The Journal of Immunology

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J Immunol 2012; 188:5935-5943; Prepublished online 18 May 2012;
doi: 10.4049/jimmunol.1102116
http://www.jimmunol.org/content/188/12/5935

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Pten Loss in CD4 T Cells Enhances Their Helper Function but Does Not Lead to Autoimmunity or Lymphoma

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PTEN, one of the most commonly mutated or lost tumor suppressors in human cancers, antagonizes signaling by the PI3K pathway. Mice with thymocyte-specific deletion of Pten rapidly develop peripheral lymphomas and autoimmunity, which may be caused by failed negative selection of thymocytes or from dysregulation of postthymic T cells. We induced conditional deletion of Pten from CD4 Th cells using a Cre knocked into the *Tnfrsf4* (OX40) locus to generate $OX40^{Cre}Pten^{f}$ mice. Pten-deficient Th cells proliferated more and produced greater concentrations of cytokines. The $OX40^{Cre}Pten^{f}$ mice had a general increase in the number of lymphocytes in the lymph nodes, but not in the spleen. When transferred into wild-type (WT) mice, Pten-deficient Th cells enhanced anti-*Listeria* responses and the clearance of tumors under conditions in which WT T cells had no effect. Moreover, inflammatory responses were exaggerated and resolved later in $OX40^{Cre}Pten^{f}$ mice than in WT mice. However, in contrast with models of thymocyte-specific Pten deletion, lymphomas and autoimmunity were not observed, even in older $OX40^{Cre}Pten^{f}$ mice. Hence loss of Pten enhances Th cell function without obvious deleterious effects. ***The Journal of Immunology*****, 2012, 188: 5935–5943.**

CD4 T cells support the coordinated activation of other leukocytes during immune responses. For instance, CD4 T cells secrete inflammatory cytokines during contact hypersensitivity (CHS) reactions, augment tumor surveillance, help B cells during the germinal center reactions, and license dendritic cells to express high levels of MHC and costimulatory ligands (1–4). Full activation of naive CD4 T cells requires persistent stimulation of the TCR and CD28 over a period of ~24 h. During this time, the T cells interact with APCs and integrate signals needed to increase metabolism, grow in size, and upregulate cytokine, chemokine, and costimulatory receptors (5). After initial activation, CD4 T cells begin to divide in response to cytokines and express additional costimulatory receptors such as ICOS and OX40. They then differentiate into different Th cell lineages that secrete cytokines such as IFN-γ, IL-4, or IL-17. Eventually, most CD4 Th cells die through apoptosis, but some survive as CD4 memory Th cells (6). Similarly, after initial activation, naive CD8 T cells differentiate to become CTLs, and the ones that survive the cycle of expansion and contraction become CD8 memory T cells (7).

The TCR and many costimulatory and cytokine receptors activate PI3Ks (8). The class I PI3Ks (p110α, p110β, p110γ, and p110δ) use $PtdIns(4,5)P_2$ as their preferred substrate to generate the second-messenger molecule $PtdIns(3,4,5)P_3$, which helps activate PH domain-containing proteins such as Akt. Akt phosphorylates Foxo transcription factors and proteins associated with mTOR complex activation (9, 10). PI3K activity is also required for optimal Erk phosphorylation in T cells (11). By these and other mechanisms, PI3Ks contribute to the proliferation, growth, survival, cytokine production, trafficking, and homeostasis of CD4 T cells (8–16). In T cells, roles for p110α and p110β have not been reported, but the p110γ isoform is activated by G protein-coupled receptors and regulates basal motility in the lymph node (LN), chemotaxis of effector T cells to sites of inflammation, and the survival of memory T cells (17–21). p110γ can also regulate chemotaxis in human T cells (22). p110δ is the functionally dominant isoform downstream of TCR, ICOS, and the IL-2R, and controls Ag-specific events such as differentiation (11, 12, 23–25). PI3K activity remains high for several days after CD4 T cell activation (26), and using acute inhibition with an isoform-selective inhibitor, we demonstrated that p110δ activity is required beyond the first 24 h after TCR activation to regulate cytokine production (16). We also demonstrated the p110δ is a major regulator of cytokine production in human T cells from healthy, atopic and arthritic individuals (16). Given the importance of Th cytokine production in supporting protective and pathological immune responses, it is important not only to understand how the PI3K pathway is activated, but also how it is sustained or curtailed.

Pten dephosphorylates $PtdIns(3,4,5)P_3$ to produce $PtdIns(4,5)P_2$, thus terminating PI3K signaling. PTEN is frequently lost or mutated in human cancers, including 20% of T cell acute lymphoblastic leukemias (27). PTEN expression is also lost in commonly used human T cell lines such as Jurkat, which may have confounded some studies where these cells are used to study T cell signaling (28, 29). Conditional deletion of *Pten* in thymocytes led to defective negative selection, progressive lymphoproliferation, autoimmunity, and CD4 T cell lymphomas (30–32). Similar effects were observed in mice that overexpressed micro-RNAs that target Pten (33). After activation, peripheral $Pten^{-/-}$ T cells proliferated more, resisted apoptosis, and failed to contract after superantigen stimulation (30, 34). $Pten^{-/-}$ T cells also could be

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Received for publication July 21, 2011. Accepted for publication April 16, 2012.

This work was supported by a core strategic grant (to K.O. and M.T.) and Project Grant BBF0154611 (to K.O.), both from the Biotechnology and Biological Sciences Research Council; a Wellcome Trust New Investigator Award (to K.O.); and a Senior Non-Clinical Fellowship from the Medical Research Council (to M.T.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: ActA-LM-OVA, OVA-secreting *Listeria monocytogenes*; CHS, contact hypersensitivity; IC, IC87114; LN, lymph node; LY, LY294002; TNCB, trinitrochlorobenzene; Treg, regulatory T cell; WT, wild-type.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1102116

activated in the absence of CD28 costimulation and resisted CTLA4-Ig–induced anergy induction, and thus Pten was suggested to impose a threshold for activation during initial TCR sensing (32). Although Pten expression levels may modulate immediate responses downstream of the TCR in thymocytes and naive T cells, the role of Pten during an ongoing immune response is not well understood.

