Conditional Deletion of PTEN in Peripheral T Cells Augments TCR-Mediated Activation but Does Not Abrogate CD28 Dependency or Prevent Anergy Induction

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Conditional Deletion of PTEN in Peripheral T Cells Augments TCR-Mediated Activation but Does Not Abrogate CD28 Dependency or Prevent Anergy Induction

Frederick L. Locke,* Yuan-yuan Zha, † Yan Zheng, † Gregory Driessens, † and Thomas F. Gajewski*‡

PTEN is thought to play a critical role in T cell activation by negatively regulating the PI3K signaling pathway important for cellular activation, growth, and proliferation. To directly eliminate PTEN in postthymic T cells for studies of functional effects, we used CAR transgenic × PTENflox/flox mice, which enabled gene deletion using a Cre adenovirus in vitro. These mice were also immunized to generate stable Th1 clones that could have PTEN deleted when desired. PTEN-deleted T cells exhibited enhanced IL-2 production, proliferation, and Akt phosphorylation upon TCR/CD28 engagement, whereas T cell survival was not potentiated. Gene expression profiling revealed a small subset of induced genes that were augmented upon PTEN deletion. However, PTEN-deficient T cells still required CD28 costimulation for IL-2 production and remained susceptible to anti-CD3-induced anergy. The absence of PTEN within the CD8 T cell compartment led to markedly increased cytolytic activity following an allogeneic MLR in vitro, without increasing autologous MLR activity. Our results indicate that deletion of PTEN can augment the activation of postthymic T cells but does not mediate CD28 independence or anergy resistance. Nonetheless, PTEN inhibition may be a viable target for immune potentiation owing to increased cytokine production by activated CD4+ cells and increased cytotoxicity by CD8+ T cells. The Journal of Immunology, 2013, 191:000–000.

PTEN protein (phosphatase and tensin homolog) is a phosphatase that plays a key role in the regulation of cellular survival and proliferation. Implicated as a tumor suppressor gene, PTEN gene loss leads to augmented cell survival (1), and it is frequently mutated or epigenetically silenced in hereditary and sporadic cancer, including T cell acute lymphoblastic leukemia/lymphoma (2). PTEN acts by dephosphorylating phosphatidylinositol (3,4,5)-triphosphate to generate phosphatidylinositol 4,5-bisphosphate and thus negatively regulates the PI3K signaling pathway. The PI3 pathway is critical for cell growth, survival, and motility signaling in numerous cell types (3). Within the T cell lineage, PTEN has been reported to negatively regulate TCR and CD28 signaling, is upregulated upon activation as a negative feedback mechanism (4), plays a role in CD4 and CD8 T cell development (5–7), affects regulatory T cell development (8), and appears to be involved in PD-1 and CTLA-4 inhibitory signaling (9).

Because of alterations in thymic development that can occur when signaling molecules are conditionally deleted using Lck-Cre or CD4-Cre transgenic (Tg) mice (10, 11), it has become desirable to develop strategies to delete genes directly in postthymic T cells, to determine functional effects directly within the peripheral T cell compartment without disturbing thymic selection. We have recently developed such a method by crossing mice Tg for the Coxsackie and adenovirus receptor (CAR) in the T cell lineage with mice bearing LoxP-targeted gene alleles, enabling specific gene deletion using a Cre adenovirus in vitro (12). Using this strategy in the current study, we have investigated the functional effects of PTEN deletion in primary T cells and Th1 clones. We find that PTEN deletion does lead to a decreased TCR signaling threshold for T cell activation, augments cytokine production, and allows for increased CTL activity in vitro. However, deletion of PTEN in peripheral T cells did not abrogate the need for CD28 and did not prevent anergy induction.

