Antigen-Induced *Pten* Gene Deletion in T Cells Exacerbates Neuropathology in Experimental Autoimmune Encephalomyelitis

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The *Pten* tumor suppressor gene is critical for normal intrathymic development of T cells; however, its role in mature antigen-activated T cells is less well defined. A genetically crossed mouse line, *Pten*^{fl/fl} GBC, in which *Pten* gene deletions could be primarily confined to antigen-activated CD8⁺ T cells, enabled us to evaluate the consequences of *Pten* loss on the course of experimental autoimmune encephalomyelitis. Compared with *Pten*^{fl/fl} controls, myelin oligodendrocyte glycoprotein (MOG) peptide-immunized *Pten*^{fl/fl} GBC mice developed more severe and protracted disease. This was accompanied by increased spinal cord white matter myelin basic protein depletion and axonal damage, as well as a striking persistence of macrophage and granzyme B-expressing cellular neuroinfiltrates in the chronic phase of the disease. This persistence may be explained by the observation that anti-CD3 activated *Pten*^{fl/fl} GBC T cells were more resistant to proapoptotic stimuli. Consistent with the predicted consequences of *Pten* loss, purified CD8⁺ T cells from *Pten*^{fl/fl} GBC mice displayed augmented proliferative responses to anti-T-cell receptor stimulation, and MOG-primed *Pten*^{fl/fl} GBC T cells exhibited a reduced activation threshold to MOG peptide. *Pten*^{fl/fl} GBC mice also developed atypical central nervous system disease, manifested by prominent cervical cord and forebrain involvement. Collectively, our findings indicate that the phosphatidylinositol 3-kinase signaling pathway is an essential regulator of CD8⁺ T-cell effector function in experimental autoimmune encephalomyelitis. (*Am J Pathol* 2008, 172:980–992; DOI: 10.2353/ajpath.2008.070892)

signaling pathway. This protein phosphatase acts by catalyzing the removal of the 3' phosphate from phosphatidylinositol-3,4,5-triphosphate (PIP₃), within the cell membrane,¹ thereby inhibiting PI3K-mediated effects on cell proliferation, differentiation, survival, metabolism, adhesion, and migration.² Mutations or cytogenetic aberrations involving the *PTEN* locus are found in multiple types of sporadic tumor,³ and heterozygosity for *PTEN*, as found in Cowden disease, predisposes individuals to malignancy.^{3,4} Although targeted disruption of *Pten* in mice results in embryonic lethality,⁵ heterozygous mice are predisposed to lymphomas as well as a variety of other neoplasms.^{5–8} In addition, with age, *Pten*^{+/-} mice developed a lymphoproliferative-autoimmune disorder accompanied by an immune complex-mediated glomerulonephritis.⁹ In keeping with the pathogenesis of this latter syndrome, *Pten*^{+/-} T cells demonstrated a defect in activation-induced cell death, as well as increased proliferative responses to T-cell receptor (TCR)-activating stimuli.

Tissue-specific conditional inactivation of *Pten* has revealed a role for this molecule in the maintenance of cell size and number,¹⁰ prevention of tumorigenesis,¹¹ and in immune system homeostasis.¹² With respect to T lymphocytes, several groups have generated thymocyte-specific deletions of *Pten*,^{12–14} revealing a role for this molecule in the maintenance of both central and peripheral tolerance. A spontaneous autoimmune syndrome was observed in mice with *Pten*-deficient T cells (after intrathymic deletion of the gene)¹²; however, it should be noted that these experiments were performed within the context of a *Pten* heterozygous background where the contributions of other *Pten*-haploinsufficient cell types (such as B lymphocytes and dendritic cells) to the observed phenotype may have been considerable. In addition to *Pten* deficiency allowing the survival of thymocytes with aberrant TCRs,¹³ it has been suggested that

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The tumor suppressor gene *PTEN* encodes a pivotal negative regulator of the phosphatidylinositol 3'-kinase (PI3K)

Pten-deficient CD4⁺ T cells are capable of becoming fully activated by antigen receptor engagement even in the absence of co-stimulatory signals.¹⁵ Intrathymic T-cell-specific deletions of *Pten* thus have the potential to lead not only to an abnormal preimmune TCR repertoire in peripheral T cells, but also an increase in the sensitivity of naïve T cells to antigen presented by dendritic cells. Although the role of *Pten* in T cell ontogeny has been well studied using intrathymic Cre expression, investigating the function of *Pten* in effector cells requires a conditional mutagenesis system in which *Pten* gene deletions can be confined to T cells after their activation by antigen.

TCRs with specificity for peptides derived from myelin-derived antigens, such as myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG), are essential for the induction of experimental autoimmune encephalomyelitis (EAE). The pathogenesis of this disease involves an array of cell types, including dendritic cells, CD4⁺ and CD8⁺ T lymphocytes,^{16,17} and elements of the innate immune system. Although considerable emphasis has been placed on the pathogenic role of CD4⁺ T cells, it has been found that granzyme B protein-expressing CD8⁺ T cells can be intimately associated with sites of axonal and oligodendrocyte injury within multiple sclerosis (MS) plaques.^{18–20} Furthermore, CD8⁺ T cells have been implicated in the pathogenesis of EAE,^{21–24} for example, Sun and colleagues²⁴ demonstrated that immunization with MOG_{35–55} peptide led to the activation of CD8⁺ α/β TCR⁺ T cells that on adoptive transfer, led to a more damaging and progressive central nervous system (CNS) disease in recipients than was evident in MOG-immunized mice. This adoptively transferred disease was also accompanied by a prominent persistence of encephalitogenic CD8⁺ T cells within recipient animals. Development of EAE after adoptive transfer of MBP-specific CD8⁺ T cells also provided evidence for the role of antigen-specific cytotoxic lymphocytes (CTLs) in the pathogenesis of this disease.²⁵ Given that the study of CD8⁺ T cells in EAE has lagged behind that of CD4⁺ T cells, gaining a better understanding of the pathogenic mechanisms used by myelin-specific encephalitogenic CD8⁺ T cells remains an important goal.

Herein, we describe the phenotypic consequences of *Pten* gene deletion within antigen-specific CD8⁺ T cells generated in response to MOG_{35–55} peptide immunization. To achieve *Pten* gene excisions in antigen-activated T cells, *Granzyme B-Cre* (*GBC*) transgenic mice in which Cre recombinase expression is directed primarily to activated CD8⁺ T cells,^{26,27} were intercrossed with *Pten*^{fllox/fllox} mice.¹² In addition to demonstrating for the first time the *in vivo* consequences of *Pten* gene deletion in mature antigen-activated T cells, this conditional mutagenesis system was able to demonstrate that *Pten* loss in CD8⁺ T cells led to an atypical EAE disease pattern associated with increased damage to CNS target tissues.

