The Pten/PI3K pathway governs the homeostasis of Vα14*i*NKT cells

Hiroyuki Kishimoto,[1] Toshiaki Ohteki,[2] Nobuyuki Yajima,[1] Koichi Kawahara,[1] Miyuki Natsui,[1] Satoru Kawarasaki,[1] Koichi Hamada,[1] Yasuo Horie,[3] Yoshiaki Kubo,[4] Seiji Arase,[4] Masaru Taniguchi,[5] Bart Vanhaesebroeck,[6] Tak Wah Mak,[7] Toru Nakano,[8] Shigeo Koyasu,[9] Takehiko Sasaki,[10] and Akira Suzuki[1]

Departments of [1]Molecular Biology, [2]Immunology, and [3]Gastroenterology, Akita University School of Medicine, Japan; [4]Department of Dermatology, Institute of Health Bioscience, Graduate School of Medicine, The University of Tokushima, Japan; [5]RIKEN Research Center for Allergy and Immunology, Yokohama, Japan; [6]Ludwig Institute for Cancer Research, London, United Kingdom; [7]Campbell Family Institute for Breast Cancer Research and Departments of Immunology and Medical Biophysics, University of Toronto, ON, Canada; [8]Department of Pathology, Medical School and Graduate School of Frontier Biosciences, Osaka University, Suita, Japan; [9]Department of Microbiology and Immunology, Keio University School of Medicine, Tokyo, Japan; [10]Department of Microbiology, Akita University School of Medicine, Japan

The tumor suppressor PTEN is mutated in many human cancers. We previously used the *Cre-loxP* system to generate mice (*LckCrePten* mice) with a Pten mutation in T-lineage cells. Here we describe the phenotype of Pten-deficient Vα14*i*NKT cells. A failure in the development of Vα14*i*NKT cells occurs in the *LckCrePten* thymus between stage 2 (CD44^{high}NK1.1^{-}) and stage 3 (CD44^{high}NK1.1^{+}), resulting in decreased numbers of peripheral Vα14*i*NKT cells. In vitro, Pten-deficient Vα14*i*NKT cells show reduced proliferation and cytokine secretion in response to αGalCer stimulation but enhanced inhibitory Ly49 receptor expression. Following interaction with dendritic cells (DCs) loaded with αGalCer, Pten-deficient Vα14*i*NKT cells demonstrate activation of PI3K. Indeed, the effects of the Pten mutation require intact function of the PI3K subunits p110γ and p110δ. In vivo, *LckCrePten* mice display reduced serum IFNγ after αGalCer administration. Importantly, Vα14*i*NKT cell–mediated protection against the metastasis of melanoma cells to the lung was impaired in the absence of Pten. Thus, the Pten/PI3K pathway is indispensable for the homeostasis and antitumor surveillance function of Vα14*i*NKT cells. (Blood. 2007; 109:3316-3324)

Introduction

Vα14*i*NKT cells, originally defined as NK1.1^{+} T cells,[1-3] are a unique lymphoid lineage characterized by the expression of several receptors that distinguish them from T, B, and natural killer (NK) cells. These receptors include an invariant rearranged T-cell receptor α (TCRα) chain, NK1.1, the IL-2/IL-15Rβ chain (CD122), and Ly49. The TCRα chain expressed by Vα14*i*NKT cells is encoded by the Vα14 and Jα18 gene segments and is usually associated with a TCRβ chain containing the Vβ8 gene segment, a combination not expressed by conventional T cells.[4] Vα14*i*NKT cells constitute more than half of the NK1.1^{+} T-cell population in the murine spleen and most NK1.1^{+} T cells in the thymus and liver.[5] Vα14*i*NKT cells respond to glycolipid antigens such as the marine sponge–derived glycolipid α-galactosylceramide (αGalCer). αGalCer is presented to NKT cells by CD1d, an antigen presentation molecule that structurally resembles major histocompatibility complex (MHC) class I. Because of their restricted specificity, Vα14*i*NKT cells can be readily identified by staining with CD1d tetramers or dimers loaded with αGalCer.[6,7]

During their development in the thymus, Vα14*i*NKT precursors at the CD4^{+}CD8^{+} (double positive [DP]) stage are positively selected by CD1d-mediated presentation of self-glycolipids (such as iGb3) by CD4^{+}CD8^{+} thymocytes.[8,9] Vα14*i*NKT precursors (CD44^{low}NK1.1^{-}; stage 1) then develop sequentially into immature NKT cells (CD44^{high}NK1.1^{-}; stage 2) and finally into mature Vα14*i*NKT cells (CD44^{high}NK1.1^{+}; stage 3).[10,11] Vα14*i*NKT cells apparently also undergo negative selection such that NKT cell maturation is severely reduced in situations in which activation signals for developing cells are enhanced to supraphysiological levels either in vitro or in vivo.[12-16]

In response to TCR ligation, mature peripheral Vα14*i*NKT cells promptly produce large amounts of Th1 cytokines (IFNγ, TNF) as well as Th2 cytokines (IL-4, IL-10, IL-13).[17,18] Vα14*i*NKT cells therefore play important roles in a variety of innate and adaptive immune responses, including those mediating autoimmunity or combating microbial infections or tumor metastasis.[5,19-21] Indeed, mice deficient in Vα14*i*NKT cells show impaired tumor rejection.[21] This observation is consistent with the antitumor effect of αGalCer administration on various tumor types, including melanomas and lung, colon, and hematopoietic cancers.[22] Despite the importance of Vα14*i*NKT cells, little is known about their intracellular signaling pathways or how these pathways might be regulated.

PTEN is a tumor suppressor gene[23] that is mutated in many human sporadic cancers and in hereditary tumor susceptibility disorders such as Cowden disease.[24] PTEN is a multifunctional phosphatase whose major substrate is phosphatidylinositol-3,4,5-triphosphate (PIP3),[25] a lipid second messenger molecule. PIP3 is generated by the action of phosphoinositide-3-kinases (PI3Ks). Four distinct class I PI3K isotypes exist in mammals: PI3Kα, β, δ, and γ.[26] The function of the PIP3 generated by PI3K is to activate

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numerous downstream targets, including the serine-threonine kinase PKB/Akt that is involved in antiapoptosis, proliferation, and oncogenesis. By using its lipid phosphatase activity to dephosphorylate PIP3, PTEN negatively regulates the PI3K-PKB/Akt pathway and thereby exerts tumor suppression.[27] Thus, murine T cells lacking Pten show enhanced proliferation and activation in vitro, and T cell–specific Pten-deficient mice develop T-cell lymphomas/leukemias and are prone to autoimmunity.[28] Conversely, mice deficient for the PI3K subunit p110γ show impaired T-cell development and activation,[29] and T cells from animals with a kinase-inactivating mutation (D910A) of p110δ have a defect in TCR-stimulated proliferation.[30] Analyses of these and other genetically modified mice have begun to provide insights into the pathways and molecules mediating intracellular signaling in Vα14*i*NKT cells. However, the specific functions of PI3K and Pten in these cells are totally unknown.

In this study, we demonstrate that mutation of Pten in murine Vα14*i*NKT cells results in developmental failure leading to increased numbers of immature Vα14*i*NKT cells and decreased numbers of mature Vα14*i*NKT cells of impaired functionality. Moreover, these defects depend on PI3K function. Our results indicate that the Pten/PI3K pathway is important for the homeostasis of Vα14*i*NKT cells and their antitumor surveillance function.

Materials and methods

Mice

LckCrePten$^{flox/flox}$,[28] Pten$^{+/-}$,[31] PI3K *p110γ*$^{-/-}$,[29] PI3K *p110δ*$^{D910A/D910A}$,[30] *Jα18*$^{-/-}$,[21] and *AktPH-GFP*$^{tg/tg}$ transgenic[32] mice (all C57BL6/J background, H-2^b) have been previously described. Because *LckCrePten*$^{flox/flox}$ mice begin to develop T-cell lymphomas at age 3 months, all experiments were performed using lymphoma-free mice younger than 8 weeks of age. *LckCrePten*$^{+/+}$ and *Pten*$^{flox/flox}$ mice[28] were indistinguishable in pilot experiments examining flow cytometry, cell proliferation, and cytokine production (data not shown), so that *Pten*$^{flox/flox}$ mice were used as representative wild-type (WT) controls in this study. The Institutional Review Board of the Akita University School of Medicine approved all animal experiments.