Materials and Methods
Mice and T cells

PTENflox/flox mice were a gift from Dr. Tak Mak of the Ontario Cancer Institute (Toronto, Canada) (10) and were crossed with CAR Tg mice expressing the extracellular domain of the CAR under control of an Lck promoter/CD2 enhancer (13). The resultant C57BL6/CAR-Tg/PTENflox/flox mice (or CAR Tg × PTENflox/flox) were homozygous for the PTENfloxp sequence. All mice were maintained under specific pathogen–free conditions in a barrier facility at the University of Chicago (Chicago, IL) according to approved protocols and National Institutes of Health guidelines. The OVA-specific CAR Tg × PTENflox/flox Th1 clone was previously described (12). T cell clones were maintained by weekly passage with OVA, IL-2, and syngeneic APCs (irradiated B6 splenocytes), as reported (14). Unless otherwise noted, T cells were cultured in complete DMEM media supplemented with 10% FCS (5% FCS for Th1 clones), penicillin, streptomycin, MOPS, 2-ME, and nonessential amino acids in an 8% CO2 incubator at 37°C.

Adenoviral transduction of CAR Tg T cells

The generation of the adenoviral vectors containing the gene expression unit of Cre recombinase (adeno-Cre) or without a coding cDNA [adeno–empty
vector (EV) and the protocol for transduction of peripheral T cells and Th1 clones was previously described (12). For adenoviral transduction, peripheral CAR Tg \( \times \) PTEN\(^{lox/lox} \) T cells (total, CD4\(^+\), or CD8\(^+\)) were isolated from splenocytes by negative selection with MACS Ab cocktails and magnetic beads (Miltenyi Biotec). Transduced CAR Tg \( \times \) PTEN\(^{lox/lox} \) T cells were rested overnight and then passaged under normal conditions, and 9 d later clones were harvested for experiments. Primary T cells were transduced, rested overnight, then cultured 6 d at 10 \( \times \) 10^6 cells per milliliter in complete medium. As naive T cells are kept alive in vitro through IL-7 signaling, we supplemented the media with 1 ng/ml IL-7 (R&D Systems) to prevent primary mouse T cells from dying precipitously in vitro (15). This step allows time for gene deletion before the cells are used for further experiments, as we have described previously (12). For primary T cell experiments we considered the possibility that PTEN deletion might have an impact on IL-7 signaling and skew the population of T cells that survive. We conducted control experiments contrasting adeno-EV– or adeno-Cre–treated splenic T cells immediately after transduction against those after the week-long rest with IL-7 to allow for gene deletion and PTEN protein degradation. We found no skewing of the population toward increased CD4 or CD8 numbers. PTEN deletion also had no substantial effect on CD127 surface expression and did not alter central memory, effector, or naive cell phenotypes (data not shown).

At the start of all experiments, 1 \( \times \) 10^6 cells were selected from each experimental condition (Adeno-Cre– or Adeno-EV–treated CAR Tg – PTEN\(^{lox/lox} \) T cells) and lysed for Western blot analysis of the PTEN protein, thus confirming Cre-mediated deletion.

**Western blotting**

Western blotting was conducted as previously described (16). For primary Ab incubation, the Abs were diluted into TBST + 5% BSA. The following Abs were used: anti-total-ERK1/2 (Zymed), anti-phospho-PTEN (139G6; Cell Signaling Technology), anti-Cre (Novagen), anti-total Akt (11E7; Cell Signaling Technology), and anti–phosphorylated (p)-Akt (C213E8; Cell Signaling Technology). Quantification was conducted using ImageJ v1.46 software (http://rsbweb.nih.gov/ij/) and a paired t test was performed for pAkt:Akt ratio in PTEN deleted compared with wild-type with the indicated conditions.

**Flow cytometry**

Abs against the following molecules coupled to the indicated fluorochromes were used—BD Pharmingen (BD) or E Biosciences: PE anti-CD3 (2C11; BD Pharmingen), PE anti-CD62L (MEL14; BD Pharmingen), APC anti-CD44 (1M7; BD Pharmingen). In general, 10^6 cells were blocked with the anti-FcR mAb 24G2, stained with the indicated Abs or appropriate isotype controls for 15 min at 4°C and then washed and resuspended for FACS analysis. Flow cytometry was performed on the FACScanto cytometer using BD FACSDivA software. Data analysis was performed using FlowJo software.