Materials and Methods

Mice

Pten^{fllox/fllox} (*Pten*^{fl/fl}) mice, generated as described previously,¹² and on a mixed 129Ola × C57BL/6 background,

were obtained from Dr. T.W. Mak (University of Toronto, Toronto, Canada) and *Tg*(GZMB-Cre)*1Jcb/J* mice,²⁶ on a mixed C57BL/6 × FVB background, were purchased from the Jackson Laboratory (Bar Harbor, ME). To generate *Pten*^{fl/fl} *GBC* mice, *Pten*^{fl/fl} mice were bred to the *GBC* line and maintained under specific pathogen-free conditions. Because mice carrying *GBC* and the floxed *Pten* mutation on both alleles (*Pten*^{fl/fl} *GBC*) were of a mixed genetic background, littermate controls, consisting of mice with floxed *Pten* alleles (*Pten*^{fl/fl}), were always used. The use of *Pten*^{fl/fl} controls was concordant with recent studies involving the conditional inactivation of *Pten*.^{28,29} *R26R-EYFP* mice³⁰ were kindly provided by Dr. F. Constantini (Columbia University, New York, NY). *GBC* and *R26R-EYFP* hemizygous mice were interbred to obtain mice with one copy each of the *EYFP* and *GBC* alleles. Colony maintenance, as well as disease induction and monitoring, were conducted within a barrier facility in accordance with the Canadian Council for Animal Care guidelines and ethics approval from the University of Calgary Animal Care Committee.

Pten Excision Polymerase Chain Reaction (PCR)

For amplification of the excised allele, primers: *Pten*flF4908 (5'-AAAAGAGTAAAGGTCTGGCTTACAA-3') and *Pten*flR8980 (5'-TCTGACACAGCCTACTTTAATTGG-3') were used. The presence of the excised allele (because of loss of *Pten* exons 4 and 5) was demonstrated by the identification of an 850-bp product on agarose gel electrophoresis.

EAE Induction and Scoring

Mice (10 to 13 weeks old) were injected with either 50 μ g, or 12.5 μ g, of synthetic peptide comprising residues 35 to 55 of the murine MOG sequence (Peptide Synthesis Core Facility, University of Calgary, Calgary, Canada) emulsified 1:1 in complete Freund's adjuvant H37Ra (Difco Laboratories/BD Biosciences, San Jose, CA), with 50 μ l being injected into each hind flank subcutaneously. Mice were also injected intraperitoneally with 300 ng of pertussis toxin (List Biological Laboratories Inc., Campbell, CA) on days 0 and 2.³¹ After immunization, mice were weighed and then scored on a scale of 0 to 14 using a previously reported method³²: a score of 0 reflected no clinical signs of disease, 1 represented a loss of tone in the tail, and a score of 2 reflected complete tail paralysis. Each limb was also assessed separately on a 0 to 3 scale: 0, no signs; 1, limb weakness or altered gait; 2, overt limb paresis; 3, limb paralysis. The term "days" in the text refers to days after immunization, with the immunization starting on day 0.

Lymph Node Cell Proliferation Assay

Mice were immunized with MOG/complete Freund's adjuvant and pertussis toxin as described above, and on day 10 cells were isolated from rear-flank, axillary, brachial, and cervical lymph nodes using a modified proto-

col.³³ After lymph node dissociation with a 40- μ m mesh sieve, and red blood cell lysis, samples were resuspended at a final concentration of 1×10^6 cells/ml; 2×10^5 cells/well were then placed in 96-well plates and stimulated with one of the following: 1 μ g/ml anti-CD3 ϵ and anti-CD28 antibodies (BD Biosciences PharMingen, San Diego, CA), 1, 10, or 100 μ g/ml of MOG_{35–55} peptide in phosphate-buffered saline (PBS), or media containing interleukin (IL)-2 alone. Samples stimulated with IL-2 alone were used to normalize the proliferative results of stimulation with either CD3/CD28 antibodies (plus IL-2) or MOG_{35–55} (plus IL-2) stimulation. Cells were resuspended in enriched RPMI media either in the presence or absence of 3% IL-2-conditioned media. Cells were cultured at 37°C for 48 hours before the addition of 1 μ Ci of ³H-thymidine (10 μ Ci/ml in RPMI 1640), and then incubated for an additional 24 hours before harvesting and counting on an LS 3801 liquid scintillation counter (Beckman Instruments, Fullerton, CA).

Splenocyte and CD8⁺ T-Lymphocyte Cultivation

Spleens from *Pten*^{fl/fl} GBC and control *Pten*^{fl/fl} mice or, *ROSA-EYFP* GBC and control *ROSA-EYFP* mice were dissociated and the cells subjected to ACK lysis. *ROSA-EYFP* or *ROSA-EYFP* GBC splenocytes were cultured in 24-well plates at 5×10^6 cells/ml and stimulated with 1 μ g/ml phorbol 12-myristate 13-acetate (Sigma-Aldrich, Oakville, Canada) plus 2 μ g/ml of ionomycin, calcium salt (Sigma-Aldrich). Stimulations were performed for 48 hours before washing the cells in sterile PBS and returning them to plates containing IL-2 (3% conditioned media) and 2 μ mol/L β -mercaptoethanol (β -ME)-supplemented AIM V (Invitrogen Canada Inc., Burlington, Canada) media for an additional 5 days. Cells were collected into Dulbecco's phosphate-buffered saline and washed in preparation for flow cytometry. *Pten*^{fl/fl} and *Pten*^{fl/fl} GBC CD8⁺ T cells were positively selected using a magnetic separation system, as recommended by the manufacturer (Miltenyi Biotec Inc., Auburn, CA). Isolated CD8⁺ T lymphocytes were plated at 2.5×10^6 cells/ml in enriched RPMI media [RPMI 1640, 10% fetal calf serum, 1% L-glutamate, 1% minimum essential medium-nonsessential amino acids, 2 μ mol/L β -ME, 1% penicillin-streptomycin, 1% sodium pyruvate] in 96-well plates with varying concentrations of added anti-CD3 ϵ (145-2C11; BD PharMingen, San Diego, CA). The cells were then cultured at 37°C for 24 hours before the addition of 1 μ Ci of ³H-thymidine (10 μ Ci/ml in RPMI 1640), and after 18 hours of incubation, counting was performed as above.

Apoptosis Assay

Purified splenic T cells (5×10^6), were subjected to red blood cell lysis, and then activated for 48 hours at 37°C in 24-well plates [previously coated with 2.5 μ g/ml of anti-CD3 ϵ (145-2C11) antibody (BD PharMingen) and 2.5 μ g/ml anti-CD28 (37.51) antibody (BD PharMingen)] in IL-2 and β -ME-supplemented AIM V media (Invitrogen).

Activated T cells were collected using Lympholyte-M; after washing, the cells were plated (1×10^6 cells/ml) in RPMI complete media with 3% IL-2-containing supernatant in 96-well plates for an additional 48 hours. Cell viability was assessed by annexin V/propidium iodide (PI) staining as per the manufacturer's instructions (Annexin: FITC Apoptosis Kit, BD PharMingen) to determine the percentage of live cells both before and 24 hours after the addition of the following proapoptotic stimuli: 5 μ g/ml soluble anti-CD3, IL-2 withdrawal, IL-2 and serum withdrawal, and 1 μ g/ml anti-Fas/CD95 (BD PharMingen).