To address the role of Pten in mature CD4 Th cells, we conditionally deleted the *Pten* gene in Th cells using a Cre-recombinase gene knocked into *Tnfrsf4* (the gene that encodes OX40) (35). OX40 is transiently expressed ~24 h after activation in the majority of CD4 T cells, but rarely in CD8 T cells (36). OX40 is also constitutively expressed on regulatory T cells (Tregs) (36). We found that deletion of *Pten* after TCR stimulation regulated the magnitude and duration of T cell responses, but not apoptosis or contraction. Furthermore, contrary to when Pten was deleted in thymocytes, lymphoma did not develop when the deletion occurred in activated Th cells. Instead, overproduction of cytokines in these mice leads to altered homeostasis of the lymphocyte compartment and enhanced inflammatory, antibacterial, and antitumor responses.

Materials and Methods

Mice

All mice were maintained under specific pathogen-free conditions. All experiments were performed in accordance with U.K. Home Office regulations. OT2 (37), $Rag1^{-/-}$ (38), $Rag2^{-/-}$ (39), $OX40^{Cre}$ (35), $Pten^{f}$ (40), and $R26^{YFP}$ (41) and $R26^{tdRFP}$ (42) mice were described previously.

Reagents

Unless otherwise stated, all chemicals were from Sigma-Aldrich. IC87114 (IC) was synthesized as previously described (16) and anti-CD3 (2C11) was purified in-house. IL-2 was synthesized by GlaxoSmithKline. Cells were cultured in RPMI 1640 supplemented with 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, 10 mM HEPES, 20 μM 2-ME, and 5% FCS at 5% CO_2.

Abs, flow cytometry, and Western blot

All Abs were from eBioscience except for Fas and CD4-V500 (BD); Pten, p-Akt, and p-Erk (Cell Signal Technologies); and anti-rabbit Ig Alexa 647 (Invitrogen). To stain for transcription factors, Pten or intracellular cytokine, we used Foxp3 staining buffer set or IC Fixation Buffer Kits (both eBioscience), respectively. CD4 T cells were purified with a Naive CD4 T cell Kit (Miltenyi) and activated with 1 μg/ml anti-CD3 on irradiated APCs to detect signaling molecules. Every 24 h, an aliquot of cells was treated with Fix Buffer I and Permeabilization Buffer 3 (BD), according to manufacturer's instruction, and cells were stained at the end of the time course. Apoptotic cells were detected with 7-aminoactinomycin D ± Annexin V (BD), according to manufacturer's instructions. Flow cytometry data were acquired with FACSCalibur or LSRII instruments (BD) and analyzed with FlowJo (Tree Star). Cells were sorted using a FACSAria (BD) machine. Cell counts were performed either using a CASYCounter or FlowCount Fluorospheres (Beckman Coulter).

Cell purification, proliferation, and cytokine assays

CD4 T cells were purified, stimulated, and proliferation and cytokine production measured as described previously (12, 16). To measure cytokines by intracellular FACS or by Mouse Cytokine Array Panel A (R&D Systems), we stimulated cells with 1 μg/ml PDB and 5 nM ionomycin for 5 h and 10 μg/ml brefeldin A was added for the final 2 h. For the Mouse Cytokine Array Panel A, lysates then were made from 5×10^7 cells and cytokines detected according to manufacturer's instructions. Pixel density was analyzed using Aida Image Analysis software.

Treg suppression assay

Suppression assays with anti-CD3–coated APCs were performed as described previously (43). Suppression was calculated as the amount of proliferation when Tregs were present compared with when they were absent: % suppression = (cpm responders with Tregs/cpm responders alone) * 100.

Autoantibody detection

Autoantibodies were detected using a Hep2 ANA kit (The Binding Site). Positive control serum from an MRL mouse was a gift from L. Martensson-Bopp (University of Gothenburg, Sweden).

Transwell assays

OT2 $Pten^{f}$ and OT2 $OX40^{Cre}Pten^{f}$ cells were activated in vitro with OVA peptide for 3 d; then live CD4 T cells were purified. Next, CD4 T cells were cocultured at various ratios with $CD45.1^{+}$ naive wild-type (WT) splenocytes, either in direct contact or separated by 0.4-μM Transwell inserts (Fisher). After 3 d, the numbers of $CD45.1^{+}$ CD4, CD8, and B220 cells were calculated.

CHS assays

CHS assays were done as described previously (16). In brief, trinitrochlorobenzene (TNCB)-sensitized mice were rechallenged with TNCB and dosed twice daily with 30 mg/kg IC or 1% methylcellulose vehicle control for 2 d. Twenty-six days after the first challenge, the same ear was rechallenged to examine secondary responses. These mice had not received drug during the primary elicitation. Ear size was measured with a micrometer (Kroeplin).

Adoptive transfer assays

CD45.1 hosts were injected i.v. with 10^6 OT2 $Pten^{f}$ or OT2 $OX40^{Cre}Pten^{f}$ CD4 T cells and the next day injected s.c. with PBS or 50 μg LPS O26:B6 + 1 mg OVA. Skin draining LNs were harvested on day 3, 6, or 9 after immunization.

Listeria monocytogenes *assays*

CD45 mismatched hosts were injected i.v. with PBS or 1.5×10^6 OT2 $Rag2^{-/-}$ or OT2 $OX40^{Cre}Pten^{f}Rag2^{-/-}$ cells. The following day, hosts were i.v. injected with 10^7 CFU attenuated OVA-secreting *Listeria monocytogenes* (ActA-LM-OVA) (44). After 3 d, splenocytes were stained for intracellular cytokines. APC-labeled SIINFEKL-MHC class I tetramer (Beckman-Coulter) were used to detect OVA-specific CD8 T cells.

Tumor assays

$CD45.1^{+}$ hosts were injected i.v. with PBS or 2×10^5 $CD45.2^{+}$ OT2 $Pten^{f}$ $Rag2^{-/-}$ or OT2 $OX40^{Cre}Pten^{f}Rag2^{-/-}$ cells. The following day, 5×10^5 EG7 were injected s.c. into hosts. Tumors were palpable between days 10 and 11. On day 14 after tumor injection, mice were culled and tumors were weighed.

Statistics

Statistics were calculated with GraphPad or SSCS software. The following symbols are used on graphs and tables: $*0.01 \leq p \leq 0.05$, $**0.001 \leq p \leq 0.01$, $***p \leq 0.001$.