**T cell stimulation and anergy induction**

T cells were activated with beads (Dynal) coated overnight at 4°C with anti-CD3 (145-2C11) at 1 \( \mu \)g/ml and anti-CD28 (PV1) at 1 \( \mu \)g/ml unless otherwise indicated. For stimulation, T cells were incubated with the Ab-coated beads in 96-well flat-bottom plates, in triplicate, at 1 \( \times \) 10^6 cells per well in 200 \( \mu \)l complete medium (or 24-well plates at 1 \( \times \) 10^6 cells per well in 1 ml for gene array experiments) for the indicated time periods at 37°C, at a 5:1 bead/T cell ratio. Previously, we have shown that concentrations of anti-CD3 mAb at 0.1–1.0 \( \mu \)g/ml give optimal responses corresponding to the level of functional output and signaling events seen with Ag/APC stimulation, and at higher levels anti-CD3 can actually be inhibitory (17). For anergy induction, first Th1 CAR Tg \( \times \) PTEN\(^{lox/lox} \) cells were made anergic by initially stimulating for 24 h with plate-bound anti-CD3, then were collected and allowed to “rest” for 24 h in culture medium alone as described (18). Cells were then collected, washed, and restimulated with anti-CD3 + anti-CD28–coated beads for functional analysis.

**Cytokine ELISA**

For cytokine concentration analysis, culture supernatants were removed from wells at the indicated time points. Mouse IL-2 and IFN-\( \gamma \) Abs were obtained from BD, and cytokine production was measured by ELISA, using NUNC MaxiSorp 96-well plates according to the manufacturer’s protocol. Cytokine concentrations were determined with the SoftMax Pro data analysis program (Molecular Devices). Three identical experimental wells were tested and analyzed for each condition, and results are expressed as mean \( \pm \) SD, with the data shown representative of three replicated experiments.

**Proliferation assay**

For the \( ^{3} \)Hthymidine incorporation assay, cells were stimulated in 96-well plates with Ab-coated beads, as described. After 24 or 48 h of incubation, wells were pulsed with 1 \( \mu \)Ci tritiated thymidine, incubated for a further 8–16 h, then frozen until harvested. The wells were harvested for the determination of \( ^{3} \)Hthymidine incorporation, using a Packard Filtermate Harvester and TopCount NXT (PerkinElmer Life Sciences). Three identical wells were analyzed for each condition, and results (in counts per minute) are expressed as mean \( \pm \) SD, with the data shown representative of three replicated experiments.

**Quantitative RT-PCR**

Reactions were performed in 25-\( \mu \)l volumes and carried out in 96-well optical plates. An ABI 7700 thermal cycler (Applied Biosystems) was used for amplification. Each gene was evaluated in duplicate, using a specific primer/probe set purchased from Applied Biosystems and labeled with FAM dye. The Ct of a particular gene was normalized against the Ct of 18S. Data were analyzed using SDS Software (Applied Biosystems).

**MLR**

MLR stimulation to generate effector cells for proliferation and CTL analysis was adapted from a previously published protocol (19). Total T cells, or separate CD4\(^+\) and CD8\(^+\) fractions, were purified from spleens by negative selection with Abs and magnetic beads from Miltenyi (MACS) according to the manufacturer’s protocol. In 24– or 96-well tissue culture plates, responder cells were plated at 1 \( \times \) 10^6 per well (or 5 \( \times \) 10^5 per well), along with stimulator cells consisting of allogeneic T cell–depleted irradiated (5000 rad) splenocytes at 1 \( \times \) 10^5 per well (or 5 \( \times \) 10^4 per well). For proliferation assays, 96-well plates were used, and thymidine was added after 72 h, for overnight incorporation, each condition performed in triplicate. For chromium-release assays, 24-well plates were used, and after 5 d, the resulting cells were collected and counted prior to exposure to target cells at equivalent proportions.