Reagents and melanoma cell line

A synthetic form of αGalCer (KRN7000) was provided by the Kirin Brewery (Gumma, Japan). Antibodies to TCRβ-FITC (H57-597), NK1.1-FITC or -PE (PK136), CD24/HSA-biotin (30-F1), and PE-Cy5-streptavidin were purchased from eBioscience (San Diego, CA). Antibodies to TCRβ-APC (H57-597), CD45R/B220-APC (RA3-6B2), Ly49C/I-biotin (5E6), Ly49A-biotin (A1), CD44-FITC (IM7), mouse IgG1–biotin (A85-1), and mouse CD1d dimmer–Ig fusion protein were purchased from BD PharMingen (San Diego, CA). The B16F10 mouse melanoma cell line was supplied by the Cell Resource Center for Biomedical Research, Tohoku University, Japan.

Flow cytometry and cell sorting

Mononuclear cells (MNCs) from the thymus, liver, and spleen were obtained as previously described.[33] For routine staining of Vα14*i*NKT cells, MNCs were first preincubated with CD16/32 mAb to prevent nonspecific binding of antibodies to FcγR. The cells were then incubated with CD1d dimmer–Ig (αGalCer-CD1d)[7] followed by anti–mouse IgG1–biotin, PE-Cy5-streptavidin, and anti-TCRβ–APC. To analyze NKT cell development, cells were also stained with anti-NK1.1–PE and anti-CD44–FITC. To detect Ly49 expression, cells were stained with anti-NK1.1–FITC, anti-TCRβ–APC, CD1d dimmer–Ig (αGalCer-CD1d), anti–mouse IgG1–PE, anti-Ly49C/I–biotin (or anti-Ly49A–biotin), and streptavidin-PE-Cy5. All flow cytometry and sorting experiments were performed using MoFlo and Summit software (Dako Cytomation, Glostrup, Denmark).

PCR and Western blotting

For analyzing the deletion frequency of the *Pten* gene at the DNA level, genomic DNA samples from sorted TCRβ$^+$NK1.1$^+$HSA^{low} thymocytes of 8-week-old *Pten*$^{flox/flox}$ and *LckCrePten*$^{flox/flox}$ mice were amplified by polymerase chain reaction (PCR) as described.[34] Sense primer (5′-GTGAAAGTGCCCCAACATAAGG-3′) and antisense primer (5′-CTCCCACCAATGAACAAACAGT-3′) were used to detect the WT and floxed *Pten* alleles; sense primer (5′-GGCCTAGGACTCACTAGATAGC-3′) and antisense primer (5′-CTCCCACCAATGAACAAACAGT-3′) were used to detect the deleted *Pten*Δ allele. Amplified fragments of 514 bp (*Pten*^{flox} allele) and 705 bp (*Pten*Δ allele) were obtained.

Total lysates (10 μg) of sorted TCRβ$^+$NK1.1$^+$HSA^{low} thymocytes from 8-week-old *Pten*$^{flox/flox}$ and *LckCrePten*$^{flox/flox}$ mice were analyzed by Western blotting using antibodies directed against Pten (Cascade Bioscience, Winchester, MA) or actin (Santa Cruz Biotechnology, Santa Cruz, CA).

In vitro and in vivo activation of NKT cells

For in vitro activation of NKT cells, thymocytes were isolated from 8-week-old *LckCrePten*$^{flox.flox}$ or *Pten*$^{flox.flox}$ mice, and NK1.1$^+$TCRβ$^+$HSA^{low} NKT cells were purified by cell sorting. αGalCer-loaded dendritic cells (DCs) were prepared from spleens as described.[35] Briefly, spleens from WT mice were digested using collagenase and erythrocytes, and MNCs were collected by step centrifugation using 28% bovine serum albumin and serumfree RPMI medium. Isolated MNCs were cultured for 2 hours, and adherent cells were further cultured overnight with or without 100 ng/mL αGalCer. Nonadherent CD11c$^+$ cells were plated at 2×10^4 per well in a 96-well plate and γ-irradiated (30 Gy). These cells were used as the αGalCer-loaded DCs. Purified NKT cells (1×10^4 per well) were incubated with αGalCer-loaded DCs in 96-well plates in RPMI 1640 medium containing 10% FCS. The αGalCer-stimulated NKT cells were harvested on day 2, and their proliferation was assayed by pulsing for 12 hours with 0.5 μCi (0.0185 MBq) [^{3}H]-thymidine (Amersham, Arlington Heights, IL) per well and measuring [^{3}H]-thymidine incorporation by standard methods. Culture supernatants were assayed for the production of IFNγ and IL-4 by ELISA (Pierce Endogen, Rockford, IL).

For in vivo activation of NKT cells, αGalCer (2 μg) was intraperitoneally injected into 8-week-old *Pten*$^{flox/flox}$, *Pten*$^{+/-}$, and *LckCrePten*$^{flox/flox}$ mice. Serum IL-4 and IFNγ levels were examined by enzyme-linked immunosorbent assay (ELISA) at 2 hours and 12 hours after αGalCer injection, respectively.

NKT cell maturation in FTOC

Fetal thymus organ culture (FTOC) was performed as described[11] with some modifications. Fetal thymus lobes from embryonic day 14.5 *Jα18*$^{-/-}$ fetal mice were cultured for 3 days on Nucleopore membrane (Whatman, Middlesex, United Kingdom) in RPMI medium supplemented with 10% FCS, nonessential amino acids, pyruvate, and β-mercaptoethanol (all from Invitrogen, Carlsbad, CA). Purified CD44^{high}NK1.1$^-$αGalCer-CD1d$^+$TCRβ$^+$ immature Vα14*i*NKT cells (1×10^5) isolated from the thymus of a 3-week-old *LckCrePten*$^{flox/flox}$ or *Pten*$^{flox/flox}$ mouse were microinjected into each thymic lobe of a *Jα18*$^{-/-}$ embryo and cultured for additional 9 days. The maturation of the injected NKT cells was evaluated by flow cytometry as described.

Tumor immunity

The B16F10 melanoma cell line was maintained in the same culture medium as that used for FTOC. *Jα18*$^{-/-}$ mice (8 weeks old) were intravenously inoculated with B16F10 cells (4×10^5). After 3 hours, these mice were intravenously injected with sorted αGalCer-CD1d$^+$TCRβ$^+$ thymocytes (1×10^6) from 8-week-old *Pten*$^{flox/flox}$, *LckCrePten*$^{flox/+}$, or *LckCrePten*$^{flox/flox}$ mice. After 30 minutes, 2 μg αGalCer was intraperitoneally injected into the mice. On days 4 and 8 after cell transfer, the mice received an additional 2 μg αGalCer. The mice were killed on day 14, and

the number of surface lung metastases was evaluated with aid of a dissecting microscope. The data were recorded as the mean number of metastases per lung ± SEM.

Confocal video microscopy

TCRβ$^+$NK1.1$^+$HSA^{low} NKT cells (1×10^4) purified from thymocytes of 8-week-old *AktPH-GFP$^{tg/tg}$* mice and DCs (1×10^5) either loaded or not with αGalCer were coplated in fibronectin-coated glass-base dishes for 10 minutes. In some experiments, the PI3K inhibitor wortmannin (10 nM; Sigma, St Louis, MO) was added before the cells were mixed. Time-lapse studies to detect PIP3 identified by the AktPH-GFP bioprobe[32] were performed at 37°C using a heating device (Harvard Apparatus, Holliston, MA) adapted to a DM IRE2 Leica (Wetzlar, Germany) microscope fitted with a confocal imaging system (Yokogawa, Tokyo, Japan) and equipped with a Leica HCX APO 100×/1.30 numerical aperture objective. Confocal fluorescent or differential interface contrast (Nomarski optics) images were acquired using an Orca-ER cooled charge-coupled-device (CCD) camera (Hamamatsu Photonics, Hamamatsu, Japan) driven by IPLab software version 3.0.4 (BD Bioscience).

Statistical methods

Statistical differences were determined using the Student *t* test.