Results

PI3K signaling in $OX40^{Cre}Pten^{f}$ T cells is sustained

In $OX40^{Cre}$ mice, Cre is expressed almost exclusively in activated CD4 T cells and Tregs, and only 2–5% of CD8 T cells (35). Using reporter mice, we detected Cre activity in 20% of $CD4^{+}$ T cells 1 d after activation, increasing to 80% after 3 d (Supplemental Fig. 1A). PCR analysis demonstrated nearly complete recombination of the *Pten* locus in YFP^{+}, $OX40^{Cre}Pten^{f}$ CD4 T cells, whereas no deletion was observed in YFP^{-} cells. This showed that the YFP accurately reported Cre activity on the $Pten^{f}$ gene (Supplemental Fig. 1B). Flow cytometry showed loss of Pten protein in $OX40^{Cre}$ $Pten^{f}$ $CD4^{+}CD25^{+}$ and $CD4^{+}CD44^{hi}$ T cells, but not in $CD4^{+}$ $CD44^{lo}$ T cells (Fig. 1A). These results confirm that *Pten* was deleted in Tregs and memory T cells, but not in naive T cells. When stimulated with anti-CD3 and APCs, Akt and Erk phosphorylation were sustained for longer in $OX40^{Cre}Pten^{f}$ CD4 T cells compared with controls (Fig. 1B). This was consistent with the kinetics observed for Cre expression after activation (Supplemental Fig. 1A).

Pten regulates lymphocyte homeostasis

Pten deletion led to enhanced activation of CD4 T cells as evidenced by increased proportion of cells expressing high levels of

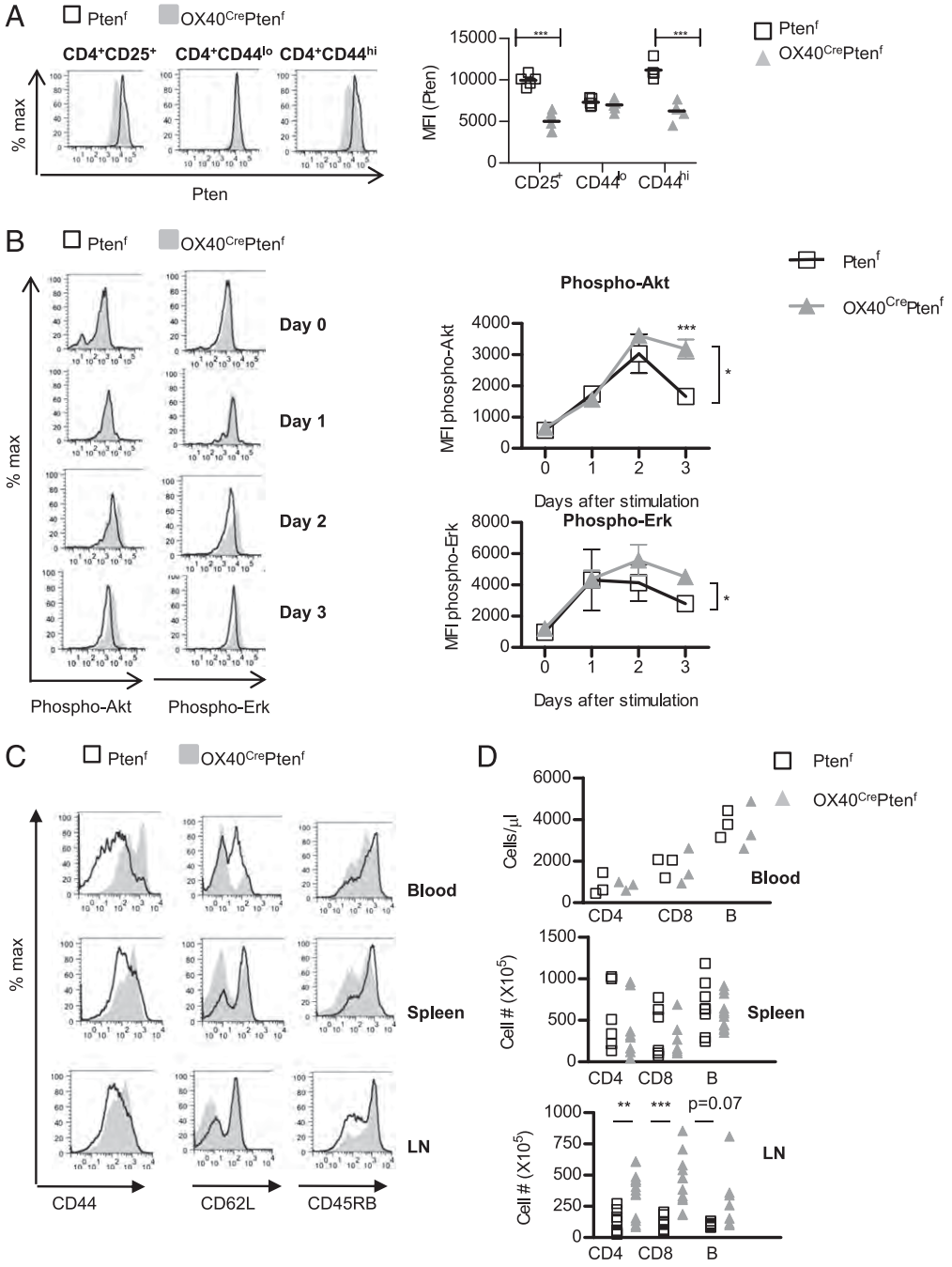


FIGURE 1. Loss of Pten leads to altered signaling and LN hyperplasia. (**A**) Pten was detected ex vivo by intracellular flow cytometry in $CD25^+$, $CD44^{lo}$, and $CD44^{hi}$ CD4 T cell mice. $n \geq 5$ for each genotype. The p values were determined with Student t test. (**B**) $CD62L^+CD4^+$ T cells were activated by irradiated APCs and anti-CD3, and the levels of Akt and Erk phosphorylation were measured at 24-h intervals. Representative histograms and graphs of median fluorescence intensities (MFI) are on the *right*. Data show mean ± SD for three mice from each genotype, and represents one of three independent experiments. Repeated-measures ANOVA shows statistical significance over the whole time course of $0.05 \geq p \geq 0.01$ for both phospho-Akt and phospho-Erk. The results of Bonferroni posttests also showed that phospho-Akt was strongly increased in $OX40^{Cre}Pten^f$ cells at day 3. (**C** and **D**) Blood, spleen, and LN cells from 5- to 10-wk-old $Pten^f$ and $OX40^{Cre}Pten^f$ mice were stained for lineage and activation markers, and analyzed by flow cytometry. (C) Representative histograms of activation markers in CD4 cells. $n \geq 5$ for each genotype. (D) Total numbers of B cells and CD4 and CD8 T cells. Student t test was used to calculate p values. $*0.01 < p \leq 0.05$, $**0.001 < p \leq 0.01$, $***p \leq 0.001$.