**51Cr-release assay**

Chromium-release assays were performed as previously described (20). Briefly, \( ^{51} \)Cr-labeled targets (2 \( \times \) 10^6) were plated with PTEN-deleted or control CD4\(^+\) and CD8\(^+\) T cells at varying concentrations in a 96-well V-bottom plate (ICN Biomedicals), in triplicate. After 4 h of incubation at

FIGURE 1. Deletion of PTEN, using adenoviral delivery of Cre recombinase in peripheral T cells, leads to augmented TCR-mediated Akt phosphorylation. Following adenoviral transduction of CAR Tg \( \times \) PTEN\(^{lox/lox} \) T cells with Cre EV, Western blot revealed that (A) total splenic T cells and (B) CD4\(^+\) Th1 clones exhibited a significant diminution of PTEN protein expression, compared with the ERK positive control. A representative of six experiments for each is shown. (C) After transduction, Th1 clones were cocultured for 30 min with anti-CD3– or anti-CD28–coated beads at the indicated concentrations, and Western blot analysis was performed for phosphorylated versus total Akt and ERK. A representative of two independent experiments is shown.
37˚C, 25 μl supernatant was transferred to a LumaPlate-96 (PerkinElmer Life Sciences) and allowed to dry overnight. Plates were then counted using the TopCount NXT (PerkinElmer Life Sciences). Percent-specific lysis was calculated using standard methods.

**Gene array analysis**

The indicated T cell populations were stimulated as described. At the times indicated, total RNA was isolated by the TRIzol reagent protocol (Invitrogen), followed by RNeasy Mini column purification (Qiagen) according to the manufacturer’s instructions. RNA integrity was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies). The concentration/purity was determined using a NanoDrop 1000 (Thermo Scientific). All RNA samples used for hybridization had an OD 260/280 and OD 260/230 ratio > 1.8 and an RNA Integrity Number > 8.0. Total RNA was processed to cDNA synthesis, cRNA synthesis, fragmentation, and hybridization to expression arrays according to the GeneChip Expression Analysis Technical Manual (Affymetrix). Briefly, 2 μg total RNA was used to synthesize double-stranded cDNA using the GeneChip Expression 3’ Amplification One-Cycle cDNA Synthesis Kit (Affymetrix). First-strand cDNA synthesis was primed with an oligo(dT)24 primer that contains T7 promoter sequences. From the cDNA purified by the GeneChip Sample Cleanup Module (Affymetrix), biotin-labeled antisense RNA (cRNA) was synthesized using the GeneChip Expression 3’ Amplification IVT Labeling Kit (Affymetrix). After cleanup of cRNA with the GeneChip Sample Cleanup Module, 20 μg cRNA was fragmented in fragmentation buffer for 35 min at 94˚C. The fragmented cRNA (12 μg) was hybridized to Affymetrix MG-430 2.0 expression arrays for 16 h at 45˚C and 60 rpm in an Affymetrix Hybridization Oven 640. Arrays were washed and stained with streptavidin PE, and the fluorescent signal was amplified using a biotinylated Ab solution in an Affymetrix Fluidics Station 450 according to the Affymetrix GeneChip protocol. The arrays were scanned with the Affymetrix GeneChip Scanner 3000 7G. CEL intensity files generated by GeneChip Operating Software v. 1.4 (MicroArray Suite 5.0) were used to extract and analyze data with dChip software (Harvard University).

Array normalization and expression value calculations were performed (21). Each analysis was done in duplicate, using different primary material treated with equivalent experimental conditions. Arrays were normalized at the probe level, and the invariant set normalization method to allow the comparison of expression values computed using the model-based method (21). Measurement accuracy evaluated by SE was used to compute 90% confidence intervals of fold changes in two-sample and two-group comparisons (i.e., EV to Cre comparison done twice and compared for consistency). Increased and decreased expression of genes by > 2-fold (lower confidence bound) are presented.

The data discussed in this publication have been deposited in the National Center for Biotechnology Information’s Gene Expression Omnibus (22) and are accessible through Gene Expression Omnibus Series accession number GSE43936 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE43936).

**Results**

PTEN-deleted peripheral T cells exhibit increased Akt phosphorylation, proliferation, and cytokine production upon CD3/CD28 engagement but do not show increased survival.

