-2 secretion by the activated CD4<sup>+</sup> blasts, which increases the effective dose of IL-2 in each well. However, it also suggests that, in addition to PTEN, other parameters may also regulate IL-2 responsiveness of CD4<sup>+</sup> T cells.

One possible explanation for our observations of PTEN-ΔT Treg expansion in response to IL-2 alone is that these cells may have already encountered TCR stimulation in vivo before isolation, thus facilitating their IL-2 responsiveness. However, we were unable to detect any differences in phosphorylation of ZAP-70 or Erk between freshly isolated wild-type and PTEN-ΔT Tregs (data not shown), arguing against the possibility that PTEN-ΔT Tregs are intrinsically activated via TCR signaling pathways. Another consideration is that T cell developmental abnormalities that may occur in PTEN-ΔT mice (18, 19) could influence the behavior of

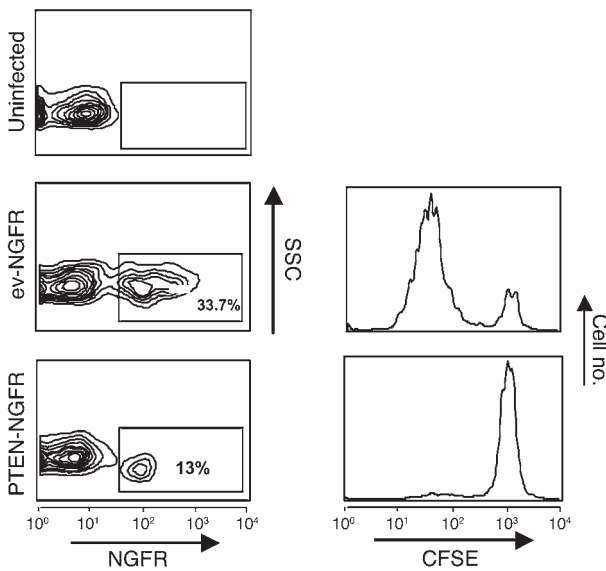


**Figure 3** Proliferation of PTEN-ΔT CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup> Tregs in response to IL-2R stimulation. (A) Purified CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup> and CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>hi</sup> cells from both wild-type and PTEN-ΔT mice were cultured at a constant density of 1 × 10<sup>5</sup>/well in the presence of rIL-2 (100 U/ml) for a 2-week period. At the indicated time points, total cell numbers were quantitated by trypan blue exclusion. Only PTEN-ΔT CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup> cells exhibited a significant increase in cell number with all other cell types showing overlapping live cell numbers over the examination period. Data are representative of 5 different experiments. (B) Purified CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup> cells from wild-type, PTEN-het (HET), and PTEN-ΔT mice were cultured in the presence of rIL-2 (100 U/ml) for 48 hours. Tritiated thymidine was added to cultures for the final 16 hours before harvesting. (C) PTEN-ΔT CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup> cells were CFSE labeled and cultured in the presence of rIL-2 (100 U/ml) for 10 days. CFSE dilution was analyzed at the indicated time points by FACS analysis. Results are representative of 4 separate experiments. FSC, forward scatter. (D) Purified wild-type and PTEN-ΔT CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup> cells and preactivated CD4<sup>+</sup> T cell blasts were stimulated with titrated doses of rIL-2 as shown for 72 hours. Tritiated thymidine was added to cultures for the final 16 hours before harvesting. (E) CD4<sup>+</sup> T cells were isolated from ER-Cre<sup>+</sup>/Pten<sup>fllox/fllox</sup> mice and lysed immediately or after culture in the presence of rIL-2 (100 U/ml) and 4-OHT (1 nM). Samples were electrophoresed on an SDS-PAGE gel, transferred to nitrocellulose membranes, and probed as indicated. Purified CD4<sup>+</sup> T cells from a littermate control mouse were used as a positive control (+ve ctrl) for PTEN expression. (F) Purified ER-Cre<sup>+</sup>/Pten<sup>fllox/fllox</sup> CD4<sup>+</sup>CD25<sup>+</sup> Tregs were CFSE labeled and cultured in the presence of rIL-2 (100 U/ml) and 4-OHT (1 nM) for 7 days. CFSE dilution was analyzed by FACS. Results are representative of 3 independent experiments. Data shown represent mean ± SD of triplicate samples.

CD4<sup>+</sup>CD25<sup>+</sup> Tregs. Although our mixed bone marrow chimera data indicate that Tregs develop normally in the absence of PTEN, to definitively exclude the possibility that potential developmental or homeostatic defects that may arise in PTEN-ΔT mice may alter the response of PTEN-ΔT Tregs to IL-2, we crossed the *Pten<sup>fllox/fllox</sup>* mice with *Cre-ER* transgenic mice, in which Cre recombinase activity occurs only in the presence of the estrogen homolog tamoxifen (20). Unlike in PTEN-ΔT mice, no defects in peripheral or central tolerance were observed in these mice, and the CD4<sup>+</sup>CD25<sup>+</sup> Treg compartments were identical to those of Cre-negative littermates (data not shown). Culture of purified CD4<sup>+</sup> T cells from *Cre-ER/Pten<sup>fllox/fllox</sup>* mice in the presence of 1 nM 4-OH tamoxifen (4-OHT) led to a significant decrease in the amount of PTEN expression

after 72 hours (Figure 3E). In addition, stimulation of *Cre-ER/Pten<sup>fllox/fllox</sup>* CD4<sup>+</sup>CD25<sup>+</sup> Tregs with IL-2 in the presence of 1 nM 4-OHT led to a robust proliferative response after 7 days in culture, as assessed by CFSE dilution (Figure 3F). Together, these data confirm that PTEN-deficient CD4<sup>+</sup>CD25<sup>+</sup> Tregs can be readily expanded upon IL-2R stimulation and that this response is not due to developmental defects as a consequence of loss of PTEN.

*Reexpression of PTEN blocks IL-2-mediated expansion of PTEN-ΔT Tregs.* As loss of PTEN expression enabled IL-2-mediated proliferation of Tregs, we hypothesized that reexpression of the PTEN gene in PTEN-ΔT Tregs would restore the normal hypoproliferative response to IL-2 that is observed in wild-type cells. We cloned the gene for human *Pten* into a bicistronic retroviral vector also



**Figure 4**

Reexpression of PTEN in PTEN-ΔT Tregs restores hypoproliferative response to IL-2. Purified PTEN-ΔT CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup> cells were CFSE labeled and retrovirally transduced as described in Methods, with either MIGR1-NGFR empty vector (ev-NGFR) or PTEN-containing virus (PTEN-NGFR). Cells were analyzed for expression of human NGFR 96 hours after infection and CFSE dilution of NGFR-positive cells analyzed by flow cytometry. Results are representative of 3 independent experiments. SSC, side scatter.