CD44 and lower levels of CD62L, and CD45RB in LNs, spleen, and blood of $OX40^{Cre}Pten^f$ mice (Fig. 1C). However, unlike mice with Pten deletion in thymocytes (30), autoantibodies were not detected in $OX40^{Cre}Pten^f$ mice even at an advanced age (Supplemental Fig. 2). Furthermore, histology of 22 organs from 19-wk-old $OX40^{Cre}Pten^f$ mice ($n = 5$) and controls ($n = 5$) showed no signs of inflammation or autoimmunity. More than 80% of mice with a germline heterozygous deletion in *Pten* form lymphomas by 24 wk of age, and 100% of mice with a thymocyte deletion of *Pten* develop lymphomas by 12 wk of age (30, 45, 46). These lymphomas derive from the thymus (47–49), but this did not exclude the possibility of lymphomas also arising in mice with postthymic deletion of *Pten*. However, none of 31 $OX40^{Cre}Pten^f$ mice culled between 25 and 37 wk of age showed evidence of lymphomas. In addition, none of five mice aged over 52 wk had lymphomas. Therefore, the pathology associated with Pten dele-

tion in all T cells was not evident when using OX40^{Cre}. Although OX40^{Cre}Pten^{f} mice did not develop lymphomas, their LNs, but not spleens, were enlarged from 5 wk after birth, with 2- to 3-fold more T and B cells in the LNs compared with littermates. In contrast, lymphocyte numbers in the spleen and blood were similar (Fig. 1D).

Because OX40^{cre} is expressed by Tregs in the thymus (35) and because Pten can affect Treg expansion in vitro (50), we enumerated Tregs in OX40^{Cre}Pten^{f} mice. There were similar numbers of Tregs in the thymi of OX40^{Cre}Pten^{f} mice, but more in the LNs (Supplemental Fig. 3A–D). Consistent with previous results (50, 51), there were no significant differences in suppression of WT responders by OX40^{Cre}Pten^{f} Tregs or in the ability of Tregs of either genotype to suppress OX40^{Cre}Pten^{f} responders (Supplemental Fig. 3E). Hence deficiencies in Tregs or susceptibility of Th cells to suppression are unlikely to have caused LN hyperplasia in OX40^{Cre}Pten^{f} mice.

Lymphocyte expansion is controlled in trans by Th cells

We next considered whether Pten-deficient Th cells altered homeostasis of Pten-sufficient bystander lymphocytes. To test this hypothesis, we generated mixed bone marrow chimeras in lethally irradiated Rag$^{-/-}$ mice, in which 50% of the donor bone marrow derived from CD45.1^{+} WT SJL mice and 50% derived from either CD45.2^{+} OX40^{Cre}Pten^{f} or CD45.2^{+} WT mice. Eight weeks after reconstitution, the number of lymphocytes in spleens of hosts receiving WT:SJL or OX40^{Cre}Pten^{f}:SJL bone marrow was similar (Fig. 2A). By contrast, there was 3- to 4-fold more B and T cells in LNs of mice receiving SJL:OX40^{Cre}Pten^{f} bone marrow than controls (Fig. 2B). When the ratio of CD45.1 to CD45.2 cells was analyzed, however, we found both donors contributed equally to cells repopulating the LNs and spleens, suggesting that OX40^{Cre} Pten^{f} CD4 T cells support general accumulation of LN cells independently of their Pten status.

To test whether a soluble factor was needed to expand lymphocytes, Pten^{f} or OT2 OX40^{Cre}Pten^{f} CD4 OT2 T cells were activated using the OVA-derived peptide recognized by the OT2 TCR. The activated OT2 T cells were then purified and cocultured with unfractionated WT lymphocytes. After 3 d, the number of WT lymphocytes was counted (Fig. 2C). OT2 OX40^{Cre}Pten^{f} CD4 T cells maintained WT lymphocytes better than controls when effectors were in direct contact with responders. This difference between genotypes was unaffected by inclusion of a Transwell filter, demonstrating that expansion of responders was due to secretion of a soluble factor by OX40^{Cre}Pten^{f} effector cells.

Pten regulates cytokine production by activated CD4 T cells

In an effort to determine which cytokines might be responsible for the hyperplasia, LN cells were stimulated with PDB and ionomycin to stimulate cytokine production. Next, lysates made from equal numbers of cells were used to probe a cytokine array testing 40 cytokines and chemokines, and the signal from OX40^{Cre}Pten^{f} cells was divided by the signal from control cells (Supplemental Fig. 4A). This chemiluminescent array is a relative measure of cytokine production, where a small difference in pixel density between genotypes can indicate a large difference in the absolute amount of cytokine. Although we could not identify a single factor responsible for hyperplasia, we found