To understand the role of PTEN in T cell activation, we used a model that allows specific deletion of genes directly within peripheral T cells (12). Mice homozygous for conditionally targeted PTEN alleles (10)
were interbred with CAR-Tg mice under the control of an Lck promoter/CD2 enhancer cassette (13, 23). This model system allows for deletion of targeted alleles using a Cre adenovirus in vitro, without a need to induce cell proliferation (12). To determine the effects of PTEN deletion in peripheral T cells, either splenic T cells or Th1 clones generated by immunization of these mice with OVA (24) were employed. In both cases, transduction with Adeno-Cre led to nearly complete elimination of PTEN protein as detected by Western blot analysis, compared with transduction with an empty vector (Fig. 1A, 1B). In all functional experiments, Western blot analysis was similarly performed to confirm efficient PTEN deletion.

Diminished PTEN levels would be expected to augment PI3K pathway activity. We examined the proximal downstream event of this pathway, Akt phosphorylation, in response to CD3/CD28 ligation. As shown in Fig. 1C, a significant increase in Akt phosphorylation was indeed observed upon deletion of PTEN. The increase in pAkt:tAkt was not constitutive (p = 0.6 for unstimulated cells) but still required TCR ligation (p < 0.005 for stimulated cells). Furthermore, the fold induction of pAkt:tAkt in stimulated cells compared with that in unstimulated cells was increased with PTEN deletion (p < 0.005). This observation supports the idea that PTEN deletion alone is not sufficient for Akt activation. It is also noteworthy that the augmentation in Akt phosphorylation was seen with ligation of the TCR complex alone, without CD28 costimulation (p < 0.05 for anti-CD3–stimulated cells), indicating that PTEN deletion is largely augmenting TCR-based signaling rather than costimulatory receptor effects. Erk phosphorylation was slightly increased in the experiment shown; however, this increase was not reproducible across experiments (data not shown).

To begin to address the functional effects of PTEN loss, T cells were stimulated with anti-CD3/anti-CD28–coated beads, and proliferation was assessed. As expected, PTEN-deleted splenic T cells and Th1 clones showed increased proliferation as measured by thymidine incorporation (Fig. 2A, 2B). As increased Akt activation has been associated with augmented cell survival in some model systems (25), the percentage of apoptotic cells was also assessed. In fact, no difference was detected in the fraction of viable cells upon PTEN deletion (Fig. 2C, 2D), indicating that the increased thymidine incorporation observed was directly due to increased proliferation and not via decreased cell death.

The increased cell proliferation observed after PTEN deletion suggested that IL-2 production may have been increased. Therefore, cytokine production was assessed in response to CD3/CD28

**FIGURE 3.** PTEN-deleted T cells reveal increased cytokine production, which is reflected at the mRNA level. PTEN-deleted versus control Th1 clones were stimulated with anti-CD3 + anti-CD28 mAb–coated beads. (A and B) Supernatant was collected at 24 h for IL-2 and IFN-γ quantification by ELISA. (C and D) Cells were collected 6 h after stimulation, and mRNA was isolated for IL-2 and IFN-γ mRNA quantification. A representative of three individual experiments is shown for each. (E) Analysis of IL-2 mRNA induction by quantitative RT-PCR over time in CD4+ T cell clones. A representative of three independent experiments is shown. Results are expressed as mean ± SEM. Two-tailed t test differences with p > 0.05 were considered insignificant, *p < 0.05, **p < 0.005.
ligation. As shown in Fig. 3A, IL-2 production was indeed augmented in PTEN-deleted Th1 clones, and a modest increase in IFN-γ production was also observed (Fig. 3B). Increased cytokine secretion was accompanied by augmented cytokine mRNA expression (Fig. 3C, 3D), arguing for potentiation at the level of gene transcription. Kinetic analysis revealed that the augmentation of IL-2 mRNA expression was detected as early as 3 h following CD3/CD28 stimulation (Fig. 3E), consistent with the notion that PTEN for the most part negatively regulates proximal TCR-mediated signaling events.