Results

Pten deficiency in NKT cells of *LckCrePten$^{flox/flox}$* mice

We previously reported a conditional *Pten* knockout mouse in which the *Pten* gene was deleted by *Lck,* a T cell–specific promoter (*LckCrePten$^{flox/flox}$* mice).[28] Genomic PCR examination of DNA from purified TCRβ$^+$NK1.1$^+$HSA^{low} thymocytes from 8-week-old *Pten$^{flox/flox}$* and *LckCrePten$^{flox/flox}$* mice showed that highly efficient Cre-mediated recombination had occurred not only in conventional T cells but also in NKT cells (Figure 1A). The deletion of Pten was confirmed at the protein level by Western blotting (Figure 1B).

Impaired development of NKT cells in Pten-deficient mice

To examine the role of Pten in NKT cell development, we analyzed the NK1.1$^+$TCRβ$^+$ NKT population in the thymus, spleen, and liver of 7- to 8-week-old *LckCrePten$^{flox/flox}$* and *Pten$^{flox/flox}$* mice. *LckCrePten$^{flox/flox}$* mice showed reductions not only in the percentages of NK1.1$^+$TCRβ$^+$ cells present in all 3 tissues (Figure 2A) but also in absolute numbers of these NKT cells in the thymus (4.5-fold decrease), spleen (2.1-fold), and liver (4.3-fold) (Figure 2B).

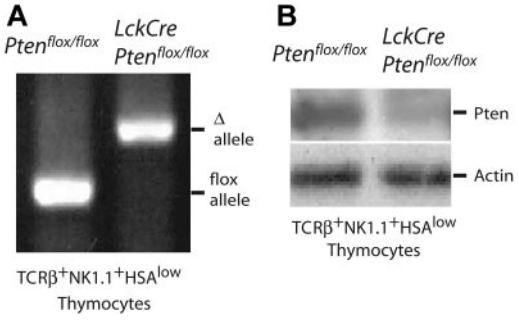

Figure 1. *Pten* gene deletion in NKT cells in *LckCrePten$^{flox/flox}$* mice. (A) Genomic DNA from TCRβ$^+$NK1.1$^+$HSA^{low} thymocytes of the indicated genotype was analyzed by PCR. In *LckCrePten$^{flox/flox}$* mice, most TCRβ$^+$NK1.1$^+$HSA^{low} thymocytes contained the deleted *Pten* allele (Δ) rather than the WT Pten allele (flox). (B) Western blot analysis of Pten protein expression by the thymocytes in panel A. Actin loading control.

We next investigated numbers of Vα14*i*NKT cells, a major subset of NKT cells, using the reagent CD1d dimer–Ig (αGalCer-CD1d).[7] The thymi of WT and *LckCrePten$^{flox/flox}$* mice contained equal numbers of αGalCer-CD1d$^+$TCRβ$^+$NKT cells (Vα14*i*NKT cells) (Figure 2C-D, left panels), indicating that Pten deficiency has no effect on total Vα14*i*NKT cell numbers. We then determined CD44/NK1.1 profiles[10,11] to follow the development of Vα14*i*NKT cells in thymus. While about 80% of thymic Vα14*i*NKT cells in *Pten$^{flox/flox}$* mice were mature and had reached stage 3 (CD44^{high}NK1.1$^+$), only 9% of Vα14*i*NKT cells in *LckCrePten$^{flox/flox}$*mice were at stage 3 and most had arrested at stage 2 (immature CD44^{high}NK1.1$^-$ cells) (Figure 2C, right panel, and E). No differences were observed between the WT and mutant thymus in the percentage of stage 1 NKT precursors (CD44^{low}NK1.1$^-$) present. When proliferative capacity was examined, there was no difference between the WT and the mutant in the percentage of BrdU-positive cells in the stage 2 population (Figure S1A, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). Thus, a block in differentiation is the most likely explanation for the observed increase in stage 2 cells in the mutant thymus. In the periphery, Vα14*i*NKT cells in *LckCrePten$^{flox/flox}$* mice were decreased in the spleen and liver to 51% and 48% of WT values, respectively (Figure 2C-D). These decreases had to be due to reductions in mature Vα14*i*NKT cells because no increases were observed in the percentage of NK1.1$^-$ Vα14*i*NKT cells in mutant spleen and liver (Figure S1B).

Vα14*i*NKT cells are positively selected in the thymus via the interaction of their TCRs with CD1d expressed by DP cortical thymocytes and negatively selected by CD1d expressed by antigen-presenting cells such as DCs.[8,12] Thus, normal CD1d expression on DP thymocytes and thymic/peripheral DCs is required for proper NKT cell development.[8,9] Flow cytometric analysis revealed no differences in CD1d levels expressed by DP thymocytes or thymic or splenic DCs from *Pten$^{flox/flox}$* and *LckCrePten$^{flox/flox}$* mice (Figure S1C). These data indicate that the impaired development of Pten-deficient Vα14*i*NKT cells is not due to altered CD1d expression by antigen-presenting cells.

The developmental failure of Vα14*i*NKT cells in *LckCrePten$^{flox/flox}$* mice is cell autonomous

The *Pten* gene is deleted in both NKT cells and T cells in *LckCrePten$^{flox/flox}$* mice, and DP T cells are an important stromal component for normal NKT cell development in the thymus.[8,9,12] To rule out the possibility that it was the Pten mutation in DP T cells that caused the decrease in NKT cells in *LckCrePten$^{flox/flox}$ mice*, purified CD44^{high}NK1.1$^-$αGalCer-CD1d$^+$TCRβ$^+$ immature Vα14*i*NKT cells (stage 2) from *Pten$^{flox/flox}$* and *LckCrePten$^{flox/flox}$* mice were transferred into *Jα18$^{-/-}$* fetal thymus (which lacks all Vα14*i*NKT cells) and cultured for 9 days using the FTOC method.[11] As shown in Figure 3, more than 60% of immature *Pten$^{flox/flox}$* Vα14*i*NKT cells developed into mature Vα14*i*NKT cells (CD44^{high}NK1.1$^+$αGalCer-CD1d$^+$TCRβ$^+$; stage 3) in this environment. In contrast, almost all transferred immature *LckCrePten$^{flox/flox}$* Vα14*i*NKT cells remained at stage 2. This result clearly indicates that the reduced number of Vα14*i*NKT cells in *LckCrePten$^{flox/flox}$* mice was due to a cell-autonomous defect.

Impaired activation of Pten-deficient NKT cells in vitro and in vivo

We next investigated the functional status of Pten-deficient Vα14*i*NKT cells by determining their proliferation and cytokine