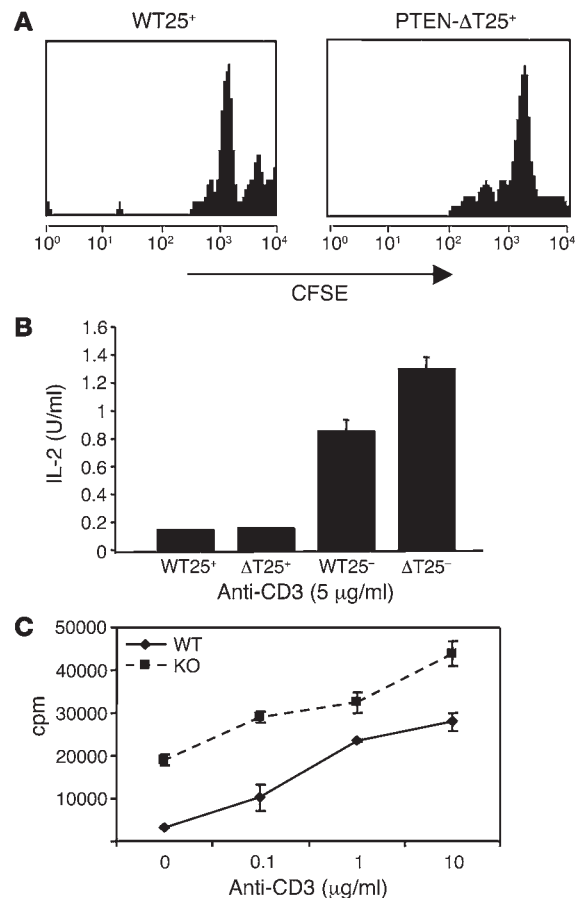
expressing the extracellular portion of human nerve growth factor receptor (NGFR) as a marker of cell transduction. Transduction of CFSE-labeled PTEN-ΔT Tregs with NGFR/MSCV-Ires-Gfp-EcoR1 (NGFR/MIGR1) empty vector typically resulted in 30–40% transduction efficiency, as determined by flow cytometric analysis of NGFR expression. In contrast, the *Pten*-containing retrovirus consistently resulted in 5–15% transduction efficiency (Figure 4). Since efficient retroviral transduction requires target cells to be progressing through the cell cycle, the significantly lower efficiency of transduction observed with the PTEN-containing virus is consistent with PTEN inhibiting cell cycle progression. After transduction, cells were cultured in the presence of rIL-2 (100 U/ml) for 4 days, and levels of CFSE dilution of NGFR<sup>+</sup> cells were examined. As shown in Figure 4, although transduction of PTEN-ΔT Tregs with empty vector did not affect IL-2-induced proliferation, reexpression of PTEN resulted in complete inhibition of IL-2-mediated proliferation, restoring the hypoproliferative response observed in wild-type Tregs.

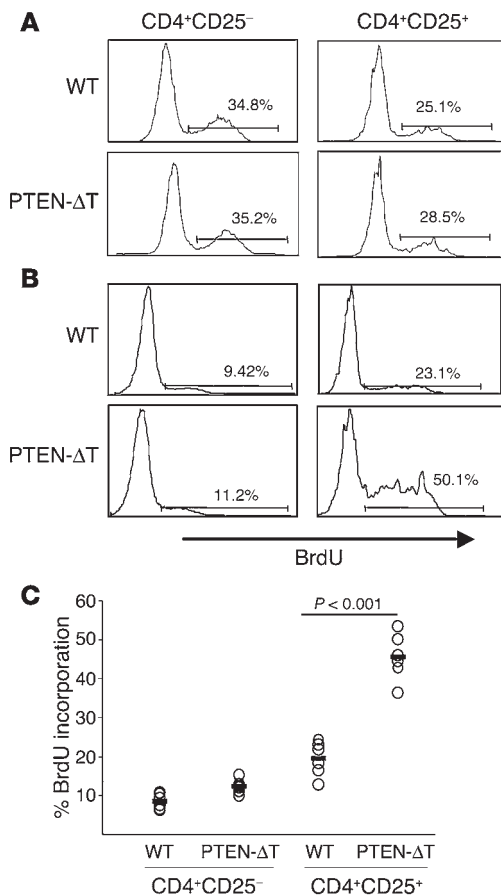
*PTEN-deficient CD4<sup>+</sup>CD25<sup>+</sup> Tregs do not proliferate in response to TCR stimulation.* In addition to their hypoproliferative response to IL-2R stimulation, it has also been established that CD4<sup>+</sup>CD25<sup>+</sup> Tregs do not divide after stimulation with anti-CD3 antibody alone. This is most likely related to the relative inability of Tregs to produce IL-2

(10). Thus, we also asked whether PTEN activity regulates the hyporesponsiveness of Tregs to TCR stimulation. Similar to their wild-type counterparts, stimulation of PTEN-ΔT Tregs with plate-bound anti-CD3 did not induce proliferation or any significant level of IL-2 production (Figure 5, A and B). It has also previously been demonstrated that the hypoproliferative response of Tregs can be broken by TCR stimulation in the presence of a relatively high dose of IL-2 (5, 10). Therefore, we examined the response of PTEN-ΔT Tregs upon stimulation with graded doses of anti-CD3 in the presence of rIL-2 (100 U/ml). PTEN-ΔT Tregs exhibited a more robust proliferative response in the presence of rIL-2 at all concentrations of anti-CD3 used in comparison with their wild-type counterparts (Figure 5C). Importantly, the differences in proliferation observed at each concentration of anti-CD3 tested were equivalent to the difference seen in the presence of rIL-2 alone (i.e., the basal level of IL-2-induced proliferation in PTEN-ΔT Tregs). This suggests that while PTEN plays a significant role in regulating the IL-2 responsiveness of Tregs, the basal level of TCR responsiveness is unaltered in these cells.