Immunofluorescence and Laser Confocal Microscopy

Paraffin-embedded lumbar and cervical cord (CC) sections (4 μ m) were processed and deparaffinized as reported previously.³⁴ Antigen retrieval was performed by boiling slides in 0.01 mol/L tri-sodium citrate, pH 6.0, for 10 minutes. Sections were preincubated with 10% goat serum, 2% bovine serum albumin, pH 5.2, and 0.2% Tween 20 in PBS, and incubated overnight at 4°C. Primary antibodies: rabbit anti-mouse Iba-1 (01-1974, 1:500; Wako, Richmond, VA), anti-CD3- ζ (6B10.2, 1:25; Santa Cruz Biotechnology Inc., Santa Cruz, CA), rabbit polyclonal anti-granzyme B (GZMB, ab4059; Abcam Inc., Cambridge, MA) or mouse anti-MBP 1:1000 (SM194; Sternberger Monoclonals, Lutherville, MD) antibodies were applied in 5% block (5% goat serum) and incubated overnight at 4°C. Slides washed three times with PBS were overlaid with secondary antibody (Cy3-conjugated Affinipure goat anti-mouse IgG (1:500; Jackson ImmunoResearch, West Grove, PA) or Alexa Fluor 488 goat anti-rabbit IgG (1:500; Molecular Probes/Invitrogen, Carlsbad, CA) in 5% block for 2 hours at room temperature in the dark. Slides were mounted in FluorSave reagent (Calbiochem/EMD Biochemicals, San Diego, CA) and examined using a LSM-510 META confocal microscope (Carl Zeiss, Thornwood, NY) to obtain digital images. Randomly selected fields from the ventral columns (VC), dorsal columns (DCs), and the lateral white matter (LWM) were collected from at least three individual mice from three separate sections at $\times 10$ magnification and scored by an observer blinded as to the genotype of the mice. The percentage of MBP-positive (+) area or GZMB⁺ area in the white matter of spinal cords was calculated using Adobe Photoshop (San Jose, CA) in which MBP⁺ area was selected and converted to a grayscale image for quantification by NIH-ImageJ (<http://rsb.info.nih.gov/ij/>).

Histopathology

Paraffin-embedded sections (4 μ m) were deparaffinized as above and stained with hematoxylin and eosin, Bielschowsky silver stain (to show axonal fibers),³¹ or with the chloroacetate esterase (CAE) stain for neutrophils (revealed by bright granular red color). Fields from $\times 10$ magnification images of VC, DCs, and LWM were scanned to provide digital images and for quantification

of axonal fibers/mm²; data were acquired as above using Adobe Photoshop and ImageJ.

Flow Cytometry of CNS-Infiltrating Cells

Mice were anesthetized using 100 mg/ml ketamine hydrochloride injection USP (Animal Health Inc., Bimeda-MTC, Cambridge, Canada), 100 mg/ml xylazine (Animal Health Inc., Bimeda-MTC), and then subjected to cardiac perfusion with 12 ml of Dulbecco's phosphate-buffered saline. The brain and spinal cords were isolated and dissociated using 40 μ m and 50 μ m mesh sieves. Cells were isolated by isotonic Percoll (Sigma-Aldrich) centrifugation, and 1×10^6 cells/ml were resuspended and incubated with the following antibodies (all purchased from BD PharMingen) in preparation for flow cytometry: Fc block (anti-mouse CD16/CD32, 2.4G2) and 5 μ g/ml of PE-conjugated anti-mouse CD4 antibody (L3T4) and/or 5 μ g/ml of PerCP-conjugated anti-mouse CD8a antibody (Ly-2, 53-6.7). Live cells were collected and gated on the FACSCalibur using CellQuest software (BD Biosciences, San Jose, CA) and quantified using FlowJo (version 3.6; TreeStar, Ashland, OR) software.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism version 4.01 (GraphPad Software, San Diego, CA) for both the parametric and nonparametric assessments. For the analysis of the disease score, a Mann-Whitney *U*-test was performed and the unpaired *t*-test and one-way analysis of variance was used for phenotypic and histopathological data analyses, the flow cytometric analyses, and the proliferation assays. Significance was attributed to values in which *P* < 0.05.

Results

Ascending Spinal Cord Involvement in *Pten*^{fl/fl} GBC Mice with EAE

To gain insight into the role of *Pten* within CD8⁺ T-cell populations engaged in the development of MOG-induced EAE, we used a system in which gene excisions would be primarily confined to activated CD8⁺ T cells

Figure 1. *Pten*^{fl/fl} GBC mice show an altered EAE disease course. **A:** Graphic representation of the *Pten*^{fl/fl} GBC genetic model for *Pten* excision in antigen-activated T lymphocytes. Cellular activation leads to expression of the Cre-recombinase enzyme. Cre then directs excision of loxP site-flanked exons 4 of 5 of *Pten* in the *Pten*^{fl/fl} GBC animals. **B:** *Pten*^{fl/fl} GBC mice contained *Pten*-deficient cells within their thoracic cord. DNA was extracted from the thoracic cords of two *Pten*^{fl/fl} and *Pten*^{fl/fl} GBC mice at 30 days after EAE induction. PCR analysis was performed to detect the 850-bp product indicative of *Pten* alleles lacking exons 4 to 5. **C:** Neurobehavioral analysis during EAE induced by immunization with 50 μ g of MOG₃₅₋₅₅ at day 0 revealed a trend toward delayed disease onset in *Pten*^{fl/fl} GBC mice accompanied by **(D)** a similar average maximum score at the peak of disease as compared to *Pten*^{fl/fl} mice. **P* < 0.05 Mann-Whitney *U*-test. Error bars represent SE.

Table 1. EAE Signs in Control and *Pten^{fl/fl}* GBC Mice

Group	MOG ₃₅₋₅₅	CDI*	Disease onset†	Disease incidence‡
Control (n = 27)	50 µg	156.5 ± 8.565	7	100
<i>Pten fl/fl</i> GBC (n = 12)	50 µg	136.2 ± 13.66	9	100
Control (n = 12)	12.5 µg	44.23 ± 4.771 SEM§	8	100
<i>Pten fl/fl</i> GBC (n = 7)	12.5 µg	87.20 ± 6.529 SEM§	8	100

*Cumulative disease index (CDI) represents the mean of the sum of daily clinical scores observed between days 7 and 28 [corrected for n (numbers) of mice].

†The day of disease onset represents the 1st day of 2 consecutive days with a clinical score of two or more (including SE).

‡Disease incidence is the percentage of mice displaying clinical signs of disease.

§P < 0.0001 for control and *Pten^{fl/fl}* GBC mice injected with 12.5 µg of MOG₃₅₋₅₅.