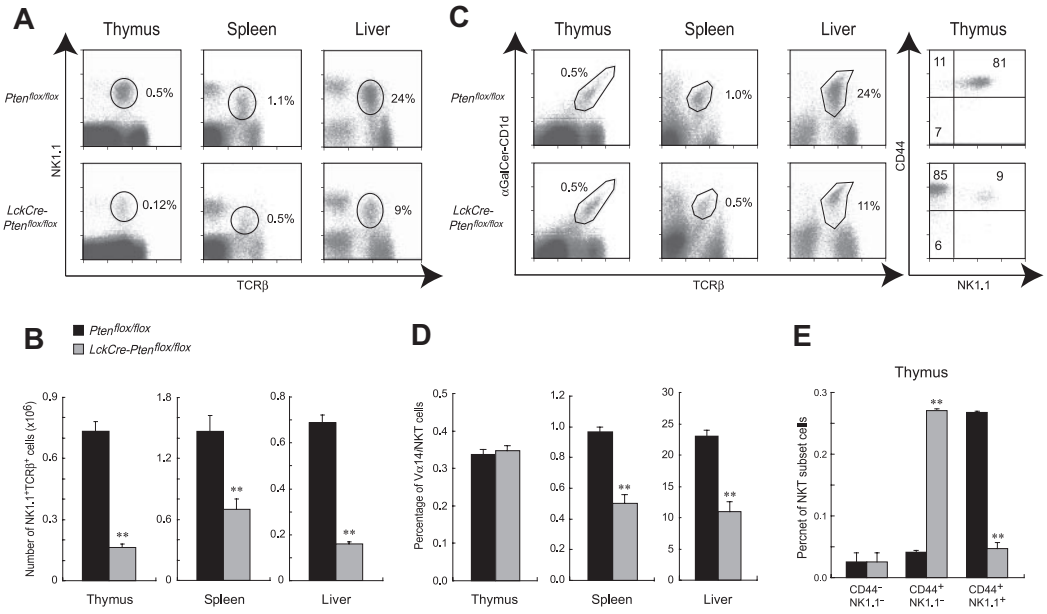

Figure 2. Altered NKT cell populations in *LckCrePten*$^{flox/flox}$ mice. (A-B) Decreased numbers of NK1.1$^+$TCRβ$^+$ cells in *LckCrePten*$^{flox/flox}$ mice. (A) MNCs were isolated from the thymus, spleen, and liver of mice of the indicated genotypes, and NK1.1$^+$TCRβ$^+$ cells were analyzed by flow cytometry. Numbers within the panels indicate the relative percentages of NK1.1$^+$TCRβ$^+$ cells in total lymphocyte populations. (B) Absolute numbers of the NK1.1$^+$TCRβ$^+$ cells in panel A. (C-E) Altered maturation and numbers of Vα14*i*NKT cells. (C, left panel, and D) The percentage of Vα14*i*NKT cells in total MNCs is normal in the thymus of *LckCrePten*$^{flox/flox}$ mice but decreased in spleen and liver. (C, right panel, and E) Stage 2 (CD44$^+$NK1.1$^-$) Vα14*i*NKT cells were increased, but stage 3 (CD44$^+$NK1.1$^+$) cells were decreased in the thymus of *LckCrePten*$^{flox/flox}$ mice. The percentages of NKT subsets in αGalCer-CD1d$^+$TCRβ$^+$-gated thymocytes (C, right panel) and in total MNCs in thymus (E) are shown. For panels B, D, and E, results are expressed as the mean ± SEM for 3 mice per group. $^{**}P < .01$.

production. When total cells from WT thymus were stained with CD1d dimer–Ig and anti-TCRβ antibody, the Vα14*i*NKT fraction was found to be slightly activated prior to any exogenous stimulation. We therefore purified NK1.1$^+$TCRβ$^+$ cells from *Pten*$^{flox/flox}$ and *LckCrePten*$^{flox/flox}$ thymi and activated these cells using DCs that had been loaded in vitro with αGalCer. In response to this activation, NK1.1$^+$TCRβ$^+$ mature NKT cells from *Pten*$^{flox/flox}$ thymus showed high levels of proliferation (Figure 4A) as well as vigorous production of IL-4 (Figure 4B) and IFNγ (Figure 4C). In contrast, NK1.1$^+$TCRβ$^+$ mature NKT cells from *LckCrePten*$^{flox/flox}$ thymus showed severely impaired proliferation and cytokine production (Figure 4A-C). These results suggest that Pten is required not only for the normal development of NKT cells but also for their functions.

To examine cytokine production by Vα14*i*NKT cells in response to αGalCer in vivo, we intraperitoneally injected αGalCer into *Pten*$^{flox/flox}$, *Pten*$^{+/-}$, and *LckCrePten*$^{flox/flox}$ mice. Administration of αGalCer to WT mice led to a rapid increase in serum IL-4 and IFNγ levels within 2 hours and 12 hours, respectively (Figure 4D-E). It is known that Vα14*i*NKT cells are solely responsible for the production of these cytokines at these time points.[22] Accordingly, in both *Pten*$^{+/-}$ and *LckCrePten*$^{flox/flox}$ mice that received intraperitoneal injection of αGalCer, serum levels of IFNγ were severely decreased. αGalCer-treated *LckCrePten*$^{flox/flox}$ mice also showed a significant decrease in serum IL-4 whereas *Pten*$^{+/-}$ mice showed only a slight (insignificant) reduction. These data indicate that, as well as reducing their numbers, mutation of Pten functionally impairs mature Vα14*i*NKT cells both in vitro and in vivo.

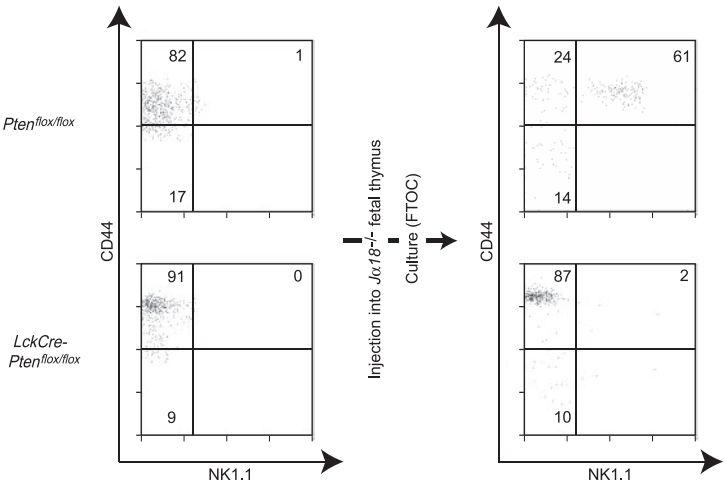

Figure 3. Failure of Pten-deficient immature Vα14*i*NKT cells to mature in vitro. CD44^{high}NK1.1$^-$ (stage 2, immature) Vα14*i*NKT cells were purified from the thymi of *Pten*$^{flox/flox}$ or *LckCrePten*$^{flox/flox}$ mice and injected into FTOCs derived from a *Jα18*$^{-/-}$ embryo. (Left) Flow cytometric analysis of cell populations injected. (Right) At 9 days after injection, Vα14*i*NKT cells in the FTOC were isolated and analyzed by flow cytometry to detect stage 3 (mature) cells. Mature Vα14*i*NKT cells were very rare in FTOCs receiving Pten-deficient stage 2 NKT cells. One result representative of 3 experiments is shown.

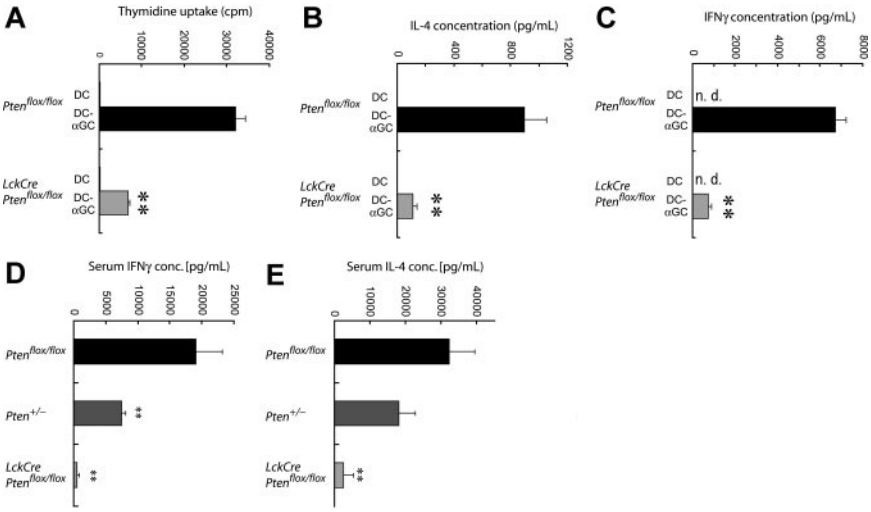

Figure 4. Impaired activation of Pten-mutant NKT cells in vitro and in vivo. (A) Impaired proliferation. NK1.1$^+$TCRβ$^+$ cells were purified from the thymi of *Pten*$^{flox/flox}$ or *LckCrePten*$^{flox/flox}$ mice and cocultured with DCs loaded with vehicle (DC) or αGalCer (DC-αGC). After 48 hours, cells were pulsed with [^{3}H]-thymidine for 12 hours and thymidine uptake was measured. (B-C) Impaired cytokine production. IL-4 and IFNγ in the supernatants of the cultures in panel A were measured by ELISA. (D-E) Impaired response of Vα14*i*NKT cells to αGalCer administration in vivo. αGalCer was intraperitoneally injected into *Pten*$^{flox/flox}$, *Pten*$^{+/-}$, or *LckCrePten*$^{flox/flox}$ mice, and serum concentrations of IL-4 (D) and IFNγ (E) were evaluated at 2 hours and 12 hours after αGalCer injection, respectively. For all panels, data are expressed as the mean value ± SEM for 3 mice per group. $^{**}P < .01$.