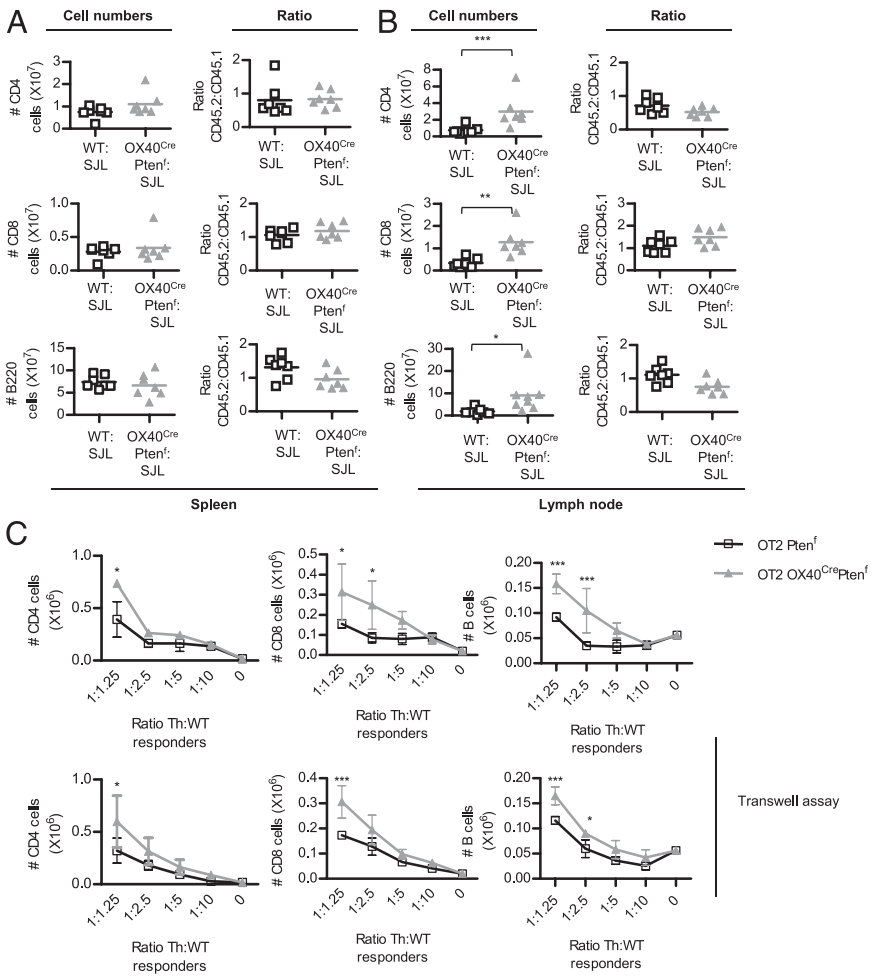


FIGURE 2. OX40^{Cre}Pten^{f} CD4 T cells support expansion of lymphocytes. Lethally irradiated Rag2$^{-/-}$ were reconstituted with a 50:50 mix of CD45.1^{+} WT SJL bone marrow and either CD45.2^{+} WT (n = 7) or OX40^{Cre}Pten^{f} (n = 9) bone marrow. (**A**) Spleen and (**B**) LN were analyzed 8 wk later for the number of lymphocytes and the relative contribution (ratio) of CD45.1 and CD45.2 bone marrow. Student t test was used to calculate p values. (**C**) Activated CD4 cells from CD45.2^{+} OT2 Pten^{f} (n = 3) and OT2 OX40^{Cre}Pten^{f} (n = 3) mice were cocultured at various ratios with CD45.1^{+} splenocytes while in direct contact (*top panels*) or while separated by a Transwell filter (*bottom panels*). After 3 d, the number of CD45.1^{+} lymphocytes was calculated. Data show mean ± SD. Three-way ANOVA was used to calculate statistical significance in number of responder cells recovered. OT2 OX40^{Cre}Pten^{f} versus WT OT2 without Transwell: CD4 and CD8 T cells *$0.01 < p \leq 0.05$; B cells: ***$p \leq 0.001$. Transwell versus Transwell: no significant difference in either OT2 OX40^{Cre}Pten^{f} or WT OT2. Results of significant Bonferroni posttests for each titration point are indicated on the graph. **$0.001 < p \leq 0.01$.

a limited range of factors were overproduced in OX40^{Cre}Pten^{f} LN, and these included cytokines made by different Th subsets and those that could be induced indirectly by Th cells, such as the chemokine CXCL9 (also known as monokine induced by IFN-γ). Therefore, we postulate that an environment rich in multiple Th cell-dependent cytokines resulted in LN hyperplasia.

OT2^{+}OX40^{Cre}Pten^{f} showed enhanced proliferation and produced greater amounts of IL-2, IL-4, and IFN-γ when stimulated with peptide (Supplemental Fig. 4B–E). The enhanced proliferation and cytokine production could be blocked by inhibitors against p110δ, Akt, and Erk added 24 h after activation (Supplemental Fig. 4B–E). The results from Supplemental Fig. 4 could be affected by the increased number of CD4^{+} T cells with an activated phenotype (32). We therefore purified naive CD62L^{+} and Ag-experienced CD62L^{-} CD4 T cells from OX40^{Cre}Pten^{f} mice. Both populations showed a 2- to 3-fold increase in proliferation and cytokine production when stimulated with anti-CD3 (Fig. 3A–D). High concentrations of the pan-PI3K inhibitor LY294002 or the p110δ-selective inhibitor IC reduced proliferation and cytokine production in OX40^{Cre}Pten^{f} and WT cells to near-background levels. At lower concentrations of IC, OX40^{Cre}Pten^{f} T cells were less sensitive than WT T cells, suggesting that the activity of PI3K isoforms other than p110δ are also normally restrained by Pten (Fig. 3A–D). To examine Th cell differentiation, CD4 T cells were activated in vitro in the presence of IL-12 to produce IFN-γ–secreting Th1 cells. A greater proportion of OT2 OX40^{Cre}Pten^{f} T cells had divided and produced IFN-γ than controls (Fig 3E–G). In addition, IFN-γ^{+} OX40^{Cre}Pten^{f} T cells stained more brightly for IFN-γ than did WT cells (Fig. 3E, 3H). Together, these data demonstrate that Pten acts to restrain the number of cells dividing, the number of those cells producing cytokine, and the amount of cytokine produced by individual cells.

Pten regulates the magnitude and kinetics of immune responses

To understand whether altered homeostasis caused by Pten-deficient Th cells affected immune responses, we used a CHS model where increased ear thickness can be used as a measure of T cell-dependent inflammation. Mice were sensitized by application of TNCB on their abdomens and then rechallenged 6 and 32 d later on their ear to elicit primary and secondary hypersensitivity responses, respectively. Mice were dosed orally with either IC or vehicle control during rechallenges (Fig. 4A). The magnitude and duration of the primary response was greater in OX40^{Cre}Pten^{f} than in WT mice. The enhanced inflammation was partially p110δ dependent because IC reduced ear swelling in WT and OX40^{Cre}Pten^{f} mice. The magnitude and duration of the secondary responses was also greater in OX40^{Cre}Pten^{f} than WT mice (Fig. 4B). IC again reduced ear swelling in both groups. We conclude that OX40^{Cre}Pten^{f} mice experience a greater inflammatory immune response, which nonetheless can be resolved and which can be attenuated by oral administration of p110δ inhibitors.

To follow the activation kinetics of OX40^{Cre}Pten^{f} T cells in vivo, we adoptively transferred OT2 Pten^{f} or OT2 OX40^{Cre}Pten^{f} CD4 T cells into WT hosts. After immunization with LPS and OVA protein, there were significantly more OT2 OX40^{Cre}Pten^{f} donor cells than controls after 6 d of activation. However, 9 d after activation, both donor cell types had returned to baseline levels (Fig.