(Figure 1A). Thus, in response to cellular activation, Cre expression from the GBC transgene leads to *loxP*-dependent genetic recombinations primarily within antigen-specific mature effector CD8⁺ T cells. This system was originally designed to mark activated effector and memory CD8⁺ T lymphocytes after antigenic challenge via the Cre-mediated activation of a reporter gene.²⁶ Using this system, Maris and colleagues²⁷ also demonstrated Cre-mediated gene excisions within all antigen-specific memory CD8⁺ T cells responding to an *in vivo* viral challenge. In the current study we interbred the control *Pten^{fl/fl}* and GBC lines to obtain *Pten^{fl/fl}* GBC female mice for the EAE experiments. Between 7 and 9 days after MOG₃₅₋₅₅/complete Freund's adjuvant immunization, both the control *Pten^{fl/fl}* and experimental *Pten^{fl/fl}* GBC female mice developed an ascending paralysis that began in the tail and spread over time to involve the limbs. Motor function was assessed daily for 28 days for each mouse and a disease score assigned as previously described³² (see Materials and Methods for the scoring system). As shown in Figure 1C, control *Pten^{fl/fl}* mice developed the first clinical signs by day 7, with all animals showing disease by day 14. The peak of disease at day 15 was accompanied by tail and hind limb paralysis (Figure 1D). *Pten^{fl/fl}* GBC mice showed a delay in disease onset and a significantly lower score at days 9 and 11 after immunization (Figure 1C), but all mice were ill by day 18, with the average peak of disease occurring at day 19 (Figure 1D). This was manifested by tail and hind limb paralysis as well as forelimb paresis. Complete Freund's adjuvant (minus MOG peptide) plus pertussis toxin-injected control *Pten^{fl/fl}* mice, displayed no clinical signs of neurological disease throughout the course of the 28-day observation period (Figure 1D). Assessed by the cumulative disease index, the severity of disease was not significantly different between the control and experimental groups (156.5 versus 136.2, Table 1). The presence of Pten-deficient T lymphocytes in the CNS after MOG immunization was assessed by locus-specific *Pten*-excision PCR. Samples taken from the thoracic cord at day 28 after immunization of *Pten^{fl/fl}* GBC mice demonstrated the presence of *Pten* Δexon 4-5 alleles (Figure 1B), consistent with the presence of infiltrating lymphocytes that had undergone GBC-mediated gene recombinations. In summary, both *Pten^{fl/fl}* and *Pten^{fl/fl}* GBC mice exhibited similar levels of spinal

cord motor dysfunction, with a trend for delayed disease onset being evident in *Pten^{fl/fl}* GBC mice.

GBC-Mediated Excision of a Floxed Reporter Gene within Activated CD8⁺ T Lymphocytes

To verify that GBC transgene-bearing CD8⁺ cells were able to undergo Cre-*loxP* mediated gene excisions in response to an activating signal, we conducted a study in which GBC mice were crossed with the ROSA-EYFP Cre-reporter strain. This reporter system is composed of a *loxP*-flanked gene segment immediately downstream of the ROSA locus promoter, that on excision by Cre recombinase leads to expression of the EYFP gene.³⁰ Expression of EYFP then allows the frequency of excisions within a cell population to be determined by flow cytometry. Analysis of EYFP⁺ splenocytes before activation revealed background levels of 30% EYFP⁺ CD4⁺ cells, and 14% EYFP⁺ CD8⁺ T cells (Figure 2). Some proportion of the EYFP⁺ CD4⁺ cells was likely attributable to the presence of NK T cells, because such cells have previously been reported to express the Cre reporter gene in the GBC model, even in the absence of cell activation.²⁷ It was also possible that a fraction of the EYFP⁺ CD4⁺ T cells, as well as the 14% of EYFP⁺ CD8⁺ cells, were

Figure 2. GBC directs gene excision within antigen-activated CD8⁺ T lymphocytes. **A:** Splenocytes isolated from ROSA-EYFP GBC mice prestimulation were assessed for EYFP fluorescence by flow cytometry; cells were then gated to obtain the percentage of the EYFP⁺ population that was either CD4⁺ or CD8⁺. **B:** Stimulation of splenocytes *in vitro* for 48 hours with phorbol 12-myristate 13-acetate and ionomycin revealed a 4.5-fold increase in the percentage of CD8⁺ T cells that were EYFP⁺ compared to cells analyzed before stimulation. Graphs are representative of n = 3 mice, from two separate experiments.

reflective of memory cells generated during environmental antigen-stimulated activation of the GBC transgene in responding T-cell populations. Importantly, and relevant to our experiments, after 48 hours of splenocyte stimulation with the combination of phorbol 12-myristate 13-acetate and ionomycin, only CD8⁺ cells from the *ROSA-EYFP GBC* mice displayed a significant (~4.5-fold) increase in the percentage of EYFP⁺ cells (Figure 2A). The CD4⁺ population exposed to this potent activating stimulus, in contrast, failed to show a significant increase in the percentage of EYFP⁺ cells (Figure 2B). These results demonstrated the *GBC* system was functional, and also that activation-induced excisions were limited primarily to CD8⁺ cells.

Pten^{fl/fl} GBC T Lymphocytes Demonstrate Resistance to Proapoptotic Stimuli

Because loss of *Pten* confers resistance to cell killing, including activation-induced cell death, we compared the responses of *Pten^{fl/fl} GBC* and *Pten^{fl/fl}* splenic T cells to several different proapoptotic stimuli. To accomplish this, splenocytes were stimulated for 48 hours with anti-CD3/CD28 antibodies plus IL-2 to induce *Pten* gene deletions, before application of the following proapoptotic stimuli: anti-CD3 (to induce activation-induced cell death), IL-2 withdrawal, IL-2 plus serum withdrawal, and anti-Fas antibody. As assessed by annexin V/PI staining, *Pten^{fl/fl} GBC* T cells demonstrated increased resistance to apoptosis induction by anti-CD3 antibody, IL-2 withdrawal, and anti-Fas antibody (Figure 3A). Given the anti-apoptotic effect of *Pten* loss, these results were attributed to anti-CD3/CD28 triggered deletions of *Pten* within *Pten^{fl/fl} GBC* T cells.

*Primed *Pten^{fl/fl} GBC* T Cells Show Increased Proliferative Responses to IL-2 and MOG Peptide*

Previously, intrathymic *Pten* loss was shown to augment the proliferation of lymphocytes to cytokine and anti-TCR stimulation.^{12–15} We therefore evaluated the MOG-peptide-specific responses of T cells obtained from the lymph nodes of MOG-primed *Pten^{fl/fl} GBC* mice at day 10. Control *Pten^{fl/fl}* and experimental *Pten^{fl/fl} GBC* lymph node cells were plated in the presence of: IL-2 alone (Figure 3B), anti-CD3/CD28 plus IL-2 (as a positive control) (Figure 3C), and 1, 10, or 100 μ g/ml of MOG_{35–55} peptide with supplemental IL-2 (Figure 4A). The responses of the *Pten^{fl/fl}* and *Pten^{fl/fl} GBC* T cells to anti-CD3/CD28 plus IL-2 stimulation indicated that the maximal proliferative potential of the T cells was equivalent between the two genotypes (Figure 3C). Stimulation with IL-2 alone, in contrast, revealed that *Pten^{fl/fl} GBC* lymph node T cells responded to a significantly greater degree than control T cells (Figure 3B). This could have been attributable to either the triggering of MOG-specific *Pten*-deficient memory cells, or to the stimulation of cells already actively engaged in anti-MOG clonal expansions that may have