Pten-deficient Vα14*i*NKT cells show defective antitumor immunity

Because Vα14*i*NKT cells were abnormal in number and function in *LckCrePten*$^{flox/flox}$ mice, we investigated whether these animals had a defect in Vα14*i*NKT-mediated antitumor immunity in vivo. First, we purified αGalCer-CD1d$^+$TCRβ$^+$ (Vα14*i*NKT) cells from *Pten*$^{flox/flox}$ mice and adoptively transferred them into αGalCer-treated *Jα18*$^{-/-}$ (Vα14*i*NKT cell–deficient) recipients that had been inoculated with B16F10 melanoma cells (Figure 5A). The presence of WT Vα14*i*NKT cells markedly inhibited the metastasis of B16F10 cells to the lungs (about 60% reduction; Figure 5B-C), consistent with previous observations.[36] In contrast, only a minor inhibition of B16F10 metastasis was achieved using Vα14*i*NKT cells from αGalCer-treated *Pten*$^{+/-}$ mice (16% reduction; Figure 5C) or αGalCer-treated *LckCrePten*$^{flox/flox}$ mice (8% reduction; Figure 5B). These findings show that mutation of Pten in Vα14*i*NKT cells impairs antitumor immunity.

PI3K signaling is responsible for the phenotypes of Pten-deficient Vα14*i*NKT cells

To determine whether PI3K was activated in Vα14*i*NKT cells that interacted with αGalCer-loaded DCs, we analyzed levels of the PI3K product PIP3. Purified Vα14*i*NKT cells expressing the fusion bioprobe AktPH-GFP[32] were cultured with αGalCer-loaded DCs in

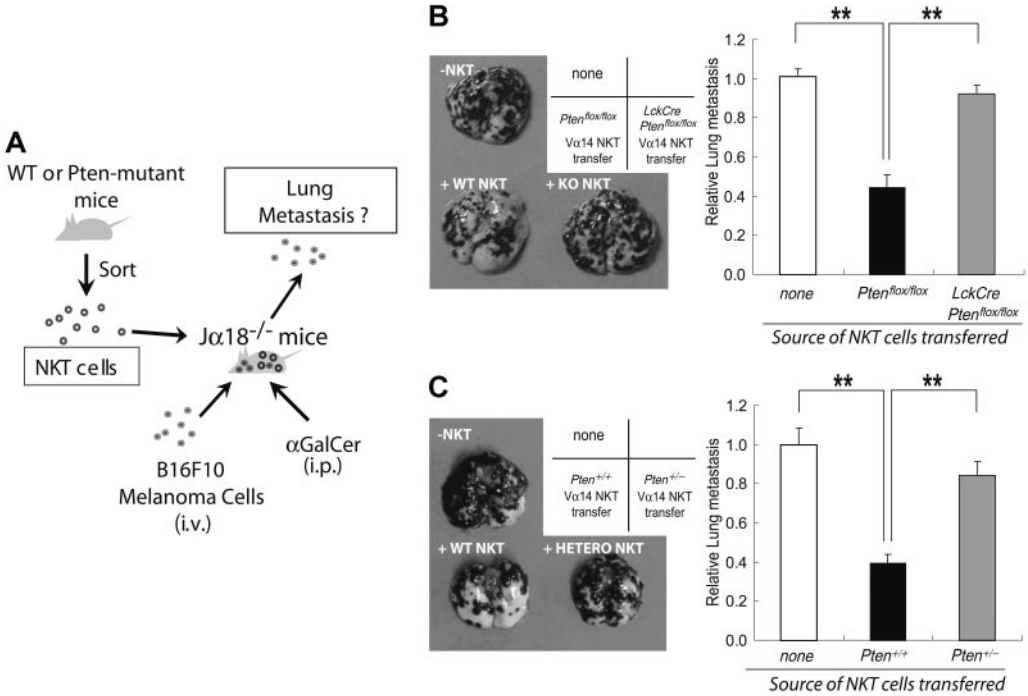

Figure 5. Defective antitumor surveillance by Pten-deficient Vα14*i*NKT cells. (A) Scheme describing the experimental protocol used to evaluate antitumor surveillance by NKT cells in vivo. Vα14*i*NKT cells were purified from mice of the indicated genotypes and adoptively transferred into *Jα18*$^{-/-}$ mice that had been inoculated with B16F10 melanoma cells. The mice were then injected with αGalCer at 0, 4, and 8 days after NKT cell transfer. After 14 days, numbers of focal lung metastases were counted. (B-C, left panels) Increased lung metastasis. Quadrant scheme in the upper corners shows the labeling of the cell images. "None" indicates controls in which no cells were transferred. Representative images of lung metastases 2 weeks after the transfer of *Pten*$^{flox/flox}$ or *LckCrePten*$^{flox/flox}$ Vα14*i*NKT cells (B) or after the transfer of *Pten*$^{+/+}$ or *Pten*$^{+/-}$ Vα14*i*NKT cells (C) are shown. (B-C, right panels) Relative numbers of the lung metastases identified in the left panels. Results shown are mean ± SEM of 18 mice that received no cells and 6 mice each that received Vα14*i*NKT cells. $^{**}P < .01$.

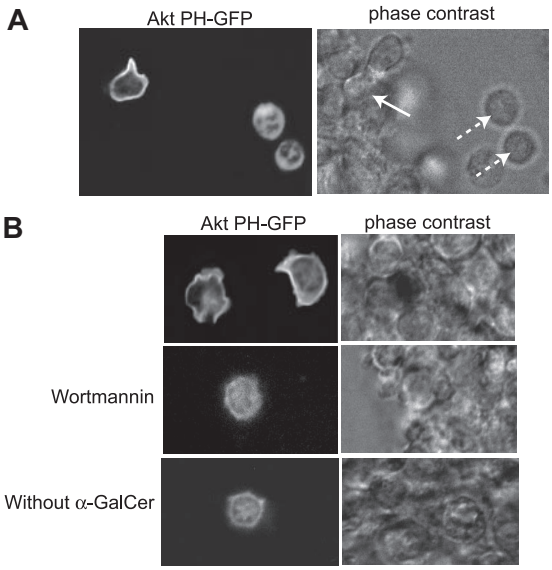

Figure 6. PI3K is activated in Vα14*i*NKT cells that interact with αGalCer-loaded DCs. Localization of PIP3 to the NKT cell membrane. TCRβ$^+$NK1.1$^+$HSA^{low} NKT cells were purified from thymocytes of 8-week-old *AktPH-GFP$^{tg/tg}$* mice (WT for Pten) expressing the PIP3-binding AktPH-GFP bioprobe I (left). These Vα14*i*NKT cells were incubated with αGalCer-loaded DCs, and PIP3 localization was determined by confocal fluorescence (left) and phase contrast (right) microscopy. (A) Vα14*i*NKT cells that made contact with αGalCer-loaded DCs showed membrane localization of PIP3 (solid arrow), while PIP3 remained in the cytoplasm of Vα14*i*NKT cells that did not make contact with DCs (dotted arrows). (B) AktPH-GFP bioprobe localization to the plasma membrane of Vα14*i*NKT cells was inhibited by wortmannin or by contact with DCs lacking αGalCer.

fibronectin-coated glass-base dishes. As shown in Figure 6A, the AktPH-GFP bioprobe was recruited to the plasma membrane of WT Vα14*i*NKT cells that interacted with αGalCer-loaded DCs (solid arrow) but remained in the cytoplasm of WT Vα14*i*NKT cells that did not make contact with the DCs (dotted arrows). In addition, the bioprobe was not recruited to the plasma membrane of WT Vα14*i*NKT cells that interacted with αGalCer-loaded DCs in the presence of wortmannin (a pan-PI3K inhibitor) or in Vα14*i*NKT cells that contacted DCs lacking αGalCer (Figure 6B). These findings suggest that PI3K is activated in Vα14*i*NKT cells when these cells are stimulated by interaction with αGalCer-loaded DCs.