PTEN deletion in peripheral T cells lowers the TCR threshold for activation, but does not abrogate the need for CD28 costimulation and does not prevent anergy induction

It has previously been suggested that the PTEN–PI3K axis represents a signaling event predominantly regulated by CD28 costimulation (26). We therefore examined closely whether deletion of PTEN directly in peripheral T cells appeared to bypass a need for CD28 ligation. A dose titration of anti-CD3 mAb was performed with or without the addition of anti-CD28 mAb. As shown in Fig. 4A and 4B, a shift in the dose–response curve was observed

**FIGURE 4.** Following PTEN deletion, the threshold for TCR-mediated signaling is lowered, yet CD28 dependency and anergy susceptibility are not affected. (A) PTEN-deleted versus control T cells were stimulated with the indicated concentrations of anti-CD3 ± anti-CD28 mAb. IL-2 production (A) from total peripheral T cells and IFN-γ production (B) from Th1 clones were assessed by ELISA. In parallel experiments, proliferation was measured in (C) total peripheral T cells and (D) CD4+ Th1 clones. (E) Following anergy induction using plate-bound anti-CD3 Ab, Th1 cells were restimulated with anti-CD3/anti-CD28 mAb–coated beads, and IL-2 production was assessed by ELISA. Representatives of three independent experiments are shown for each. Results are expressed as mean ± SEM. Two-tailed t test differences with \( p > 0.05 \) were considered insignificant, \( *p < 0.05, **p < 0.005 \).
with PTEN-deleted T cells. Approximately 10-fold less anti-CD3 mAb was required in PTEN-deleted T cells to generate comparable levels of IL-2 production to nondeleted cells for both primary splenic T cells (Fig. 4A) and Th1 clones (data not shown). A similar shift in dose titration was observed for Th1 helper production of IFN-γ (Fig. 4B) and for the dose of anti-CD3 mAb required to induce proliferation (Fig. 4C 4D). However, it is important to note that CD28 ligation was still required to induce substantial IL-2 production, even when PTEN was deleted (Fig. 4A, 4E). Therefore, deletion of PTEN directly in postthymic T cells did not bypass the requirement for CD28 costimulation.

We next examined whether PTEN deletion in peripheral T cells would prevent those cells from being capable of anergy induction. This idea was examined in Th1 clones, which represents the classical cellular model of T cell anergy (27–29). Anergy was induced using immobilized anti-CD3 mAb, as we and others have reported previously (12, 30). As shown in Fig. 4E, Th1 clones were equivalently rendered hyporesponsive to CD3/CD28 ligation under anergizing conditions, whether or not PTEN was deleted. Cells in both conditions remained viable, as indicated by their ability to secrete IL-2 in response to PMA and ionomycin. Therefore, deletion of PTEN directly in postthymic T cells failed to prevent anergy induction in vitro.

Gene expression profiling reveals a small subset of induced genes influenced by PTEN expression

Our results thus far indicated that conditional deletion of PTEN in peripheral T cells led to a marked augmentation of IL-2 production and proliferation in response to CD3/CD28 ligation. It was therefore of interest to determine the spectrum of transcripts regulated by PTEN, using gene expression profiling. To this end, CAR Tg x PTENflox/flox Th1 clones were treated with either adeno-Cre or adeno-EV, then stimulated for 6 h with anti-CD3/anti-CD28 mAb–coated beads. We found that deletion of PTEN resulted in augmented expression of a remarkably limited set of transcripts after activation (Fig. 5, Table I). The top two differentially expressed genes were CDK19 and the aryl hydrocarbon receptor nuclear translocator 2, which could theoretically influence cell proliferation. The third most differentially expressed gene was IL-2, which was already defined through analysis of candidates. Most other differentially expressed genes were near the 2-fold cutoff threshold. These results suggest that the major role of PTEN in terms of influencing gene expression in peripheral CD4+ T cells is likely regulation of IL-2 production and proliferation.

PTEN deletion in CD8 T cells yields markedly augmented cytolytic activity in vitro

The PI3K pathway is thought to be an important signaling event in CD8+ T cell function as well, and its blockade has been proposed as an immunosuppressive strategy to prevent alloreactivity activity after transplantation. Conversely, mechanisms to augment signaling through the PI3K axis could improve CD8+ T cell–based immunity against tumors or infectious agents. To examine the effect of PTEN deletion in CD8+ T cell function, we performed an allogeneic MLR using total splenic T cells from CAR Tg x PTENflox/flox mice transduced with adeno-Cre (PTEN deleted) or adeno-EV (control). Stimulators were T cell–depleted splenocytes from either allogenic DBA/2 mice or autologous C57BL/6 mice as a control. Thymidine incorporation was measured, and PTEN deletion led to a significant increase in proliferation against allogeneic stimulator cells compared with control cells (Fig. 6A).