Figure 3. *In vitro* responses of *Pten^{fl/fl} GBC* splenocytes to various stimuli, and *Pten^{fl/fl} GBC* CD8⁺ T-cell proliferative responses to anti-CD3 ϵ -stimulation. **A:** Splenic T cells were activated for 48 hours with anti-CD3 ϵ , anti-CD28, and IL-2 before application of the following proapoptotic stimuli: anti-CD3 ϵ (5 μ g/ml), IL-2 withdrawal, IL-2 and serum withdrawal, and anti-Fas (1 μ g/ml). Levels of apoptotic cells were determined after 24 hours by annexin V/PI staining and flow cytometry. The results were expressed as the percentage of annexin V/PI-negative cells remaining compared to untreated cells at 24 hours. Quantitative analysis was performed on the data obtained from $n = 3$ mice for each group. **B and C:** For the lymph node cell proliferation assay, cells were isolated from MOG-injected mice at day 10 and restimulated *in vitro* with IL-2 alone (**B**) or plate-bound anti-CD3 and anti-CD28 antibodies plus IL-2 for 48 hours before addition of ³H-thymidine (**C**). After an additional 24 hours of incubation, ³H-thymidine incorporation was quantified (cpm). Analysis was performed from the data of $n = 5$ mice per group. **D:** Increased proliferation of *Pten^{fl/fl} GBC* CD8⁺ T cells. Purified CD8⁺ T cells were incubated with the indicated concentrations of anti-CD3 ϵ , and proliferation was measured by thymidine uptake. Mean thymidine uptake for four mice per group $\pm$ SEM is shown. * $P < 0.05$; ** $P < 0.01$; unpaired *t*-test. Error bars represent SE.

Figure 4. *Pten*^{fl/fl} GBC mice displayed a decreased threshold in response to MOG₃₅₋₅₅ stimulation that was also reflected in the clinical disease scores after low-dose MOG immunization. **A:** Whole lymph node cell proliferation assay. Cells were isolated from MOG-injected mice at day 10 and restimulated *in vitro* with plate-bound anti-CD3/CD28 antibodies plus IL-2 for 48 hours before addition of ³H-thymidine, and then they were incubated for an additional 24 hours before quantification (cpm). *Pten*^{fl/fl} GBC mice displayed significantly more proliferation in response to treatment with MOG₃₅₋₅₅ peptide plus IL-2 at the indicated concentrations. Quantitative analysis was performed from the data of *n* = 5 mice for each group. ***P* < 0.01; ****P* < 0.001 unpaired *t*-test. **B:** *Pten*^{fl/fl} GBC and *Pten*^{fl/fl} mice were immunized with 12.5 μg of MOG₃₅₋₅₅ peptide and monitored for development of EAE throughout a 28-day period. **C:** Neurobehavioral scoring. *Pten*^{fl/fl} GBC mice showed a significantly greater disease severity in the chronic stages of disease when immunized with the lower dose of MOG, when compared to *Pten*^{fl/fl} mice. **P* < 0.05 Mann-Whitney unpaired *t*-test. Error bars represent SE.

still been ongoing at day 10. Importantly, *Pten*^{fl/fl} GBC cells from lymph nodes of MOG-primed animals exhibited higher levels of proliferation at lower MOG concentrations than did T cells from *Pten*^{fl/fl} controls. MOG-primed *Pten*^{fl/fl} GBC lymphocytes thus demonstrated a greater level of sensitivity to their cognate antigen, manifested by greater proliferative responses in the presence of limiting concentrations of MOG peptide (Figure 4A). This latter result would be in keeping with *Pten* gene

deletions (brought about during *in vivo* priming) leading to a reduction in T-cell activation thresholds, and possibly to an increased sensitivity to the mitogenic effect of IL-2, in MOG-specific memory T cells.

CD8⁺ Pten^{fl/fl} GBC T Cells Show Increased Proliferative Responses to Anti-CD3 Stimulation

To determine whether *Pten*^{fl/fl} GBC CD8⁺ T cells would exhibit an altered proliferative response and/or increased sensitivity to an anti-TCR stimulus, purified CD8⁺ cells were stimulated with varying concentrations of anti-CD3 antibody. We found that CD8⁺ T cells isolated from *Pten*^{fl/fl} GBC mice displayed increased proliferation when exposed to 0.25, 1.0, and 2.5 μg/ml of anti-CD3 as compared to control *Pten*^{fl/fl} CD8⁺ T cells (Figure 3D). Perhaps in agreement with the findings of Buckler and colleagues¹⁵ who demonstrated that stimulation of *Pten*-deficient CD4⁺ T cells with limiting concentrations of anti-CD3 resulted in higher levels of proliferation than controls, we found that *Pten*^{fl/fl} GBC CD8⁺ T cells displayed an increased proliferative response to anti-CD3 at all concentrations evaluated. These results suggested that increased proliferation of *Pten*^{fl/fl} GBC CD8⁺ cells, resulting from *Pten* gene excision, was occurring *in vivo* after MOG immunization.

Pten^{fl/fl} GBC Mice Exhibit Increased Severity of EAE after Immunization with a Reduced Dose of MOG₃₅₋₅₅ Peptide

In view of the increased *in vitro* proliferative responses of *in vivo*-primed *Pten*^{fl/fl} GBC T cells to reduced concentrations of MOG₃₅₋₅₅ peptide (Figure 4A), we hypothesized that *Pten*^{fl/fl} GBC mice would develop more severe EAE than *Pten*^{fl/fl} controls when immunized with a reduced amount of MOG₃₅₋₅₅ peptide (ie, 12.5 μg rather than 50 μg). Both *Pten*^{fl/fl} GBC and control male and female mice immunized with the reduced dose of MOG peptide presented with disease at day 8 after immunization, however, *Pten*^{fl/fl} GBC mice displayed a significantly greater cumulative disease index from day 7 through day 28 after immunization compared to controls (87.20 versus 44.23; Table 1). In keeping with this result, *Pten*^{fl/fl} GBC mice displayed not only a trend toward higher levels of peak disease severity than *Pten*^{fl/fl} mice, but they also demonstrated a significantly greater separation of the *Pten*^{fl/fl} and *Pten*^{fl/fl} GBC clinical scores during the chronic phase (from day 21 onwards) of the disease (Figure 4, B and C). Thus, by reducing the dose of the immunizing peptide, the phenotypic difference between *Pten*^{fl/fl} GBC and *Pten*^{fl/fl} mice became even more apparent. We attribute this difference to *Pten* gene deletions having occurred within MOG peptide-specific *Pten*^{fl/fl} GBC T cells. Interestingly, the delayed onset of clinical disease seen

with the high MOG immunizing dose (Figure 1C) was not observed when the low dose of MOG was used (Figure 4C).

*CD8⁺, CD3⁺, and GZMB⁺ T Cells Are Increased in the CNS of *Pten^{fl/fl}* GBC Mice at Disease Onset and Persist into the Chronic Phase of EAE*

To quantify and phenotype the CNS-infiltrating T cells, entire brains and spinal cords from *Pten^{fl/fl}* and *Pten^{fl/fl}* GBC mice were harvested at day 10, a time coinciding with the onset of ascending paralysis. Isolated populations of cells consisted of ~10% lymphocytes with the remainder being made up of a mixture of macrophages and parenchymal cells. Flow cytometry of cells isolated from the CNS of mice with EAE demonstrated no significant differences in the levels of CD4⁺ T cells of *Pten^{fl/fl}* GBC and *Pten^{fl/fl}* mice (Figure 5A). *Pten^{fl/fl}* GBC mice, however, displayed a significant increase in the proportion of CD8⁺ cells within their CNS lymphocyte populations as compared to controls ($P < 0.01$) (Figure 5A). In addition, the *Pten^{fl/fl}* GBC mice contained equivalent numbers of infiltrating CD4⁺ and CD8⁺ T cells, whereas CD4⁺ cells outnumbered the CD8⁺ T cells in the CNS of *Pten^{fl/fl}* mice. In addition, DNA samples isolated from day 10 *Pten^{fl/fl}* GBC EAE spinal cords revealed the presence of the *Pten*Δ excision band, consistent with the presence of excised *Pten* alleles in the infiltrating T-cell populations (data not shown).