The PI3K signaling pathway leads to PKB/Akt activation, an event that is negatively regulated by Pten. Accordingly, Pten deficiency usually leads to enhanced PI3K signaling and cellular hyperactivation and hyperproliferation.[26,28] Curiously, Pten deficiency in Vα14*i*NKT cells led to impaired cell numbers and functions. We therefore examined the role of PI3K in generating the phenotypes of Pten-deficient NKT cells. The PI3K subunit p110δ is highly expressed in hematopoietic cells and is the major catalytic subunit of class IA PI3Ks in T-lineage cells.[30] The PI3K subunit p110γ, which is a unique catalytic subunit responsible for class IB PI3K activation downstream of G protein–coupled receptors (GPCRs), is also highly expressed in lymphocytes and important for the normal development of T-lineage cells.[29] To determine whether PI3K signaling was responsible for the defects of Pten-deficient Vα14*i*NKT cells, we examined double-mutant mice lacking Pten and PI3K p110γ[29] *(LckCrePten$^{flox/flox}$/p110γ$^{-/-}$)* or Pten and PI3K p110δ[30] *(LckCrePten$^{flox/flox}$/p110δ$^{D910A/D910A}$)*. The mutation of p110γ partially rescued Vα14*i*NKT cell numbers in *LckCrePten$^{flox/flox}$* mice (Figure 7A, upper panel) and completely rescued the proliferation defect of Pten-deficient Vα14*i*NKT cells (Figure 7B). A slightly weaker but still significant effect was seen upon mutation of p110δ. Moreover, numbers of peripheral Pten-deficient Vα14*i*NKT cells were restored to near-normal levels in both strains of double-mutant mice (data not shown). These results indicate that excessive PI3K signaling contributes to the phenotypes of Pten-deficient Vα14*i*NKT cells.

Enhanced expression of inhibitory Ly49 receptors by Pten-deficient Vα14*i*NKT cells

Interaction between MHC class I and inhibitory Ly49 receptors (such as Ly49A and LY49C/I) can negatively regulate the responses of T cells to alloantigens,[37] suggesting that signaling via the TCR can be regulated by a Ly49-dependent mechanism. With respect to NKT cells, NKT functions are impaired upon inhibitory

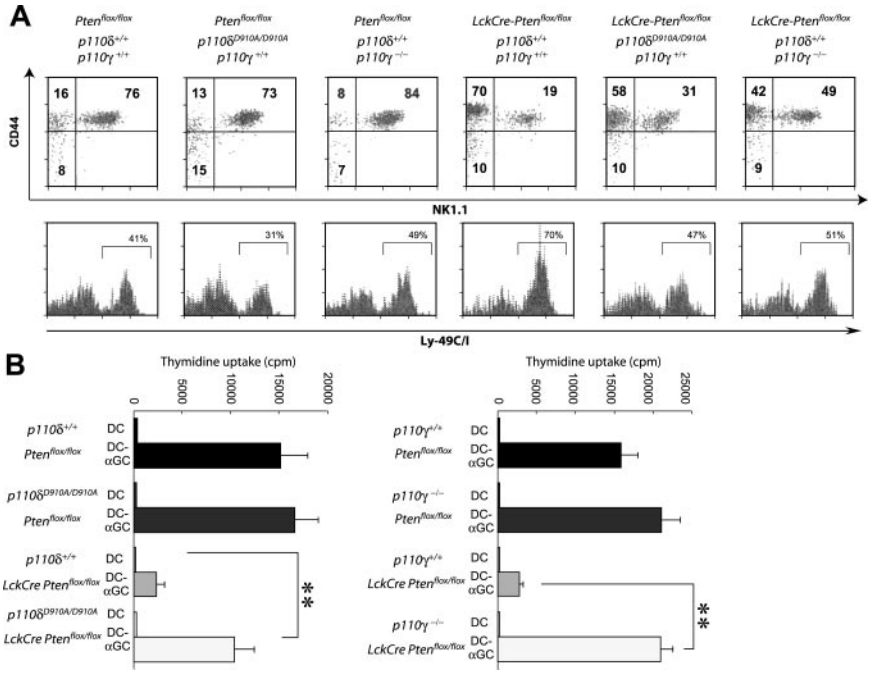

Figure 7. The phenotypes observed in Pten-deficient NKT cells are PI3K dependent. (A, top) Decreased maturation. Thymic Vα14*i*NKT cells from mice of the indicated genotypes were analyzed by flow cytometry for percentages of stage 3 cells. (A, bottom) Increased expression of Ly49C/I receptors. The αGalCer-CD1d$^+$TCRβ$^+$NK1.1$^+$ cells in the top panel were analyzed by flow cytometry to determine relative expression levels of Ly49C/I. (B) Impaired proliferation. The NK1.1$^+$TCRβ$^+$ cells from panel A were stimulated by incubation with αGalCer-loaded DCs, and [^{3}H]-thymidine incorporation was measured. Data shown are the mean ± SEM for 3 mice per group. $^{**}P < .01$. For both panels, the defects of Pten-deficient Vα14*i*NKT cells were partially rescued by mutation of a PI3K subunit (p110γ or p110δ).

Ly49–MHC class I interaction,[38] and mice transgenic for Ly49A show impaired NKT cell maturation and selection.[39] Thus, Ly49-mediated regulation is important for the development and functions of NKT cells, and this regulation may involve modulation of the strength of TCR signaling. These considerations led us to examine the expression of inhibitory Ly49 receptors by our Pten-deficient Vα14*i*NKT cells. Specifically, we determined the expression of Ly49C/I (which recognizes MHC class I, H-2K^b, H-2D^d) and Ly49A (which recognizes MHC class I, H-2D^d) by thymic Vα14*i*NKT cells of *LckCrePten$^{flox/flox}$* mice and the double mutants. As expected, the proportion of αGalCer-CD1d$^+$TCRβ$^+$NK1.1$^+$ thymocytes showing strong expression of Ly49C/I (Figure 7A, bottom) and Ly49A (Figure S2) was increased in *LckCrePten$^{flox/flox}$* mice compared with *Pten$^{flox/flox}$* mice. At least for Ly49C/I, this increased expression was mitigated by the presence of the *p110δ$^{D910A/D910}$* or *p110γ$^{-/-}$* mutation. There were no differences between *LckCrePten$^{flox/flox}$* mice and *Pten$^{flox/flox}$* mice in the H2-K^b levels expressed by thymic or splenic DCs (Figure S1C). Thus, PI3K signaling is responsible for the altered inhibitory Ly49 receptor repertoire of Pten-deficient thymic Vα14*i*NKT cells. Taken together, our results suggest that the reduced number and functions of Pten-deficient Vα14*i*NKT cells may be linked to their increased expression of inhibitory Ly49 receptors.

Discussion

Many studies in mouse models have demonstrated that Vα14*i*NKT cells are activated in a broad range of disorders, including autoimmune diseases, microbial infections, graft rejection, and cancer progression/metastasis.[5,19-21] However, little is known about intracellular signal transduction in NKT cells, particularly the role of the Pten/PI3K signaling pathway that is crucial in so many other cell types. Our results are the first to demonstrate that mutation of Pten in murine Vα14*i*NKT cells impairs the development and functions of these cells and that these defects depend on PI3K activity. An in vivo consequence of the defective Vα14*i*NKT compartment is enhanced tumor metastasis.

The study of genetically modified mice has begun to yield important insights into the mechanisms controlling the maturation of Vα14*i*NKT cells. To date, investigations of diverse gene-deficient mice have revealed 3 general categories of Vα14*i*NKT cell abnormalities.[40] In the first category (group 1), the mutant mice show a total absence or a severe reduction in numbers of both immature (stage 2) and mature (stage 3) Vα14*i*NKT cells. In the second category (group 2), the mutant animals exhibit a block in Vα14*i*NKT cell development between stage 2 and stage 3 such that stage 2 cells are increased while stage 3 cells are decreased. In the third category (group 3), numbers of stage 3 Vα14*i*NKT cells are decreased but there is no increase in stage 2 cell numbers. Knockout mice lacking CD1d, Fyn, SAP, IL-7, IKK2, NIK, or RelB all have the group 1 phenotype, indicating that these molecules are essential for the initial development of Vα14*i*NKT cells from DP precursors.[40,41] Mice lacking T-bet[42] or Itk[43] are members of group 2. These molecules do not affect the earliest stages of NKT differentiation from DP precursors but are critical for the continued maturation of stage 2 Vα14*i*NKT cells. Mice deficient for IL-15 or transgenic for mIkBα fall into group 3 because these molecules are important for the survival of mature NKT cells.[40,44] While the phenotypes observed in the Vα14*i*NKT cells of T-bet– or Itk-deficient mice are strikingly similar to those we observed for Vα14*i*NKT cells of *LckCrePten$^{flox/flox}$* mice, there are some fundamental differences. First, although the phenotypes observed in T-bet–deficient mice can be explained by a failure to express the IL-2R/IL-15R common β subunit (CD122),[42] the proliferative response to IL-15 by mature NKT cells of *LckCrePten$^{flox/flox}$* mice was normal (Figure S3). Second, Itk-deficient Vα14*i*NKT cells show reduced expression of the inhibitory NK receptors Ly49C/I and intact IFNγ secretion,[43] but Pten-deficient Vα14*i*NKT cells exhibit enhanced Ly49C/I and Ly49A expression and reduced IFNγ secretion. In addition, because Itk is activated by Pten deficiency,[45] reduction of Itk signaling is unlikely to have occurred in our *LckCrePten$^{flox/flox}$* mice. For these reasons, we believe that the phenotypes observed in Vα14*i*NKT cells of *LckCrePten$^{flox/flox}$* mice cannot be explained by effects attributable to altered T-bet or Itk function. Finally, in all the previously reported mutant mice, the molecules deleted by the gene targeting are known to activate cells. No other mutant mouse to date features the targeting of an intracellular signaling molecule that negatively regulates cellular activation in Vα14*i*NKT cells.