It was of interest to determine the effects of PTEN deletion on the development of cytotoxicity and whether the effect of PTEN deletion was within the CD4+ or CD8+ T cell compartment. To examine this possibility, CD4+ and CD8+ T cell populations were first purified from CAR Tg x PTENflox/flox mice and then separately transduced with either adeno-Cre or adeno-EV. An MLR was performed, and CTL activity was assessed against chromium-labeled P815 (H-2b) tumor cells. As shown in Fig. 6B, a markedly increased level of cytolytic activity was observed upon deletion of PTEN, and this effect was completely recapitulated when PTEN deletion was performed selectively within the CD8+ T cell compartment. Thus, in addition to augmenting cytotoxic production and proliferation by CD4+ T cells, PTEN deletion can potentiate cytolytic activity by CD8+ T cells.

Discussion

In this article we show that deletion of PTEN directly in peripheral T cells having undergone normal thymic development decreases the activation threshold for TCR-mediated signaling. PTEN deletion in peripheral T cells retains dependency on CD28 costimulatory signaling for activation and does not generate an anergy-resistant phenotype. Nonetheless, PTEN deletion augmented cytokine production and proliferation in CD4+ T cells, and additionally potentiated cytolytic activity by CD8+ T cells. These functional effects were associated with augmentation in activation of the PI3K pathway, as evidenced by increased TCR-induced phospho-Akt. Thus, developing pharmacologic strategies to potentiate the PI3K pathway in T cells could serve as an attractive immune-potentiating platform.

Previously published reports of T cell PTEN deletion during thymic development required analysis of T cells while the mice remained young, as lymphoproliferation, autoimmunity, and lymphoma ultimately developed by 16 wk. The differences between those results and our current data are most likely due to the timing of PTEN deletion during T cell development, although a direct
Table I. Genes with 2-fold (lower confidence bound) increased or decreased expression, 6 h following stimulation, in PTEN-deleted T cells (Adeno-Cre) as compared with wild-type T cells (Adeno-EV)

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That being said, we acknowledge some limitations of our present model as well. First, the adenovirus itself used for Cre transduction may alter T cell function, although we have shown previously that empty, nonreplicating, adenoviral vectors do not affect the T cell functions analyzed in the assay systems used in this study (13, 23, 35). The use of an empty vector control allows for evaluation of Cre expression as the single variable tested. We also have done extensive analysis of the potential off-target effects of Cre on T cells (using CAR Tg T cells without a floxed allele) and have not detected any such effects in our in vitro assays (12, 16, 36) (and data not shown). Second, for gene deletion in primary T cells, coculture with IL-7 was required to prevent cell death in vitro, similar to the requirement for IL-7R signaling for naive T cell survival in vivo (15). Although PTEN deletion might affect IL-7R signaling, we did not find any skewing of T cell populations following this culture; did not detect any differential expression of the IL-7R in PTEN-deleted cells; and, following withdrawal of IL-7, did not observe any expansion or contraction of PTEN-deleted T cell population. It should be noted that any in vivo Cre system (such as CD4-Cre or Lck-Cre Tg mice) similarly would potentially have an impact on many different receptor-mediated signaling pathways, including cytokine receptors such as the IL-7R. Third, although PTEN protein absence is complete in the deleted Th1 clones, a very low level of residual PTEN protein persists in the T cell population. It should be noted that any in vivo Cre system (such as CD4-Cre or Lck-Cre Tg mice) similarly would potentially have an impact on many different receptor-mediated signaling pathways, including cytokine receptors such as the IL-7R.