Although lumbar cord sections demonstrated similar levels of Iba-1⁺ cell infiltrates at days 15 and 30 in the mice (data not shown), at the CC level, inflammatory cell infiltrates in *Pten^{fl/fl}* GBC mice were qualitatively more dense than those of *Pten^{fl/fl}* mice (Figure 5B). In the CC white matter, including the DCs, VCs, and LWM, the sections from *Pten^{fl/fl}* GBC mice displayed increased numbers of co-localizing Iba-1⁺ cells (green) and CD3⁺ T lymphocytes (red), as compared to control *Pten^{fl/fl}* mice. It was notable that T lymphocyte infiltrates in the *Pten^{fl/fl}* GBC CC were most prominent at the periphery of the LWM. MBP staining (red fluorescence) white matter-rich regions of CC revealed that *Pten^{fl/fl}* GBC mice had sustained greater levels of MBP signal depletion at both days 15 and 30 than the control *Pten^{fl/fl}* mice (Figure 6B). This was consistent with the greater extent of the infiltrates observed in the *Pten^{fl/fl}* GBC CC sections.

Given the prediction that *Pten* gene excisions were occurring in MOG-activated CD8⁺ T cells, and hence, within CTL effector populations, we performed immunohistochemistry to detect granzyme B (GZMB) protein expression in the CC. Although nonimmunized (*Pten^{fl/fl}*) mice (Figure 6AI) lacked GZMB⁺ cells, both EAE control *Pten^{fl/fl}* as well as *Pten^{fl/fl}* GBC mice contained collections of GZMB⁺ cells at day 15 that were particularly evident in areas of MBP depletion (Figure 6A, II and III). Quantification of GZMB⁺ regions demonstrated that cells expressing this molecule were more plentiful in the *Pten^{fl/fl}*

Figure 5. *Pten^{fl/fl}* GBC mice display more CD8⁺, CD3⁺, and Iba-1⁺ CNS infiltrates at disease onset and also during the chronic phase of EAE. **A:** Flow cytometry analysis of CD4⁺ and CD8⁺ T cells isolated from the CNS of *Pten^{fl/fl}* GBC and *Pten^{fl/fl}* mice at day 10. *Pten^{fl/fl}* GBC mice display similar numbers of CD4⁺ and CD8⁺ infiltrates whereas *Pten^{fl/fl}* mice display significantly more CD4⁺ T cells than CD8⁺ T cells within the CNS. * $P < 0.05$, ** $P < 0.01$, Tukey's multiple comparisons test. Results are compiled from $n = 5$ for each *Pten^{fl/fl}* and *Pten^{fl/fl}* GBC mice. **B:** Sections of representative DCs, VCs, and LWM of CCs stained for T cells (anti-CD3 antibody, red fluorescence) and macrophages/microglia (Iba-1, green fluorescence). At day 30 there were higher levels of Iba-1⁺ cells in all three CC areas within *Pten^{fl/fl}* GBC mice (J–L), as compared to *Pten^{fl/fl}* controls (G–I). In addition, higher levels of CD3⁺ cells were observed in *Pten^{fl/fl}* GBC mice (D–F) in each area, as compared to *Pten^{fl/fl}* mice (A–C). In images G through L, it can be seen that both cell types tend to co-localize, with infiltrate density being highest in the *Pten^{fl/fl}* GBC mice.

GBC CNS on days 15 and 30 (Figure 6C). A dramatic loss of the GZMB⁺ cell population (green fluorescence) was evident in the day 30 controls (Figure 6AV), when compared to the *Pten^{fl/fl}* GBC samples (Figure 6AVI). In the latter, the GZMB⁺ cells had persisted and were particularly evident within regions of MBP depletion. Examination of the CNS at day 60 demonstrated the persistence of dense GZMB⁺ cell infiltrates in the *Pten^{fl/fl}* GBC mice with EAE (data not shown). The accumulation of GZMB⁺

Figure 6. Loss of *Pten* results in increased CC LWM MBP loss and infiltration by GZMB-expressing cells at the peak of disease and at day 30. **A:** *Pten^{fl/fl}* GBC mice (III, VI) exhibit greater demyelination and increased infiltration by GZMB⁺ cells as assessed by loss of myelin basic protein staining (red), and immunofluorescence staining for GZMB⁺ (green) cells, as compared to *Pten^{fl/fl}* (II, V) and intact mice (I, IV) at days 15 and 30. **B:** Quantification of MBP⁺ area within the LWM of the CC revealed significant myelin loss in *Pten^{fl/fl}* GBC mice at day 15 compared to *Pten^{fl/fl}* mice and at day 30 compared to intact mice. **C:** Quantification of GZMB⁺ cells in the LWM of the CC revealed that both *Pten^{fl/fl}* GBC and *Pten^{fl/fl}* mice displayed a significant increase in GZMB⁺ infiltrates at days 15 and 30 as compared to intact mice; whereas *Pten^{fl/fl}* GBC displayed increased numbers of GZMB⁺ cells at day 15 that persisted to day 30 compared to *Pten^{fl/fl}* mice. Analyses represented the results of *n* = 4 per group; **P* < 0.05 ***P* < 0.01; ****P* < 0.001 Tukey's multiple comparisons test. Error bars represent SE.

cells in *Pten^{fl/fl}* GBC mice, likely representing CTL that had undergone *Pten* gene excisions, could have been attributable to either the ongoing recruitment and/or an increase in the longevity of the T cells within the CNS, the latter possibility being in accordance with the anti-apoptotic effect of *Pten* loss.

Pten^{fl/fl} GBC Mice Display Increased Inflammatory Infiltrates in their CC and Forebrains

In addition to having increased numbers of GZMB⁺, CD3⁺, and CD8⁺ infiltrates in their upper CNS and brains, *Pten^{fl/fl}* GBC mice displayed an altered pattern of cell infiltration. As shown in Figure 7A, there was a difference in the extent of cell infiltration within the forebrains of *Pten^{fl/fl}* GBC and *Pten^{fl/fl}* mice; the *Pten^{fl/fl}* GBC sections revealed greater levels of inflammatory cell infiltration and invasion into forebrain and CC parenchyma. Specifically, infiltrates were observed along the fimbria (FI), hippocampus (HC), thalamus (Th), hypothalamus (HypT) (Figure 7A, c and d), as well as both the DC and LWM of the CC (Figure 7A, g and h). *Pten^{fl/fl}* control forebrain and CC sections displayed less substantial infiltrates consisting primarily of perivascular collections of cells at day 15 with less evidence of the parenchymal invasion seen in the *Pten^{fl/fl}* GBC mice (Figure 7A; a, b, e, and f). To assess the contribution of neutrophils to the inflammatory lesions, chloroacetate esterase staining was performed on day 15 sections (Figure 7B); this revealed the presence of increased numbers of neutrophils within inflammatory foci of the CC white matter in *Pten^{fl/fl}* GBC mice. *Pten^{fl/fl}* GBC mice thus exhibited more severe inflammatory lesions of their upper CNS than controls.