**Figure 5**

PTEN-ΔT CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup> cells remain hypoproliferative to TCR stimulation. (A) Purified PTEN-ΔT or wild-type CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup> cells were CFSE labeled and stimulated with plate-bound anti-CD3 (5 μg/ml) for 72 hours. (B) Supernatants were harvested under the conditions described above after 24 hours stimulation, and levels of IL-2 were determined by ELISA. Data shown represent the mean ± SD of triplicate samples. Data are representative of 3 separate experiments. (C) Purified PTEN-ΔT or wild-type CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup> cells were stimulated with IL-2 (100 U/ml), irradiated APCs, and varying doses of anti-CD3 (2C11) as shown for 72 hours. Tritiated thymidine was added to cultures for the final 16 hours before harvesting. Data shown represent the mean ± SD of triplicate samples. WT25<sup>+</sup>, CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup> cells from wild-type mice; ΔT25<sup>+</sup>, CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup> cells from PTEN-ΔT mice; WT25<sup>-</sup>, CD4<sup>+</sup>CD25<sup>-</sup>CD45RB<sup>hi</sup> cells from wild-type mice; ΔT25<sup>-</sup>, CD4<sup>+</sup>CD25<sup>-</sup>CD45RB<sup>hi</sup> cells from PTEN-ΔT mice.





**Figure 6**

PTEN-ΔT Tregs exhibit enhanced homeostatic expansion in the periphery. PTEN-ΔT mice and littermate controls were administered 1 mg BrdU every 12 hours for 3 days, at which time they were sacrificed and thymus and spleen cells were stained for BrdU. (A) Levels of BrdU incorporation in CD4<sup>+</sup>CD8<sup>-</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD8<sup>-</sup>CD25<sup>-</sup> thymocytes. (B) Levels of BrdU incorporation in CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> splenocytes. (C) Percentage BrdU-positive CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> splenocytes from PTEN-ΔT mice (n = 6) and littermate controls (n = 6).

in regulating the peripheral homeostasis of Tregs in vivo, without any significant effects on Treg development in the thymus.

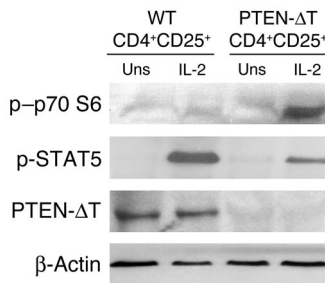
*Deletion of PTEN in Tregs facilitates downstream activation of PI3K-dependent signaling through the IL-2R.* Previous studies on IL-2R signaling have shown that activation of both the JAK/STAT and PI3K/Akt pathways are critical for IL-2-induced proliferation (11). We have demonstrated that activation of signaling pathways downstream of PI3K does not occur in Tregs in response to IL-2R stimulation and have identified negative regulation of this signaling pathway by PTEN as a possible mechanism for this observation. Therefore, we examined whether, in the absence of PTEN, IL-2R stimulation could activate signaling downstream of PI3K.

To this end, freshly isolated Tregs from wild-type mice or Tregs isolated from PTEN-ΔT mice and expanded for 8 days with IL-2 (100 U/ml) to obtain necessary cell numbers (see Methods) were rested overnight in complete media then stimulated for 30 minutes with 100 U/ml rIL-2. Cell lysates were subsequently tested for activation of both JAK/STAT and PI3K-dependent signaling pathways. As reported previously (12), stimulation of wild-type Tregs resulted in robust but isolated activation of JAK/STAT signaling, as shown by phosphorylation of STAT5, without any detectable activation of PI3K-dependent signaling through phosphorylation of p70 S6 kinase. In contrast, while activation of JAK/STAT signaling was also clearly detectable after IL-2R stimulation of PTEN-ΔT Tregs, we additionally observed phosphorylation of p70 S6 kinase, indicating that IL-2R stimulation activated PI3K-dependent signaling pathways in these cells (Figure 7).

*Overexpression of PTEN inhibits IL-2-mediated expansion of activated CD4<sup>+</sup> T cells.* The results shown above suggest that PTEN is neces-

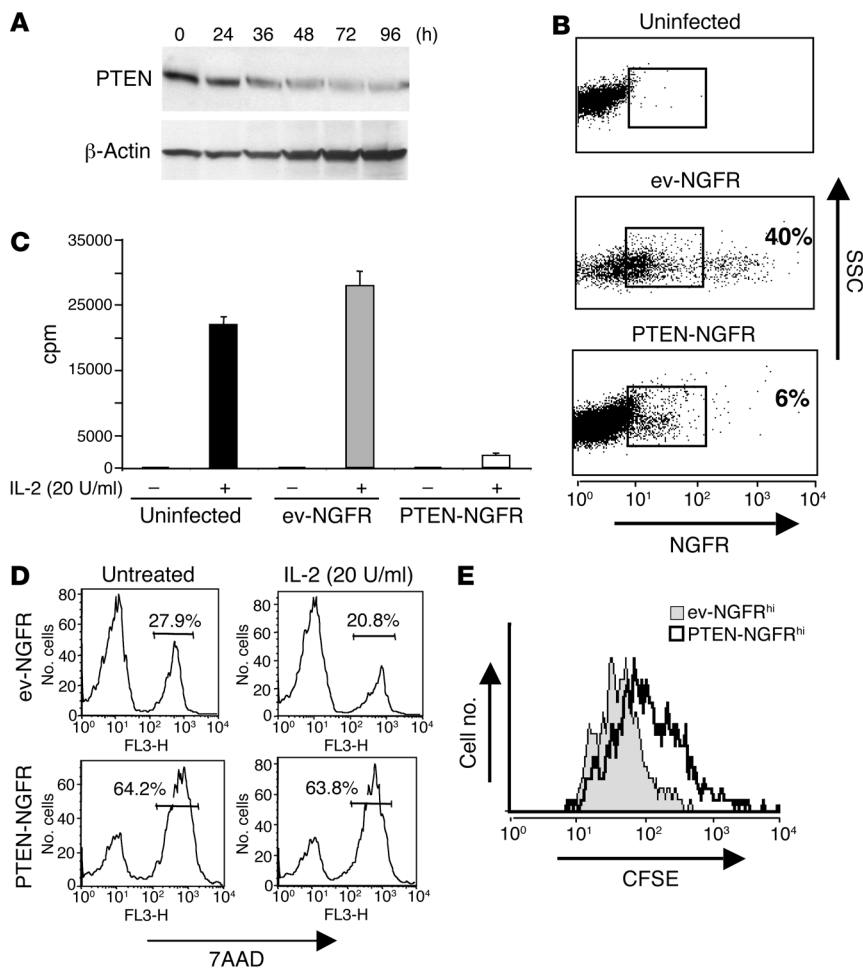
Taken together, these data confirm that Tregs can be expanded more readily in the absence of PTEN. However, they also indicate that the hypoproliferative responses of Tregs to IL-2R or TCR stimulation alone are mediated, at least in part, through distinct mechanisms.