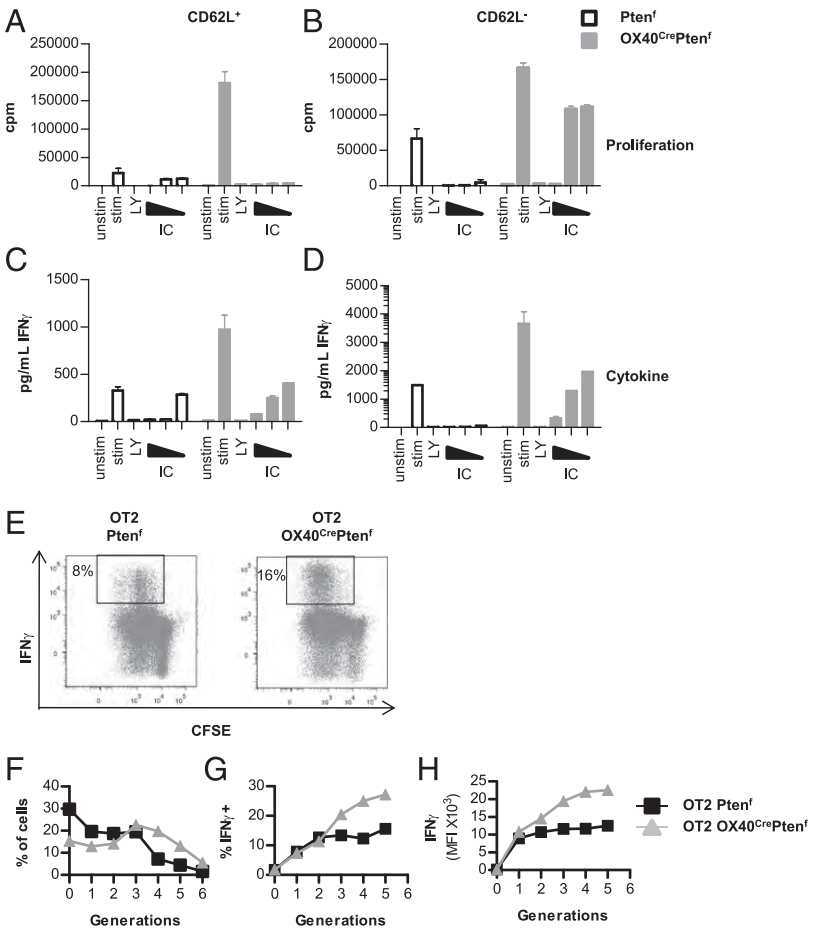
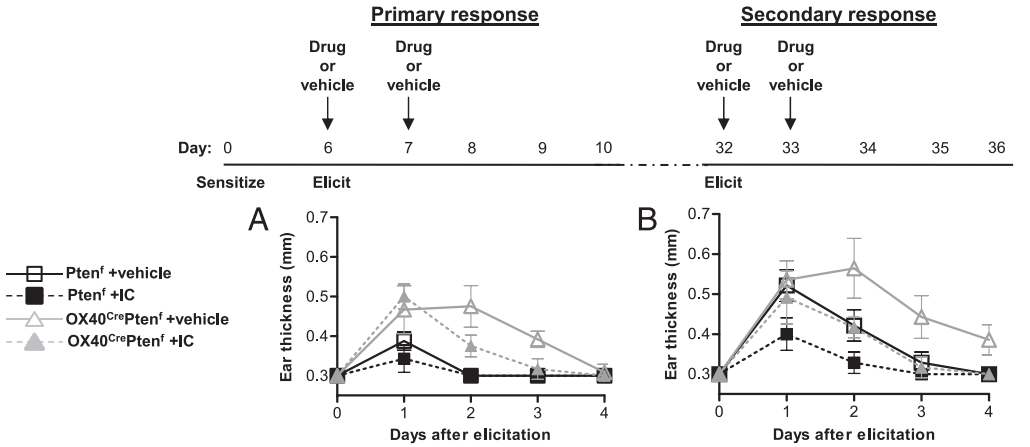


FIGURE 3. Pten antagonizes p110δ-regulated proliferation and cytokine production. (**A**–**D**) CD4 T cells from Pten^{f} (n = 3) and OX40^{Cre}Pten^{f} (n = 3) were sorted into (A, C) CD62L^{+} and (B, D) CD62L^{-} populations, and stimulated with anti-CD3 and either DMSO, 10 μM LY294002, or 10, 1, or 0.1 μM IC (doses represented by triangle). Two days later, (A, B) proliferation and (C, D) IFN-γ production were analyzed. Data show mean ± SEM. Data represent three independent experiments. (**E**–**H**) CFSE-labeled OT2 Pten^{f} and OT2 OX40^{Cre}Pten^{f} were stimulated with peptide in Th1-skewed conditions, and cells were stained after 3 d for intracellular IFN-γ. Data are representative of three independent experiments. (E) Representative plots. (F) CFSE dilution was used to determine the number of mitoses/generations each cell had gone through. (G) The proportion of IFN-γ^{+} cells was determined for each generation. (H) The median fluorescence intensity (MFI) in IFN-γ^{+} cells was determined for each generation to quantify the amount of IFN-γ each cell was making.



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	Primary response			Secondary response		
Day	Ptenf vs. OX40CrePtenf	Ptenf vehicle vs. Ptenf IC	OX40CrePtenf vehicle vs. Ptenf IC	Ptenf vs. OX40CrePtenf	Ptenf vehicle vs. Ptenf IC	OX40CrePtenf vehicle vs. Ptenf IC
1	***	*	n.s.	n.s.	***	n.s.
2	***	n.s.	***	***	***	***
3	***	n.s.	***	***	n.s.	***
4	n.s.	n.s.	n.s.	***	n.s.	***

FIGURE 4. Pten limits CHS responses by a p110δ-dependent mechanism. (**A**) TNCB-sensitized WT and OX40^{Cre}Pten^{f} mice were challenged with TNCB on an ear. Mice were dosed with 30 mg/kg IC or vehicle control 1 h before challenge and twice daily for 2 d. Ear size was measured before challenge and at 24-h intervals. (**B**) TNCB-sensitized WT and OX40^{Cre}Pten^{f} mice were challenged with TNCB on an ear and then rechallenged on the same ear 24 d later. Mice were also dosed with 30 mg/kg or vehicle control and then twice daily for 2 d during the secondary response only. Data show mean ± SD of six or more mice for each group, and represent two experiments for undrugged mice and one experiment for IC-drugged mice. (**C**) Statistics were measured by repeated-measures ANOVA. Table shows results of Bonferroni posttests on each day of ear measurement. *$0.01 < p \leq 0.05$, ***$p \leq 0.001$.