Pten^{fl/fl} GBC Exhibit Greater Levels of Axonal Loss in the CC LWM than Controls

To assess CNS damage at day 30, DC, VC, and LWM of CC sections were evaluated for axonal loss using the Bielschowsky silver stain impregnation method. The representative images of CC LWM in Figure 7C reveal qualitative evidence of increased damage, with swelling of axonal sheaths and greater axonal fiber depletion in *Pten^{fl/fl}* GBC mice compared to both MOG-immunized and nonimmunized *Pten^{fl/fl}* controls. Consistent with the histological findings, quantification of axon numbers within different cord regions (Figure 7D) revealed that LWM of the *Pten^{fl/fl}* GBC CC contained decreased numbers of axons, consistent with our previous observation that the highest levels of cellular infiltrates for *Pten^{fl/fl}* GBC occurred on the periphery of the CC, and especially in superficial regions of the LWM. Although not significant, there was a trend for the CC VC of *Pten^{fl/fl}* GBC mice to show increased axonal loss as compared to control *Pten^{fl/fl}* mice (Figure 7D). In summary, consistent with the LWM of the CC being a site of prominent T-cell infiltration, axonal damage in *Pten^{fl/fl}* GBC mice was primarily localized to the LWM.

Discussion

Conditional mutagenesis experiments involving floxed genes in T cells have typically used Cre recombinase transgenes expressed during intrathymic development.^{12,13,35–37} A major drawback of this approach be-

Figure 7. *Pten*^{fl/fl} GBC mice exhibit more pronounced forebrain inflammation with prominent neutrophil infiltrates, as well as greater cord axon loss. **A:** H&E staining of *Pten*^{fl/fl} GBC sections revealed greater levels of inflammatory cell infiltration and invasiveness into forebrain and CC parenchyma as compared to *Pten*^{fl/fl} mice. Infiltration was observed along the fimbria (FI), hippocampus (HC), thalamus (Th), hypothalamus (HypT) (c, d), as well as the DC and LWM of the CC (g, h). *Pten*^{fl/fl} forebrain and CC sections in contrast displayed predominantly perivascular infiltration on day 15 with reduced parenchymal invasion (a, b, c, e, f). **B:** Neutrophil infiltration at the peak of disease (day 15) as assessed by chloroacetate esterase stain. The figure is representative of *n* = 4 mice for each sample group. **C:** Silver staining of CC sections to display axonal loss in *Pten*^{fl/fl} GBC mice, as compared to *Pten*^{fl/fl} mice at day 30. **D:** Quantification of axonal counts within the white matter (DC, VC, and LWM) in mice of both genotypes. For quantification: intact (open bars) *n* = 3, *Pten*^{fl/fl} (gray bars) *n* = 4, *Pten*^{fl/fl} GBC (filled bars) *n* = 4, and from each individual three counts from each spinal cord region. **P* < 0.05, ***P* < 0.01 Tukey's multiple comparison's test. Error bars represent SE.

comes evident when questions about gene function specifically in antigen-activated mature or effector T cells are being posed, since intrathymic gene deletions have the potential not only to skew the outcome of developmental processes such as positive and negative selection, but can also result in abnormalities of naïve peripheral T cells (such as altered activation thresholds and/or interference with the mechanisms of tolerance). Thus, to study the consequences of specific gene deletions on effector/memory T-cell populations responding to antigenic challenges, and to be able to directly compare the behavior of these cells to T-cell populations in control mice, it is critical that the naïve T-cell TCR repertoires (and activation thresholds) be similar in both of the responding populations. Without this, it becomes difficult to discern phenotypic effects stemming from an intrathymic developmental anomaly from those attributable to gene loss within antigen-activated T cells. Therefore, to study the role of *Pten* specifically within MOG-specific effector CD8⁺ populations it was critical for us to use mice having equivalent preimmune TCR repertoires and T-cell activation thresholds.

To trigger deletions of *Pten* in mature, antigen-activated T cells we applied the GBC system in which gene deletions are primarily confined to CD8⁺ cells after TCR

activation.^{26,27} In this system, T-cell activation leads to *de novo* Cre expression from the GBC transgene, thus triggering *loxP*-dependent genetic recombinations within activated CD8⁺ cells. This method has been used successfully to identify activated effector and memory CD8⁺ T lymphocytes *in situ* after antigenic challenge, by virtue of the Cre-mediated activation of the placental alkaline phosphatase reporter gene.²⁶ Maris and colleagues²⁷ also used this method to demonstrate the occurrence of a high frequency of Cre-mediated gene excisions within lymphocytic choriomeningitis virus-specific CD8⁺ cells after *in vivo* challenge with this agent.

The role of *Pten*, or of other components of the PI3K pathway for that matter, in the pathogenesis of EAE has not been previously subjected to a genetic analysis. Herein, we show that loss of *Pten* in CD8⁺ T cells alters the clinical and pathological features of MOG-induced EAE. Despite *Pten* deficiency resulting in the infiltration and persistence of large numbers of lymphocytes, especially GZMB⁺ CD8⁺ T cells and phagocytic cells (microglia and macrophages) in the CNS, we found that this was not associated with a dramatic increase in clinical disease severity at higher doses of MOG_{35–55}, at least as assessed by the conventional scoring method that is

based on the detection of spinal cord motor deficits. Thus, the observed differences in the forebrain inflammation between *Pten^{fl/fl}* GBC and *Pten^{fl/fl}* mice might not have been accurately reflected by a scoring system based primarily on the degree of tail and limb paresis. Similarly, the clinical scoring would be unlikely to reflect the increased axonal damage in the LWM region of the cord (Figure 7D), a site also heavily infiltrated by GZMB⁺ cells in *Pten^{fl/fl}* GBC mice (Figure 6A), because the LWM lies some distance away from the more centrally located corticospinal tracts. These caveats, along with the increased loss of MBP staining we observed at day 30 (Figure 6B), indicate worsening of EAE pathology that we attribute to *Pten* gene deletions in effector CD8⁺ T cells.

An alteration in either the emigration from lymphoid tissues, or a defect in transmigration into the CNS might account for the trend of delayed EAE clinical disease onset in the *Pten^{fl/fl}* GBC mice. An abnormality of chemotaxis or an altered adhesiveness to CNS blood vessels might explain the preferential infiltration of cells into the LWM of the CC and the forebrain. In support of such possibilities, *Pten* has been shown to be required for both normal motility and the chemoattractant-directed migration of cells, including lymphocytes.^{38,39} Such studies have predicted that *Pten*-deficient T cells would display an alteration in their motility toward foci of inflammation, and this provides a plausible explanation for the atypical patterns of cell infiltration we observed in the CNS of *Pten^{fl/fl}* GBC mice. An abnormality in the response to chemoattractants could promote the accumulation of cells within specific CNS sites. However, we saw no delay in disease onset when low-dose MOG immunization was used. The biological mechanism behind this apparent discrepancy is not clear, however, assuming that the high MOG dose led to a much larger population of activated, cytokine-producing T cells than the low MOG dose, then increased concentrations of cytokine might be present within the systemic circulation. Were this to result in increased endothelial adhesiveness, and lead to the margination of *Pten*-deficient T cells in multiple vascular beds, it might limit the initial numbers of circulating T cells available for entry into the CNS. Such an effect would be predicted to be less pronounced in mice immunized with the low dose of MOG. Clearly, the application of additional techniques, such as adoptive transfer of fluorescently tagged *Pten*-deficient T cells into mice, in combination with intravital microscopy, might be able to reveal subtle defects in vessel adhesion, transmigration, or chemotaxis by *Pten*-deficient CD8⁺ cells.