*PTEN-ΔT Tregs exhibit an enhanced rate of peripheral homeostatic turnover in vivo.* Although CD4<sup>+</sup>CD25<sup>+</sup> Tregs are characterized by hypoproliferative responses in vitro, it is also clear that they readily undergo expansion in vivo (21, 22). Furthermore, in unmanipulated animals, Tregs in peripheral lymphoid organs exhibit higher levels of basal proliferation compared with their CD4<sup>+</sup>CD25<sup>-</sup> counterparts, and this response is known to depend, at least in part, on IL-2 (21, 22). As we observed that PTEN-ΔT Tregs proliferate in response to IL-2 in vitro, we next examined whether the homeostasis of Tregs in vivo was altered in the absence of PTEN. PTEN-ΔT mice and littermate controls were injected with BrdU for 3 days, after which CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> T cells from both the thymi and spleens of these mice were analyzed for BrdU incorporation. While similar levels of BrdU incorporation were seen in splenic CD4<sup>+</sup>CD25<sup>-</sup> cells from control versus PTEN-ΔT mice, splenic PTEN-ΔT Tregs exhibited a significantly higher rate of BrdU staining compared with wild-type CD4<sup>+</sup>CD25<sup>+</sup> T cells (Figure 6, B and C). Elevated BrdU uptake in splenic Tregs was a result of increased peripheral turnover, as we found no difference in the levels of BrdU incorporation in either single-positive CD4<sup>+</sup>CD25<sup>-</sup> or CD4<sup>+</sup>CD25<sup>+</sup> subsets from PTEN-ΔT mice compared with wild-type littermates (Figure 6A). Together, these observations demonstrate that PTEN plays a role



**Figure 7**

Deletion of PTEN facilitates IL-2R signaling downstream of PI3K in Tregs. FACS-purified CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup> cells from PTEN-ΔT mice were expanded in the presence of rIL-2 (100 U/ml) for 8 days. Cells were washed extensively and, along with freshly isolated wild-type Tregs, were rested overnight in complete medium. Cells were subsequently left unstimulated (uns) or stimulated with 100 U/ml rIL-2 for 30 minutes. Samples were lysed and electrophoresed on an SDS-PAGE gel, transferred to nitrocellulose membranes, and probed as indicated. p, phosphorylated.

**Figure 8**

Reexpression of PTEN in activated CD4<sup>+</sup> T cells inhibits IL-2-mediated proliferation. (A) Splenocytes from DO11.10 TCR transgenic mice were stimulated with ova peptide (1 μg/ml). CD4<sup>+</sup> T cells were purified by magnetic bead separation at the indicated time points and subsequently lysed. Samples were analyzed for PTEN expression by Western blot, and membranes were stripped and reprobed for β-actin as a loading control. Results are representative of 3 independent experiments. (B) Purified CD4<sup>+</sup> T cells were retrovirally transduced, as described in Methods, with either MIGR1-NGFR empty vector or PTEN-containing virus. Cells were analyzed for expression of human NGFR 48 hours after infection. Data shown illustrate typical transduction efficiencies achieved using these vectors. The gate drawn shows NGFR-positive subsets used for comparison in subsequent experiments. (C) NGFR-positive cells were purified either by FACS sort or magnetic bead separation and cultured in the presence or absence of rIL-2 (20 U/ml) for 48 hours. Tritiated thymidine was added to cultures for the last 16 hours before harvesting. Data shown represent the mean ± SD of triplicate cultures and are representative of 4 independent experiments. (D) Cells as in C were analyzed for viability by 7-AAD incorporation. Data shown is representative of 4 independent experiments. (E) Purified CD4<sup>+</sup> T cells were CFSE labeled before stimulation and retroviral transduction. After infection, cells were cultured in the presence of rIL-2 (10 U/ml) for 48 hours, and cells expressing identical levels of NGFR were analyzed for CFSE dilution by flow cytometry.

sary and sufficient for the hypoproliferative response of Tregs to IL-2. As IL-2 is known to enhance the proliferation of activated CD4<sup>+</sup> T cells, we asked whether maintenance of its expression would have a similar inhibitory effect on IL-2R-induced cell division in these cells.

Activation of nonregulatory CD4<sup>+</sup> T cells both induces CD25 expression and downregulates PTEN (12) (Figure 8A). To examine PTEN activity following T cell activation, we again used retrovi-

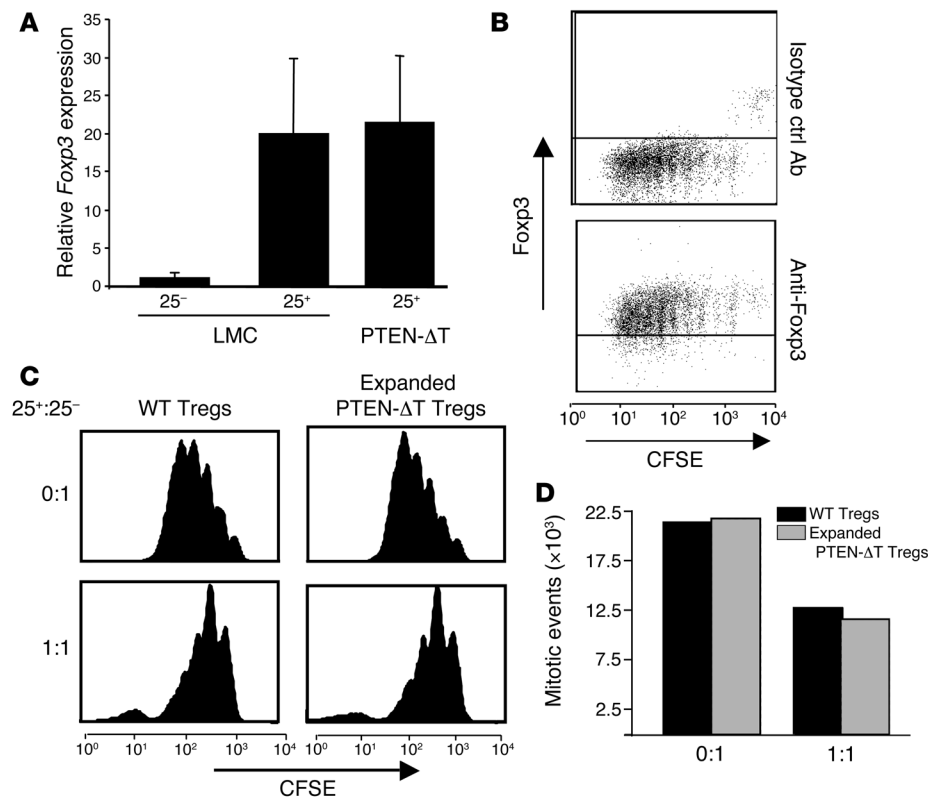
ral transduction. Similar to our studies on PTEN-ΔT Tregs, infection of activated CD4<sup>+</sup> T cells with NGFR/MIGR1 empty vector typically resulted in 40–50% transduction efficiency while the *Pten*-containing virus resulted in 5–10% transduction efficiency, again consistent with PTEN inhibiting cell cycle progression (Figure 8B). Purified NGFR-positive cells from both mock (ev-NGFR) and PTEN (PTEN-NGFR) transduced cells were subsequently cultured in the presence or absence of IL-2, and proliferation was assessed by thymidine incorporation. While cells expressing empty vector proliferated to an extent similar to that of nontransduced T cells, those expressing PTEN showed a dramatically decreased level of thymidine incorporation (Figure 8C).