Ly49 is a multigene receptor family expressed mainly by NK cells and stage 3 mature Vα14*i*NKT cells.[44] Ly49A, Ly49C/I, and Ly49G2 are inhibitory NK receptors, while Ly49D and Ly49H are activatory NK receptors. Both groups of receptors interact with proteins encoded by specific MHC class I alleles.[46] It has been clearly shown that the interaction between Ly49A and H-2D^d and between Ly49C and H-2K^b or H-2D^d negatively regulates NK effector functions such as cytotoxicity and bone marrow graft rejection.[47,48] Similarly, the appropriate inhibitory Ly49–MHC class I interactions can negatively regulate the maturation, proliferation, and functions of NKT cells.[37,38,39] In our study, we found that most αGalCer-CD1d$^+$TCRβ$^+$NK1.1$^+$ thymocytes in *LckCrePten$^{flox/flox}$* mice showed an enhancement of Ly49C/I expression that was PIP3 dependent. The continuous accumulation of PIP3 that resulted from the Pten deficiency in our mutants may have up-regulated the expression of inhibitory Ly49 receptors. Thus, the phenotypes observed in Pten-deficient Vα14*i*NKT cells can plausibly be explained by their enhanced expression of inhibitory Ly49 receptors.

Several lines of evidence suggest that developing Vα14*i*NKT cells undergo negative selection if they encounter high-avidity antigen or abundant self-antigen. First, the addition of αGalCer to early-stage FTOC prevents the development of Vα14*i*NKT cells in a dose-dependent manner.[14,16] Second, overexpression of CD1d on DCs or thymocytes in transgenic mice results in a reduced number of Vα14*i*NKT cells that exhibit both altered Vβ repertoire usage and reduced sensitivity to antigen.[12,14] Third, in mice doubly transgenic for the activatory Ly49D receptor and its ligand H2-D^d, Vα14*i*NKT cells are absent from the thymus and periphery due to developmental arrest between stage 2 and stage 3. The addition of the inhibitory Ly49A transgene to Ly49D/H2-D^d transgenic mice resulted in a partial rescue of NKT cell development.[15] A possible interpretation of these findings is that Vα14*i*NKT cells with self-recognizing Ly49 inhibitory receptors preferentially survive as a result of decreased negative selection mediated by activatory Ly49. It may be that, as well as TCR avidity, an appropriate balance of signals delivered by activatory and inhibitory Ly49 receptors is required to set the affinity threshold and regulate proper Vα14*i*NKT cell development. Pten-deficient Vα14*i*NKT cells exposed to endogenous glycolipid antigens may transmit supraphysiological levels of signaling which, in turn, may drive enhanced negative selection of Vα14*i*NKT cells. The deficit of Vα14*i*NKT cells in the peripheral organs and thymus may then be partially restored by repopulation of the mature Vα14*i*NKT compartment with cells

showing increased expression of inhibitory Ly49 receptors, altered Vβ usage, and reduced sensitivity to antigen.[14]

Pre-TCR signaling in T cells of PI3K p110$\delta^{-/-}$/p110$\gamma^{-/-}$ double-mutant mice is greatly decreased compared with signaling in T cells of PI3K$\delta^{-/-}$ or $\gamma^{-/-}$ single-mutant animals.[49] In addition, T-cell responses induced by TCR stimulation are much weaker in p110$\delta^{-/-}$[50] and p110$\gamma^{-/-}$[29] mice than in WT controls. In our study, we showed that the phenotypes observed in Pten-deficient NKT cells could be partially rescued by mutation of p110γ. These results suggest that TCR signaling may directly activate both PI3Kδ and PI3Kγ in Vα14*i*NKT cells. Alternatively, inhibitory Ly49 receptors may directly affect both PI3Kδ and PI3Kγ, which may eventually result in modulated TCR signaling. In support of the latter possibility, there is evidence that SHIP1 (another PIP3 phosphatase) as well as the p85 subunit of PI3K can both be recruited to Ly49A or Ly49C.[51]

The molecular mechanisms underlying the impaired function of Vα14*i*NKT cells in patients with autoimmune disease or cancer remain to be determined. Our work suggests that the continuous activation of the PI3K pathway in murine *LckCrePten*$^{flox/flox}$ Vα14*i*NKT cells (due to decreased Pten function) may alter the negative selection, development, function, and tolerance of these cells. In humans, a parallel scenario could lead to the onset of these diseases. It is well known that αGalCer has antitumor effects against cancer cells of various origins and that this protection depends on an initial burst of IFNγ production by NKT cells and subsequent ongoing IFNγ production by NK cells.[36] IFNγ itself is not directly toxic to tumor cells, but the IFNγ produced by NKT cells is critical for the recruitment and proliferation of NK cells (and other leukocytes) that are cytolytic to tumor cells. In addition, IFNγ has potent antiangiogenic activity[52] and induces other leukocytes to produce IFNγ-inducible factors such as IFN-inducible protein 10 (IP-10) and monokine induced by IFNγ.[53] These cytokines may indirectly inhibit tumor neovascularization and thus induce tumor hypoxia. We have demonstrated that Pten-deficient Vα14*i*NKT cells fail to produce IFNγ both in vitro and in vivo, most likely accounting for the impaired antitumor surveillance observed in *LckCrePten*$^{flox/flox}$ mice.

Our study is the first to show that engagement of the TCRs of murine Vα14*i*NKT cells by αGalCer-loaded DCs leads to the activation of PI3K in the NKT cells. We have also demonstrated that the loss of Pten or activation of PI3K signaling in Vα14*i*NKT cells leads to a failure in their development. The few mature Vα14*i*NKT cells that survive are tolerant to αGalCer, resulting in defective antitumor surveillance and accelerated metastasis. Mice heterozygous for the Pten mutation *(Pten*$^{+/-}$*)* showed a significant reduction in serum IFNγ production after administration of αGalCer in vivo. In addition, adoptive transfer of *Pten*$^{+/-}$ Vα14*i*NKT cells into αGalCer-treated, B16F10 melanoma cell–inoculated *Jα18*$^{-/-}$ recipients impaired the inhibition of B16F10 melanoma metastasis to the lungs. We speculate that an identical mechanism may be operating in humans with Cowden disease, a hereditary syndrome of cancer susceptibility caused by heterozygous mutations of *PTEN:* Namely, an individual who inherits a mutated *PTEN* allele is not only at risk for additional tumorigenic mutations due to loss of heterozygosity (LOH) of the *PTEN* gene but may also experience accelerated growth of any incipient tumors due to impaired NKT-mediated antitumor surveillance.

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Authorship

Conflict-of-interest disclosure: The authors declare no competing financial interests.

H.K. and T.O. contributed equally to this work.

Correspondence: Akira Suzuki, Hondo 1-1-1, Akita, 0108543 Japan; e-mail: suzuki@med.akita-u.ac.jp.