5A). This shows that Pten can control the magnitude of T cell response to Ag after initial TCR activation events, yet the lack of Pten expression does not interfere with the contraction of the T cell response. Consistent with this, we found no difference in apoptosis of OT2 OX40^{Cre}Pten^{f} cells compared with controls during the first 72 h after activation in vitro (Fig. 5B). Activated OT2 OX40^{Cre}Pten^{f} had a mild survival advantage in response to anti-Fas–induced death, but behaved similarly to controls in response to cytokine deprivation, anti-CD3 stimulation, and gamma irradiation (Fig. 5C). Therefore, although activation is enhanced, the absence of Pten does not necessarily interfere with apoptotic signaling in OX40^{Cre}Pten^{f} T cells.

To test how OX40^{Cre}Pten^{f} T cells respond to pathogens, we transferred naive OT2 OX40^{Cre}Pten^{f}Rag2$^{-/-}$ or OT2 Rag2$^{-/-}$ control T cells into WT hosts and infected these with attenuated ActA-LM-OVA. The numbers of adoptively transferred OT2 OX40^{Cre}Pten^{f}Rag2$^{-/-}$ cells appeared to be enhanced compared with controls in response to infection, although this difference was not statistically significant (Fig. 6A). However, twice as many of these cells produced IL-2 and IFN-γ compared with controls 3 d postinfection (Fig. 6B, 6C). We also measured the magnitude of the endogenous CD8 T cell response to ActA-LM-OVA using MHC tetramers. More OVA-specific CD8 T cells expanded in infected mice that had received OT2 OX40^{Cre}Pten^{f}Rag2$^{-/-}$ compared with those receiving OT2 Rag2$^{-/-}$ cells (Fig. 6D). In fact, WT OT2 Rag2$^{-/-}$ cells failed to increase the proportion of *L. monocytogenes*-responsive CD8 T cells, consistent with published results (52).

These results raised the possibility that OX40^{Cre}Pten^{f} CD4 T cells could promote cytotoxic immune responses in vivo under conditions where WT CD4 T cells fail to make a difference. To test this possibility further, we inoculated mice with an OVA-expressing thymoma cell line (EG7) and determined the ability of transferred OT2 cells to limit the growth of the tumors. Indeed, mice with adoptively transferred OT2 OX40^{Cre}Pten^{f}Rag2$^{-/-}$ but not OT2 Pten^{f}Rag2$^{-/-}$ T cells rejected the tumors (Fig. 6E). We conclude that OX40^{Cre}Pten^{f} CD4 T cells can promote cytotoxic immune responses potentially by activating CD8 T cells under conditions where WT CD4 T cells fail to do so.

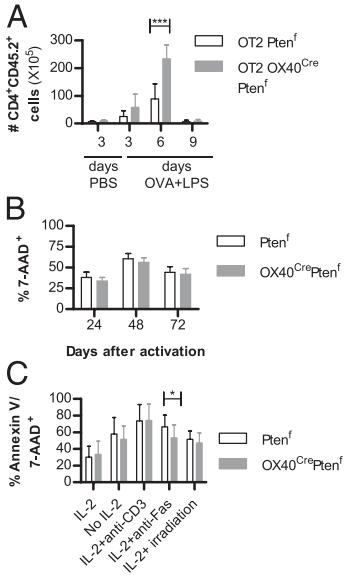

FIGURE 5. OX40^{Cre}Pten^{f} Th cells can undergo apoptosis in vitro and contract in vivo normally. (**A**) OT2 Pten^{f} or OT2 OX40^{Cre}Pten^{f} CD4 T cells were injected into SJL (CD45.1). The number of CD45.2^{+} donor cells in the draining LNs was counted at various time points after s.c. injection with either PBS or LPS+OVA. Data show mean ± SD of a minimum of four mice from three independent experiments. Student *t* test was used to calculate *p* values. (**B**) Death was assayed in CD4 T cells at 24-h intervals after in vitro activation. Data represent two independent experiments. (**C**) CD4 T cells were activated in vitro for 3 d, then cultured with no IL-2, 20 ng/ml IL-2, 20 ng/ml IL-2 + activation-induced death stimuli (10 μg anti-CD3, 1 μg/ml anti-Fas), or 20 ng/ml IL-2 + DNA damage (400 rad γ-irradiation). Death was assayed after 24 h. Data show mean ± SD for $n \geq 6$ for each genotype. Student *t* test was used to calculate *p* values.