Previous studies using ectopic expression of PTEN in T cell lines have supported a role for PTEN not only in inhibiting cell cycle progression but also in inducing apoptotic cell death (23, 24). Therefore, we also examined the viability of purified NGFR-positive cells both in the presence and absence of rIL-2. Using 7-AAD staining as a measure of cell death, we found that PTEN overexpression leads to a significant increase in activated T cell death when compared with empty vector-transduced cells (64% versus 28%). The addition of exogenous rIL-2 did not rescue this effect (Figure 8D).

To exclude the possibility that cells overexpressing PTEN failed to incorporate thymidine exclusively due to cell death, we measured CFSE dilution in live cells as an alternative means to assess proliferation after PTEN transduction. Freshly isolated CD4<sup>+</sup> T cells were CFSE labeled prior to activation with PMA and ionomycin and incubated for a period of 18 hours. These cells were then transduced with either empty vector or PTEN-expressing virus before subsequent culture with rIL-2 (10 U/ml) for a further 48 hours. Live cells were then identified based on forward- and side-scatter profiles, for NGFR expression, and CFSE dilution was determined in live NGFR-positive subsets. For comparison, cells expressing identical levels of NGFR were analyzed. As shown in Figure 8E, proliferation of PTEN-overexpressing cells was significantly less

than that observed in empty vector-transduced cells, confirming that PTEN acts to inhibit IL-2-induced proliferation of activated CD4<sup>+</sup> T cells. As an appropriate comparison, cells expressing identical levels of NGFR were analyzed (Figure 8B) although the results described were identical even when all NGFR-positive cells were included in the analysis (data not shown). While the inhibition of IL-2-induced proliferation by PTEN in recently activated CD4<sup>+</sup> cells was clear, it was far less significant than the almost complete





**Figure 9** Ex vivo expansion of PTEN-ΔT Tregs does not affect their regulatory phenotype. (A) Real-time PCR analysis of expression levels of *Foxp3* mRNA in PTEN-ΔT CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup> cells that have been expanded for 8 days ex vivo with rIL-2 compared with freshly isolated CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup> and CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>hi</sup> cells from wild-type littermate mice. Results are representative of 3 independent experiments. LMC, littermate control. (B) Expression of Foxp3 protein on CFSE-labeled PTEN-ΔT CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup> cells expanded for 8 days with rIL-2 (100 U/ml). (C) PTEN-ΔT Tregs were expanded in the presence of rIL-2 for 8 days and subsequently washed extensively before coculture at the indicated ratio with wild-type CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>hi</sup> responder cells expressing the Thy1.1 congenic marker. Freshly isolated CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup> cells from wild-type mice were used for direct comparison. Cells were stimulated in the presence of 3 × 10<sup>5</sup> irradiated APCs with soluble anti-CD3 (0.5 μg/ml) for 72 hours, at which time CFSE dilution of Thy1.1-expressing responder cells was examined by flow cytometry. 25<sup>+</sup>:25<sup>-</sup>, ratio of CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup> cells to CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>hi</sup> cells as denoted in the labels to the left of the panels. (D) Quantitative comparison of level of suppression by wild-type and PTEN-ΔT Tregs through calculation of number of mitotic events of Thy1.1<sup>+</sup> responder cells.

inhibition observed in retrovirally transduced PTEN-ΔT Tregs (Figure 4). This suggests that other factors in addition to PTEN may also regulate IL-2-mediated proliferation in activated non-regulatory CD4<sup>+</sup> T cells.

*Expanded PTEN-deficient CD4<sup>+</sup>CD25<sup>+</sup> Tregs retain their suppressor phenotype.* One of the largest drawbacks to exploiting the potential of Tregs in a therapeutic setting is the very low frequency with which these cells are found in normal, healthy individuals. Our results demonstrate that in the absence of PTEN activity, these cells can be readily expanded ex vivo using only rIL-2. These data highlight the potential for targeting this lipid phosphatase to facilitate ex vivo expansion of Tregs in response to cytokine stimulation alone. However, such strategies could only be feasible if in vitro manipulation of these cells does not alter their regulatory potential. Therefore, to determine whether PTEN-deficient Tregs retain their regulatory phenotype after expansion with rIL-2, we examined their ability to

suppress the proliferation of wild-type CD4<sup>+</sup> responder T cells in vitro as well as their ability to suppress the development of inflammatory bowel disease in vivo. As shown in Figure 9, Tregs expanded for 8 days retained expression of *Foxp3* mRNA and protein (Figure 9, A and B) and their ability to inhibit CD4<sup>+</sup> effector T cell proliferation (Figure 9, C and D). The level of suppression was similar to that observed with freshly isolated wild-type Tregs.