In addition to the increased infiltration of cells in specific CNS regions, we observed prominent GZMB⁺ staining in the CNS lesions, consistent with the presence of CTLs (Figure 6AVI). The persistence of the cells in *Pten^{fl/fl}* GBC EAE lesions may have been attributable either to a sustained influx of CTLs into the CNS and/or an increase in the longevity of these cells. In favor of the latter scenario, we found that activated *Pten^{fl/fl}* GBC T cells exhibited not only a greater resistance to IL-2 withdrawal-induced apoptosis, but also increased survival in response to either anti-CD3 antibody- or anti-Fas antibody-induced apoptosis (Figure 3A). Thus, our finding that activated *Pten^{fl/fl}* GBC T cells

were more resistant to several physiologically relevant proapoptotic stimuli provided a plausible explanation for the persistence of GZMB⁺ cells within the lesions of *Pten^{fl/fl}* GBC mice with EAE.

There is considerable evidence that *Pten* expression not only sensitizes cells to a range of proapoptotic stimuli, but that it functions as a negative regulator of cell proliferation.⁴⁰ In keeping with the latter property, we found that *Pten^{fl/fl}* GBC CD8⁺ T lymphocytes displayed increased proliferation after anti-CD3 antibody stimulation, and also T cells isolated from *in vivo* MOG-primed *Pten^{fl/fl}* GBC mice proliferated more vigorously on re-exposure to MOG peptide presented by splenic APCs (Figure 4A). Increased T-cell proliferation in the periphery might have been a factor accounting for the increased numbers of CD8⁺ T lymphocytes for the CNS of *Pten^{fl/fl}* GBC mice (Figure 5A). In addition, *Pten^{fl/fl}* GBC T lymphocytes demonstrated increased proliferation in response to IL-2 alone (Figure 2B), a result that could be interpreted in at least two ways: that *Pten^{fl/fl}* GBC T cells obtained from immunized mice were still in the process of clonal expansion and this was augmented by exogenous IL-2, or that *Pten*-deficient T cells were capable of mitogenic responses in the absence of TCR activation. The latter would be in agreement with the observation by Walsh and colleagues¹⁴ that *Pten*-deficient CD4⁺ CD25⁺ T-reg cells were able to proliferate in response to IL-2 alone. How might IL-2 be able to trigger proliferation of T cells lacking *Pten* protein? *Pten*-deficient T cell systems have revealed that *Pten* is an inhibitor of cell cycle progression,^{12,14,15} and *Pten* was shown to be able to reverse the PKB/Akt-mediated inhibition of p27^{Kip1} expression, a pivotal cell cycle inhibitor.⁴¹ Furthermore, cytokine-stimulated T-lymphocyte proliferation, but not anti-CD3 induced proliferation, has been shown to be regulated by p27^{Kip1},^{42–44} and p27^{Kip1}-deficient splenocytes were hyperproliferative in response to IL-2, suggesting that dysregulation of p27^{Kip1} expression is a key component of the altered proliferative phenotype of *Pten*-deficient T lymphocytes.⁴⁵ These results suggest that the increased proliferative response of *Pten^{fl/fl}* GBC T cells to IL-2 might be attributable to the unrestrained inhibition of this cell cycle regulator as a consequence of *Pten* loss.

The *in vitro* experiments on primed *Pten^{fl/fl}* GBC T-cell populations (probably composed primarily of MOG-specific T memory cells) showed that they were more proliferative than controls in response to downward titration of the MOG_{35–55} peptide concentration (Figure 4A). Reflective of the results obtained in these *in vitro* experiments, we found that immunization with a substantially reduced dose of MOG_{35–55} peptide still yielded EAE of a severity comparable to that of *Pten^{fl/fl}* GBC mice given the full immunizing dose of MOG-peptide. In contrast, *Pten^{fl/fl}* littermate control mice exhibited much lower disease scores when immunized with the reduced dose of MOG antigen. These results indicated that MOG-primed T cells from *Pten^{fl/fl}* GBC mice, in contrast to the controls, had retained both their sensitivity and proliferative potential even when the amount of antigen was limiting. Could the reduced threshold for activation by MOG-peptide be indicative of a decreased requirement for co-stimulation by

primed *Pten^{fl/fl}* GBC T lymphocytes? Previously, it was shown that *Pten*-deficient T lymphocytes were able to proliferate more vigorously than wild-type controls in response to anti-CD3 antibody in the absence of anti-CD28 antibody co-stimulation,¹⁵ a result in keeping with our results with the *Pten^{fl/fl}* GBC CD8⁺ T cells. Furthermore, because co-stimulation is required for effector T-cell persistence and functionality,⁴⁶ *Pten*-deficient T cells, predicted to have a reduced need for co-stimulation, might show not only an increase in effector function but also an increase in their longevity. The latter possibly being a factor in the persistence of GMZB⁺ cells in *Pten^{fl/fl}* GBC mice with EAE.

The conditional mutagenesis system we have applied is unique in that it allows analysis of gene function within mature, antigen-activated CD8⁺ T cells engaged in immune responses *in vivo*. Our study of the anti-MOG responses of *Pten^{fl/fl}* GBC mice potentially addresses some of the clinical and pathological differences between EAE and MS. For example, one criticism of the EAE model in most inbred mouse strains has been that it primarily affects the lower spinal cord, with little or no involvement of the upper CNS,⁴⁷ our model, in contrast, showed prominent forebrain involvement. Furthermore, many EAE models have focused on the encephalitogenic properties of CD4⁺ T lymphocytes, supporting the idea that MS and EAE are primarily Th₁/Th₁₇-driven diseases. Although CD4⁺ lymphocytes do play an important role in *Pten^{fl/fl}* GBC EAE (also suggested by our isolation of CD4⁺ cells from the CNS of *Pten^{fl/fl}* GBC mice), our *Pten^{fl/fl}* GBC model reinforces the idea that CD8⁺ lymphocytes make key contributions to neuropathology of EAE (and possibly MS as well).^{48–50} As an alternative to the use of adoptive transfers to answer questions about the roles of mutant CD8⁺ cells,²⁵ the genetic model we have applied herein provides a novel way to study the functional consequences of CD8⁺ T-lymphocyte-specific gene deletions in the EAE model.

Future therapeutic interventions for MS may potentially target any one of a number of different cellular pathways, including those involved in phagocytosis, chemotaxis, regulating the sensitivity of cells to apoptosis, cell activation, and responses to cytokines.⁴⁷ Because such processes often involve the PI3K pathway, studies focused on deletions of the key negative regulator, *Pten*, provide an opportunity to gauge the relative importance of PI3K in controlling the functions of the various cell types involved in mediating autoimmune demyelination.

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