Despite this low level of residual protein, we nonetheless observed a potent phenotype upon PTEN deletion: markedly augmented IL-2 production and proliferation, Akt phosphorylation, and cytolytic activity.
An implication of our findings is that CD28 costimulation relies on pathways other than augmentation of PI3K activity. It is known that CD28 ligation can augment several TCR signaling pathways; however, transfection of CD28 mutants has indicated that the ability to activate PI3K is not necessary for IL-2 production and proliferation (6, 39). We typically have seen minimal augmentation of pAkt in conventional T cells with CD28 costimulation, but, in contrast, have seen augmentation of Ras pathway signaling when suboptimal TCR ligation is used. In fact, we have recently reported evidence strongly implicating alterations in Ras pathway signaling that seem critical for the costimulatory effects of CD28. Ligation of CD28 promoted RasGRP translocation to the immunological synapse, and IL-2 production driven by CD3/CD28 costimulation was lost in RasGRP1−/− mice. Moreover, introduction of active Ras could recapitulate all the major functional effects of CD28 engagement (increased IL-2 mRNA, increased mRNA stability, prevention of anergy, potentiation of T cell survival, and augmentation of glucose uptake) (40). Together, these results strongly argue that Ras pathway (rather than PI3K pathway) signaling constitutes a major functionally important signaling event in CD28 costimulation.

Although we failed to detect any effect of PTEN deletion on anergy induction in Th1 cells, this is not a property of the model system itself. We recently have identified the transcription factor Egr2 as an important contributor to the anergic state. In a fashion analogous to the current report, we generated Th1 clones from Egr2flox/flox × CAR Tg mice, which enabled Cre-mediated deletion of Egr2 in vitro. In fact, T cell anergy was largely prevented in this system (36). Use of this model also enabled identification of other key Egr2 transcription targets in anergy (41). These data not only support the utility of this model system, but additionally support the functional role for alternative signaling pathways as key for anergy, besides PTEN.

It was striking to us that unbiased gene expression profiling on PTEN-deleted T cells revealed such a limited set of transcripts regulated by this signaling molecule. We primarily saw augmentation of IL-2 mRNA level transcription, which we had known already by examining it as a candidate target gene. Beyond effects on gene expression, PTEN deletion also markedly augmented cytolytic activity by CD8+ T cells, which may be a posttranslational effect. The increased cytolysis was not due to more T cells; rather, this seems to be an increase in cytolytic activity directly. Of note, in other model systems, important changes in membrane ruffling and adhesion following conditional deletion of PTEN have been reported (42–44). Future study should focus on these mechanisms in CD8+ T cells. Together, these results suggest that the major function of PTEN is to negatively regulate IL-2 production, proliferation, and cytolytic effector function in activated peripheral T cells.

The increased effector function of T cells upon PTEN deletion argues that pharmacologic strategies to inhibit PTEN and/or augment PI3K activity may be attractive as a method to develop immune-potentiating drugs. Although multiple small-molecule inhibitors of T cell activation have been developed for immune suppression during settings of immune-mediated disease, the rational development of immune-potentiating drugs has in general lagged behind. The first example applied clinically and achieving Food and Drug Administration approval is the anti–CTLA-4 mAb ipilimumab (45). The significant expense of protein-based therapeutics makes it potentially attractive to pursue small-molecule approaches. An additional theoretical advantage is the opportunity to discontinue such drugs in case immune-mediated adverse events arise, with potentially rapid resolution. Future development of such immune-potentiating agents that phenocopy effects seen with deletion of PTEN or other negative regulatory factors in T cells seems warranted.

Disclosures
The authors have no financial conflicts of interest.

References
5. Kane, L. P., P. G. Andres, K. C. Howland, A. K. Abbas, and A. Weiss. 2001. Akt regulates PI3K activity by CD8+ T cells, which may be a posttranslational effect. The increased cytolysis was not due to more T cells; rather, this seems to be an increase in cytolytic activity directly. Of note, in other model systems, important changes in membrane ruffling and adhesion following conditional deletion of PTEN have been reported (42–44). Future study should focus on these mechanisms in CD8+ T cells. Together, these results suggest that the major function of PTEN is to negatively regulate IL-2 production, proliferation, and cytolytic effector function in activated peripheral T cells.

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