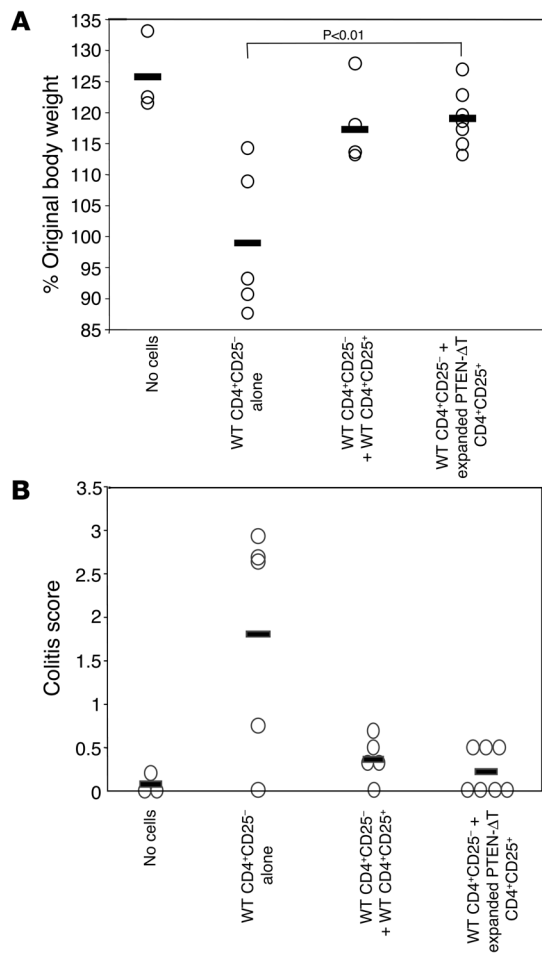
Adoptive transfer of CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>hi</sup> cells into immunodeficient hosts has been demonstrated to result in the development of colitis, which can be prevented by cotransfer of Tregs (2, 25). To examine whether ex vivo-expanded, PTEN-deficient Tregs could prevent disease in vivo, we cotransferred wild-type CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>hi</sup> cells with either wild-type freshly isolated Tregs or PTEN-ΔT Tregs after expansion for 5 days in vitro with rIL-2. Similar to our in vitro observations, expanded PTEN-ΔT Tregs prevented the development of colitis in vivo to the same extent as freshly isolated wild-type Tregs (Figure 10).

A recent report has suggested that prevention of colitis in this model may be mediated by cell competition for an existing niche in the host rather than through the direct suppressive activity of Tregs (26). If this were the case in our system, one might also expect to see prevention of colitis if Tregs were replaced in this assay by PTEN-ΔT CD4<sup>+</sup>CD25<sup>+</sup> T cells. However, coadoptive transfer of PTEN-ΔT non-Tregs did not affect the development of colitis resulting from the transfer of wild-type CD4<sup>+</sup>CD25<sup>+</sup> T cells (Supplemental Figure 2).

Together, these data confirm that CD4<sup>+</sup>CD25<sup>+</sup> Treg development is intact in PTEN-ΔT mice and demonstrate that expansion of these cells with IL-2 in vitro does not alter their suppressor phenotype.

## Discussion

Many studies have focused on the effects of IL-2 on CD4<sup>+</sup>CD25<sup>+</sup> Tregs, with several recent reports providing substantial evidence of an essential role for IL-2 in Treg development and/or peripheral homeostasis (27, 28). Despite these observations, very little is known about the intracellular pathways that regulate IL-2R signaling in CD4<sup>+</sup>CD25<sup>+</sup> Tregs, and indeed, a defining characteristic of CD4<sup>+</sup>CD25<sup>+</sup> Tregs is their relative inability to proliferate ex vivo in response to IL-2R stimulation. Recently, we characterized IL-2R signaling in Tregs and found that, unlike activated T cells, downstream mediators of the PI3K-dependent signaling pathway remain inactive following stimulation with IL-2. We associated

**Figure 10**

Prevention of colitis by ex vivo expanded PTEN- $\Delta$ T Tregs. Rag1<sup>-/-</sup> mice were injected with  $6 \times 10^6$  freshly isolated wild-type CD4<sup>+</sup>CD25<sup>-</sup>CD45RB<sup>hi</sup> cells either alone ( $n = 5$ ) or together with  $3 \times 10^6$  freshly isolated wild-type Tregs ( $n = 5$ ) or PTEN- $\Delta$ T Tregs that had been expanded for 5 days ex vivo with rIL-2 ( $n = 7$ ). (A) Body weight is represented as percentage of initial weight 8 weeks after transfer. Statistical analysis was performed using an unpaired 2-tailed Student's *t* test. (B) Severity of colitis was histologically scored as described previously (25).

have normal development of CD4<sup>+</sup>CD25<sup>+</sup> Tregs (data not shown). Together, these data suggest that JAK/STAT-dependent signaling plays a predominant role over PI3K-dependent pathways downstream of the IL-2R in promoting Treg development.

These data also suggest that the breakdown in T cell tolerance observed in mice deficient in PTEN is the result of defects within non-Treg compartments. As described previously, PTEN-deficient T cells are hyperresponsive to activation stimuli and less susceptible to activation-induced cell death when compared with wild-type controls (18, 31). As development of PTEN- $\Delta$ T Tregs appears normal, it will be of interest to determine whether the altered responsiveness of effector T cells lacking PTEN allows them to escape regulation by CD4<sup>+</sup>CD25<sup>+</sup> T cells and contribute to disease.

Despite their apparently normal thymic development, PTEN- $\Delta$ T Tregs exhibited an increased rate of BrdU incorporation in the periphery (Figure 6), and could be expanded quite readily in response to IL-2 in vitro (Figure 3). Given these observations, and as IL-2 plays an important role in regulating the peripheral homeostasis of Tregs (22), one might expect to see an increased number of peripheral Tregs in PTEN- $\Delta$ T mice. However, the frequency of Tregs in PTEN- $\Delta$ T mice before the onset of disease was identical to that observed in littermate controls (Figure 1). This indicates that, in addition to exhibiting a higher level of peripheral homeostatic expansion, PTEN- $\Delta$ T Tregs also have a higher rate of turnover compared with their wild-type counterparts. Interestingly, however, a number of recent reports have demonstrated that peripheral CD4<sup>+</sup>CD25<sup>+</sup> Tregs proliferate more readily than CD4<sup>+</sup>CD25<sup>-</sup> effector T cells in the steady state in vivo, yet under normal homeostatic conditions, the relative frequency of Treg versus effector T cell subsets remains relatively stable over time (21, 22). These observations suggest that the higher rate of turnover of CD4<sup>+</sup>CD25<sup>+</sup> Tregs (compared with CD4<sup>+</sup>CD25<sup>-</sup> T cells) may be balanced by a higher rate of cell death, perhaps due to the lack of available "space" in the periphery (21). Interestingly, it has recently been demonstrated that in vivo IL-2 therapy of lymphopenic patients results in a marked increase in the frequency of peripheral Tregs, suggesting that a preexisting niche must be present in the peripheral environment to facilitate an alteration in the relative frequency of Tregs versus effector T cells (32).

Arguably, the greatest single barrier to harnessing the therapeutic potential of naturally occurring CD4<sup>+</sup>CD25<sup>+</sup> Tregs for the treatment of immune disorders is their relative low frequency in normal, healthy individuals as well as their "anergic" phenotype ex vivo. Accordingly, several groups have reported a number of approaches aimed at overcoming these difficulties through the expansion of Tregs ex vivo for subsequent treatment in disease models (3, 7, 33, 34). Many of these strategies involve activating Tregs with a combination of stimulatory antibodies to the TCR and CD28 in conjunction with relatively high doses of rIL-2 (3, 7). Our data indicate that, by targeting PTEN activity, Tregs can be expanded in response

this distinct signaling pattern with relatively high levels of expression of the lipid phosphatase PTEN (12). Therefore, here we tested the hypothesis that in the absence of PTEN, Tregs would respond to IL-2 stimulation by proliferating in a fashion similar to that of their activated CD4<sup>+</sup> T cell counterparts.