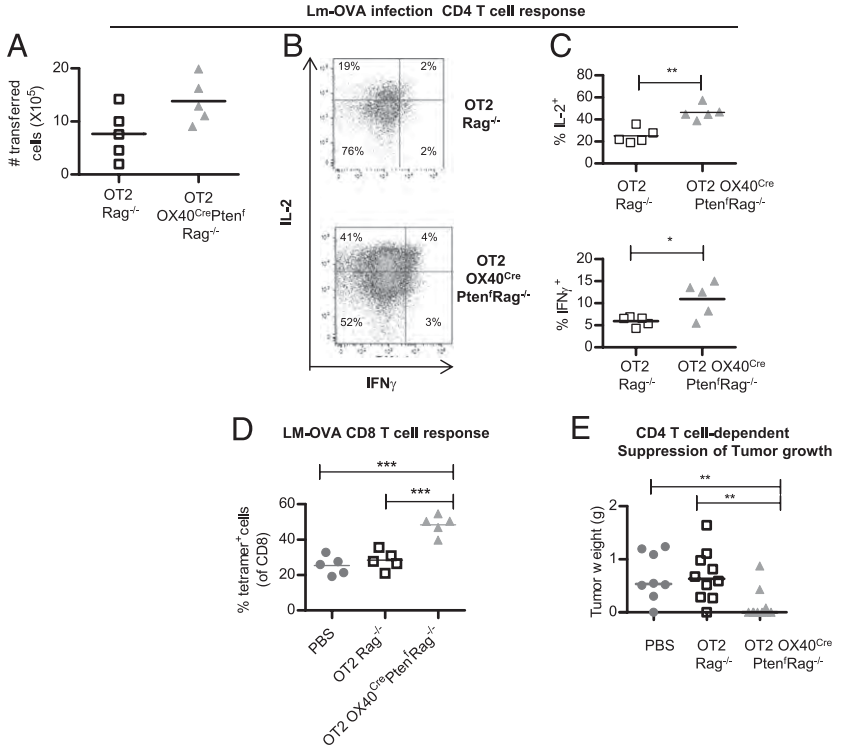

FIGURE 6. OX40^{Cre}Pten^{f} Th cells can enhance antibacterial and antitumor responses. (**A**–**C**) Mice injected with cells from OT2 Rag$^{-/-}$ (n = 5) or OT2-OX40^{Cre}Pten^{f}Rag2$^{-/-}$ (n = 5) mice were immunized with 10^7 CFU ActA-LM-Ova. After 3 d, spleens were analyzed for (A) the number of transferred cells and the proportion that produced (B and C) IL-2 or IFN-γ. (**D**) Mice injected with PBS (n = 6), OT2 Rag2$^{-/-}$ (n = 6), or OT2 OX40^{Cre}Pten^{f} Rag2$^{-/-}$ (n = 6) were immunized with 10^7 CFU ActA-LM-Ova. The percentage of endogenous OVA-tetramer^{+} CD8 T cells from blood taken on day 8 postinfection is shown. Repeated-measures ANOVA was used to calculate p values. (**E**) Mice injected i.v. with OT2 Pten^{f}Rag2$^{-/-}$ (n = 10), OT2 OX40^{Cre} Pten^{f}Rag2$^{-/-}$ (n = 10), or PBS (n = 8) were inoculated s.c. with EG7 cells. Excised tumors were weighed on day 14. Data show median values. p values were calculated using Mann–Whitney U tests. Data represent two independent experiments. *$0.01 < p \leq 0.05$, **$0.001 < p \leq 0.01$, ***$p \leq 0.001$.

Discussion

We have shown in this article that Pten is not an essential tumor suppressor in peripheral CD4 Th cells. Instead, Pten plays an important role in regulating lymphocyte homeostasis. When Pten was lost after activation, CD4 Th cells hyperproliferated and produced greater concentrations of cytokines. We postulate that cytokine overproduction turns Pten-deficient Th cells into "superhelpers" that enhance inflammatory, antibacterial, and antitumor responses. However, the enhanced responses could still be resolved and spontaneous disease did not develop, in contrast with pathology in many other mouse knockout models of negative regulatory proteins such as CTLA4 and Cbl (53). In addition, bone marrow chimera and Transwell studies suggest that a soluble factor led to a non-CD4 T cell autonomous expansion of lymphocytes in LNs. Recently, it was shown that cytokines permeate the LN and induce signaling in distant bystander lymphocytes (54). Hence excess cytokines produced by Pten-deficient Th cells might affect other naive lymphocytes and significantly alter LN homeostasis.

We confirmed in vitro and in a CHS model that Pten acts, at least in part, by antagonizing signaling by the PI3K p110δ. However, although p110δ inhibitors completely blocked activation in WT cells, they were only partially effective in Pten-deficient cells. This suggests that other PI3K isoforms could contribute. Possible candidates are p110α, which we have recently shown contributes to tonic Ag receptor signaling in B cells (55), or chemokine-dependent p110γ activity (19, 20, 56, 57).

Previous studies have shown that although lymphomas are found in the LNs and spleens of Pten-deficient mice, they are actually derived from the thymus (47–49). Mechanistically, this has been linked to c-myc translocation and overexpression (49, 58). Using converse experiments, we show in this study that Pten does not act as a tumor suppressor in mature T cells. In humans, mature T cell lymphomas are rare compared with thymic-derived lymphomas, which may reflect increased genome stability in more mature T cells. Indeed, DNA damage checkpoint regulators were dysregulated in Pten-deficient thymocytes but not peripheral T cells (48). Alternatively, there may be additional backup mechanisms to prevent lymphomagenesis in peripheral T cells. Consistent with this, leukemic oncogenes only caused transformation when ectopically expressed in hematopoietic stem cells and not mature T cells (59).

Pten has previously been shown to deter autoimmunity because when it was deleted from thymocytes, autoantibodies, autoreactive T cells, and lymphoid interstitial pneumonia developed (30). Autoantibodies and tissue infiltration still arose when young mice were thymectomized, which the authors suggested was caused by defective activation-induced cell death in peripheral T cells (49). However, autoreactivity in those studies could still have been caused by failed thymocyte negative selection in young animals (30, 32). We detected no evidence of spontaneous autoantibody production, autoimmunity, or inflammation in our model, possibly because there were no defects in Treg suppression or because apoptosis was only mildly affected. We thus conclude that Pten is not an essential repressor of apoptosis or autoimmunity stemming from mature T cells.

Although spontaneous immunopathologies did not develop in OX40^{Cre}Pten^{f} mice, heightened immune responses did. We previously showed that T-dependent humoral immune responses were enhanced in OX40^{Cre}Pten^{f} mice because of increased number and cytokine production of T_{FH} cells (24). In this article, we show that OX40^{Cre}Pten^{f} Th cells enhanced cellular immune responses to *L. monocytogenes* and prevent tumor growth. Because Pten deficiency revealed a previously unrecognized potential for Th in promoting primary CD8 T cell responses (52), we propose that OX40^{Cre}Pten^{f} T cells are "superhelpers." The concept of superhelpers has potential clinical implications because adoptive immunotherapy and prophylactic vaccination is currently aimed at modulating responding CTLs, although interest is growing in exploiting CD4 T cells as well (4). Pten-deficient superhelper Th could potentially improve clinical outcomes. Ag-specific CD4 cells have previously been shown to promote partial or complete regression in lymphopenic environments or when high numbers

were infused at regular intervals alongside Ag-specific CTLs (60–62). In this study, we demonstrate that similar results can be achieved using a single injection of Pten-deficient, Ag-specific CD4 T cells in normal hosts. Reduction of Pten activity in mature T cells using genetic or chemical means may, therefore, be both safe and desirable in some therapeutic settings such as tumor immunotherapy.

Acknowledgments

We thank Nigel Killeen, Pier Paolo Pandolfi, and Hans Jorg Fehling for mouse strains, and Hou Shen for the attenuated *Listeria*-expressing OVA. We thank Jonathan Clark for the synthesis of IC, the staff at the Biological Services Unit and Flow Cytometry facility for expert help, and Anne Segonds-Pichon for statistical advice.

Disclosures

K.O. is a consultant for GlaxoSmithKline. The other authors have no financial conflicts of interest.

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