In order to isolate Tregs deficient in PTEN expression, we used a Cre-LoxP system to generate mice with a T cell-specific deletion in PTEN. As described previously, these mice suffer a profound defect in T cell tolerance characterized by an accumulation of autoreactive T cells in the periphery, which precedes the development of a CD4<sup>+</sup> T cell lymphoma (18). Given the importance of IL-2R signaling in Treg development and homeostasis, disrupted regulation of these signals, as may occur in the absence of PTEN, may be a potential contributing factor to the autoimmunity observed in these mice. Indeed, there have been several recent reports demonstrating that genetic alteration of downstream signaling pathways activated by IL-2 can have profound effects on Treg homeostasis. For example, mice expressing a constitutively active STAT5 transgene have an approximate 5-fold increase in the number of Tregs both in the thymus and periphery (29, 30). Our observations that Treg development and homeostasis appear normal in the thymus of PTEN- $\Delta$ T mice and that a functional Treg compartment can be isolated from these mice suggest that PTEN deficiency (and its effects on PI3K-dependent signaling) does not affect the development of these cells in vivo. In support of this observation, we have also found that mice expressing a constitutively active Akt transgene (mAkt mice)



to rIL-2 alone without the need to stimulate the TCR and costimulatory receptors. Most importantly, we have found that ex vivo expansion of PTEN-deficient Tregs does not affect their regulatory phenotype, as illustrated by their ability to suppress the proliferation of effector T cells in vitro as well as their ability to prevent the development of colitis in vivo (Figures 7 and 8).

Taken together, our data provide a mechanism that explains the hypoproliferative response of CD4<sup>+</sup>CD25<sup>+</sup> Tregs to IL-2 in vitro and also identify PTEN as a negative regulator of peripheral Treg homeostasis in vivo. These data indicate that negative regulation of PTEN activity in concert with IL-2R stimulation may facilitate expansion of CD4<sup>+</sup>CD25<sup>+</sup> Tregs both ex vivo and in vivo for potential therapeutic use.

## Methods

**Mice.** *Pten*<sup>flox/flox</sup> mice have been described previously and were a kind gift from Tak Mak (University of Toronto, Toronto, Ontario, Canada) (18). *CD4-Cre* mice were a kind gift from Yongwon Choi (University of Pennsylvania, Philadelphia, Pennsylvania, USA). *Pten*<sup>flox/flox</sup> mice were crossed with *CD4-Cre* transgenic mice to generate mice with a deletion of PTEN specific to the T cell compartment. As T cell-specific deficiency of PTEN results in early lethality, *Pten*<sup>flox/+</sup> *CD4-Cre*<sup>+</sup> mice were bred with *Pten*<sup>flox/flox</sup> mice to generate offspring. Mice were genotyped as described (18), and offspring that were found to express the Cre transgene and were homozygous for the *Pten*<sup>flox</sup> allele were used for analysis as homozygous mutant mice (PTEN-ΔT) while Cre-negative littermates were used as controls. *Cre-ER* mice have been described previously (20); these were crossed with *Pten*<sup>flox/flox</sup> mice and genotyped as described above. *Rag1*<sup>-/-</sup> mice, C57BL/6 mice expressing the Thy1.1 congenic marker, and DO11.10 TCR transgenic mice were purchased from Jackson Laboratory. All colonies were maintained under specific pathogen-free conditions at the animal facilities of the University of Pennsylvania. All experiments described in this manuscript were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania.

**Media, reagents, antibodies, and flow cytometry.** All cells were grown in RPMI 1640 (Mediatech Inc.) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, 10 mM HEPES (all from Invitrogen), and 50 μM 2-mercaptoethanol (Sigma-Aldrich). Biotin-anti-CD25 (7D4), APC-anti-CD4 (RM4-5), FITC-anti-CD45RB, and streptavidin-PE were purchased from BD Biosciences – Pharmingen. Anti-Foxp3 staining kit was purchased from eBioscience. Murine recombinant IL-2 (IL-2) was purchased from R&D Systems. Cells were analyzed on a BD FACSCalibur using CellQuest 5.2 software (BD). Anti-phospho-STAT5, anti-phospho-p70 S6 kinase (all from Cell Signaling Technology), anti-PTEN (Cascade Bioscience), and anti-actin antibodies were used for Western blot analysis.

**Bone marrow chimeras.** C57BL/6 Thy1.1 mice were lethally irradiated (10 Gy) prior to reconstitution with 2 × 10<sup>6</sup> total T cell-depleted bone marrow cells at specified ratios from either wild-type (Thy1.1) or PTEN-ΔT (Thy1.2) mice. Recipient mice were treated with Neosporin (DSM Pharmaceuticals Inc.) for 1 week prior to and 2 weeks after reconstitution. Ten weeks after reconstitution, thymic subsets were analyzed by flow cytometry for expression of Foxp3 and degree of chimerism.

**CD4<sup>+</sup>CD25<sup>+</sup> T cell isolation.** Spleen and lymph node cells were isolated from 2- to 3-week-old *Pten*<sup>flox/flox</sup> *Cre*<sup>+</sup> mice before the onset of disease and from age-matched Cre-negative littermates. Cell preparations were stained with anti-CD4 APCs, anti-CD25 biotin, streptavidin-PE, and anti-CD45RB-FITC and subsequently purified into CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup> cells by flow cytometry on a FACS Vantage Cell Sorter (BD Biosciences). Cell purity was routinely greater than 95%.

**Proliferation assays.** FACS-purified CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup> cells were CFSE labeled and cultured in complete medium supplemented with 100 U/ml rIL-2 in 96-well plates at a density of 10<sup>5</sup> cells/well. For TCR stimulation, cells were cultured either with plate-bound anti-CD3 (2.5 μg/ml) and anti-CD28 (2.5 μg/ml) or in the presence of irradiated T cell-depleted splenocytes with varying doses of soluble anti-CD3. Activated CD4<sup>+</sup> T cell blasts were derived by stimulating purified CD4<sup>+</sup> cells with PMA (0.2 μg/ml) and ionomycin (1 μg/ml) for 24 hours and subsequent washing before culture in the presence of rIL-2. In the case of cells from *Cre-ER/Pten*<sup>flox/flox</sup> mice, 1 nM 4-OHT was also added to the medium. Cell number was measured every 72 hours by Trypan blue exclusion, and cells were maintained at a density of 5 × 10<sup>5</sup> cells/ml. To measure proliferation, CFSE dilution was determined by FACS analysis at the indicated time points, or alternatively, after 48–72 hours, cells were pulsed with tritiated thymidine for a further 12 hours before harvest.

**In vitro suppression assays.** A total of 1.5 × 10<sup>5</sup> Thy1.1<sup>+</sup> CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>hi</sup> T cells were CFSE labeled and cultured with 3 × 10<sup>5</sup> irradiated T cell-depleted splenocytes and anti-CD3 Abs (2C11; 0.5 μg/ml). The indicated ratios of CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup> cells from either wild-type or PTEN-ΔT mice (Thy1.2<sup>+</sup>) were added to the cultures. To measure suppression, CFSE dilution of Thy1.1-positive cells was assessed by flow cytometry after 72 hours. Alternatively, cells were pulsed with 0.5 μCi of tritiated thymidine for the final 12 hours before being harvested.

**In vivo colitis model.** *Rag1*<sup>-/-</sup> mice were injected i.v. with 6 × 10<sup>5</sup> CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>hi</sup> T cells either alone or with 3 × 10<sup>5</sup> freshly isolated wild-type CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>lo</sup> Tregs, PTEN-ΔT CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>hi</sup> T cells, or PTEN-ΔT Tregs that had been expanded in vitro with rIL-2 (100 U/ml). Mice were weighed and examined every week for signs of disease and sacrificed for tissue harvest at 8 weeks. Tissues were fixed in 10% neutral buffered formalin (Fisher Scientific), cut into 5-μm sections, and stained with H&E. Severity of colitis was scored blindly as described previously (25).

**Immunoblotting.** As a result of the necessary breeding strategies employed, the number of PTEN-ΔT mice generated per litter was typically 50% of the number of littermate controls. Therefore, in order to obtain sufficient cell numbers to analyze intracellular signaling pathways, it was necessary to expand isolated PTEN-ΔT Tregs in vitro in the presence of rIL-2 as described above while Tregs from littermate control mice were generated in numbers allowing analysis immediately subsequent to isolation and moreover were not expandable by IL-2 alone. Both cell types were subsequently rested overnight in complete medium before study. After stimulation, cells were lysed at 4 °C in lysis buffer composed of 50 mM Tris-HCL (pH 6.8), 0.2% 2-mercaptoethanol, 20% glycerol, 4% SDS, and 0.001% bromophenol blue (all from Sigma-Aldrich). Cell lysates were clarified by centrifugation at 11,000 g for 10 minutes. Supernatants were boiled for 5 minutes, separated on a 10% SDS-PAGE at 1 × 10<sup>6</sup> cell equivalents/well and blotted onto Hybond ECL nitrocellulose membranes (Amersham Biosciences). Membranes were blocked for 1 hour in blocking reagent (Roche Diagnostics) at room temperature and probed with indicated antibodies at 1:1,000 dilution overnight at 4 °C. Membranes were washed and probed with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody at 1:1,000 for 60 minutes at room temperature. Blots were visualized by enhanced chemiluminescence (Roche Diagnostics) according to the manufacturer's protocol and on Hyperfilm ECL (Amersham Biosciences). Antibodies were subsequently stripped from membranes using Restore Western Blot Stripping Buffer (Pierce Biotechnology) and reprobed as above.

**Retroviral transduction.** The cDNA for human *Pten*, a kind gift from K. Yamada (NIH, Bethesda, Maryland, USA), was cloned into the MIG-IRES truncated nerve growth factor receptor retroviral vector, which has been previously described (35). High-titer retroviral supernatants were prepared as previously described. PTEN-ΔT Tregs or primary CD4<sup>+</sup> T cells were CFSE



labeled and stimulated for 18 hours with PMA (0.2 µg/ml) and ionomycin (1 µg/ml) for 18 hours. Cells were washed and transduced by spin infection with retroviral supernatants containing 2 µg/ml polybrene (Sigma-Aldrich) at 500 g for 90 minutes. Cells were subsequently cultured for 48–96 hours in complete media supplemented with rIL-2. Cells were analyzed for transduction efficiency by measuring NGFR expression by flow cytometry.

**Quantitative real-time PCR.** Total RNA was extracted from FACS-purified CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> T cells with an RNeasy kit (QIAGEN) and reverse transcription performed with the Superscript first-strand synthesis system (Invitrogen). Quantitative real-time PCR was performed with PRISM 7700 (Applied Biosystems) using primers, with an internal fluorescent probe specific for Foxp3 and GAPDH obtained from Applied Biosystems.

**In vivo BrdU labeling.** Mice were injected i.p. with 1 mg BrdU (BD Biosciences – Pharmingen) every 12 hours for 3 days (22). Mice were then sacrificed, and 2 × 10<sup>6</sup> splenocytes or thymocytes were surface stained as above for CD4, CD8, and CD25. BrdU staining with anti-BrdU FITC was performed using a BrdU labeling kit (BD Biosciences – Pharmingen) per the manufacturer's instructions.

**Fluorescence-linked immunosorbent assay.** A total of 10<sup>7</sup> 5-µm latex beads (interfacial dynamics) were coated with anti-IL-2 capture Abs (10 µg/ml) (BD Biosciences – Pharmingen) in PBS for 90 minutes at 37°C. Then, 2 × 10<sup>5</sup> beads were added to 100 µl of test supernatant or titrated amounts of rIL-2 in 100 µl complete medium as standards. Bead-bound IL-2 was detected using PE-labeled anti-IL-2 (BD Biosciences – Pharmingen) and subsequent FACS analysis.

**Statistics.** As a measure of colitis progression, body weight was compared using a 2-tailed Student's *t* test, and differences were considered statistically significant at *P* < 0.01.

## Acknowledgments

We thank the University of Pennsylvania Cancer Center Flow Cytometry Core for expert assistance with FACS and the Morphology Core, Center for Molecular Studies in Digestive and Liver, for assistance with histology. We also thank Scott Adler, Somia Perdow-Hickman, Aaron Neumann, David Neujahr, Chuangqi Chen, and Margaret Lucitt for helpful discussions. This work was supported by NIH grant AI-43620 (to L.A. Turka). P.T. Walsh is supported by an American Society of Transplantation basic science fellowship grant.

Received for publication January 27, 2006, and accepted in revised form June 20, 2006.

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