References

1. Imai K, Kanno M, Kimoto H, Shigemoto K, Yamamoto S, Taniguchi M. Sequence and expression of transcripts of the T-cell antigen receptor alpha-chain gene in a functional, antigen-specific suppressor-T-cell hybridoma. Proc Natl Acad Sci U S A. 1986;83:8708-8712.
2. Budd RC, Miescher GC, Howe RC, Lees RK, Bron C, MacDonald HR. Developmentally regulated expression of T cell receptor beta chain variable domains in immature thymocytes. J Exp Med. 1987;166:577-582.
3. Fowlkes BJ, Kruisbeek AM, Ton-That H, et al. A novel population of T-cell receptor alpha beta-bearing thymocytes which predominantly expresses a single V beta gene family. Nature. 1987;329:251-254.
4. Taniguchi M, Koseki H, Tokuhisa T, et al. Essential requirement of an invariant V alpha 14 T cell antigen receptor expression in the development of natural killer T cells. Proc Natl Acad Sci U S A. 1996;93:11025-11028.
5. Kronenberg M, Gapin L. The unconventional lifestyle of NKT cells. Nat Rev Immunol. 2002;2:557-568.
6. Matsuda JL, Naidenko OV, Gapin L, et al. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. J Exp Med. 2000;192:741-754.
7. Schumann J, Voyle RB, Wei BY, MacDonald HR. Cutting edge: influence of the TCR V beta domain on the avidity of CD1d:alpha-galactosylceramide binding by invariant V alpha 14 NKT cells. J Immunol. 2003;170:5815-5819.
8. Bendelac A. Positive selection of mouse NK1+ T cells by CD1-expressing cortical thymocytes. J Exp Med. 1995;182:2091-2096.
9. Coles MC, Raulet DH. NK1.1+ T cells in the liver arise in the thymus and are selected by interactions with class I molecules on CD4+CD8+ cells. J Immunol. 2000;164:2412-2418.
10. Benlagha K, Kyin T, Beavis A, Teyton L, Bendelac A. A thymic precursor to the NK T cell lineage. Science. 2002;296:553-555.
11. Pellicci DG, Hammond KJ, Uldrich AP, Baxter AG, Smyth MJ, Godfrey DI. A natural killer T (NKT) cell developmental pathway involving a thymus-dependent NK1.1(-)CD4(+) CD1d-dependent precursor stage. J Exp Med. 2002;195:835-844.
12. Schumann J, Pittoni P, Tonti E, Macdonald HR, Dellabona P, Casorati G. Targeted expression of human CD1d in transgenic mice reveals independent roles for thymocytes and thymic APCs in positive and negative selection of Valpha14i NKT cells. J Immunol. 2005;175:7303-7310.
13. Xu H, Chun T, Colmone A, Nguyen H, Wang CR. Expression of CD1d under the control of a MHC class Ia promoter skews the development of NKT cells, but not CD8+ T cells. J Immunol. 2003;171:4105-4112.
14. Chun T, Page MJ, Gapin L, et al. CD1d-expressing dendritic cells but not thymic epithelial cells can mediate negative selection of NKT cells. J Exp Med. 2003;197:907-918.
15. Voyle RB, Beermann F, Lees RK, et al. Ligand-dependent inhibition of CD1d-restricted NKT cell development in mice transgenic for the activating receptor Ly49D. J Exp Med. 2003;197:919-925.
16. Pellicci DG, Uldrich AP, Kyparissoudis K, et al. Intrathymic NKT cell development is blocked by the presence of alpha-galactosylceramide. Eur J Immunol. 2003;33:1816-1823.
17. Kawano T, Cui J, Koezuka Y, et al. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. Science. 1997;278:1626-1629.
18. Burdin N, Brossay L, Koezuka Y, et al. Selective

ability of mouse CD1 to present glycolipids: alpha-galactosylceramide specifically stimulates V alpha 14+ NK T lymphocytes. J Immunol. 1998; 161:3271-3281.

19. Taniguchi M, Harada M, Kojo S, Nakayama T, Wakao H. The regulatory role of Valpha14 NKT cells in innate and acquired immune response. Annu Rev Immunol. 2003;21:483-513.
20. Godfrey DI, Kronenberg M. Going both ways: immune regulation via CD1d-dependent NKT cells. J Clin Invest. 2004;114:1379-1388.
21. Cui J, Shin T, Kawano T, et al. Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. Science. 1997;278:1623-1626.
22. Elewaut D, Kronenberg M. Molecular biology of NK T cell specificity and development. Semin Immunol. 2000;12:561-568.
23. Li J, Yen C, Liaw D, et al. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science. 1997;275:1943-1947.
24. Liaw D, Marsh DJ, Li J, et al. Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nat Genet. 1997;16:64-67.
25. Maehama T, Dixon JE. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem. 1998;273:13375-13378.
26. Vanhaesebroeck B, Ali K, Bilancio A, Geering B, Foukas LC. Signalling by PI3K isoforms: insights from gene-targeted mice. Trends Biochem Sci. 2005;30:194-204.
27. Stambolic V, Suzuki A, de la Pompa JL, et al. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell. 1998;95:29-39.
28. Suzuki A, Yamaguchi MT, Ohteki T, et al. T cell-specific loss of Pten leads to defects in central and peripheral tolerance. Immunity. 2001;14:523-534.
29. Sasaki T, Irie-Sasaki J, Jones RG, et al. Function of PI3Kgamma in thymocyte development, T cell activation, and neutrophil migration. Science. 2000;287:1040-1046.
30. Okkenhaug K, Bilancio A, Farjot G, et al. Impaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice. Science. 2002;297:1031-1034.
31. Suzuki A, de la Pompa JL, Stambolic V, et al. High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice. Curr Biol. 1998;8:1169-1178.
32. Nishio M, Watanabe K, Sasaki J, et al. Control of cell polarity and motility by the PtdIns(3,4,5)P(3) phosphatase SHIP1. Nat Cell Biol. 2007;9:36-44.
33. Ohteki T, MacDonald HR. Major histocompatibility complex class I related molecules control the development of CD4+8- and CD4–8- subsets of natural killer 1.1+ T cell receptor-alpha/beta+ cells in the liver of mice. J Exp Med. 1994;180: 699-704.
34. Hamada K, Sasaki T, Koni PA, et al. The PTEN/PI3K pathway governs normal vascular development and tumor angiogenesis. Genes Dev. 2005; 19:2054-2065.
35. Ohteki T, Suzue K, Maki C, Ota T, Koyasu S. Critical role of IL-15-IL-15R for antigen-presenting cell functions in the innate immune response. Nat Immunol. 2001;2:1138-1143.
36. Smyth MJ, Crowe NY, Pellicci DG, et al. Sequential production of interferon-gamma by NK1.1(+) T cells and natural killer cells is essential for the antimetastatic effect of alpha-galactosylceramide. Blood. 2002;99:1259-1266.
37. Held W, Cado D, Raulet DH. Transgenic expression of the Ly49A natural killer cell receptor confers class I major histocompatibility complex (MHC)-specific inhibition and prevents bone marrow allograft rejection. J Exp Med. 1996;184: 2037-2041.
38. Ortaldo JR, Winkler-Pickett R, Mason AT, Mason LH. The Ly-49 family: regulation of cytotoxicity and cytokine production in murine CD3+ cells. J Immunol. 1998;160:1158-1165.
39. Robson MacDonald H, Lees RK, Held W. Developmentally regulated extinction of Ly-49 receptor expression permits maturation and selection of NK1.1+ T cells. J Exp Med. 1998;187:2109-2114.
40. Matsuda JL, Gapin L. Developmental program of mouse Valpha14i NKT cells. Curr Opin Immunol. 2005;17:122-130.
41. Pasquier B, Yin L, Fondaneche MC, et al. Defective NKT cell development in mice and humans lacking the adapter SAP, the X-linked lymphoproliferative syndrome gene product. J Exp Med. 2005;201:695-701.
42. Townsend MJ, Weinmann AS, Matsuda JL, et al. T-bet regulates the terminal maturation and homeostasis of NK and Valpha14i NKT cells. Immunity. 2004;20:477-494.
43. Gadue P, Stein PL. NK T cell precursors exhibit differential cytokine regulation and require Itk for efficient maturation. J Immunol. 2002;169:2397-2406.
44. Stanic AK, Bezbradica JS, Park JJ, et al. NF-kappa B controls cell fate specification, survival, and molecular differentiation of immunoregulatory natural T lymphocytes. J Immunol. 2004;172: 2265-2273.
45. Shan X, Czar MJ, Bunnell SC, et al. Deficiency of PTEN in Jurkat T cells causes constitutive localization of Itk to the plasma membrane and hyperresponsiveness to CD3 stimulation. Mol Cell Biol. 2000;20:6945-6957.
46. Yokoyama WM, Seaman WE. The Ly-49 and NKR-P1 gene families encoding lectin-like receptors on natural killer cells: the NK gene complex. Annu Rev Immunol. 1993;11:613-635.
47. Karlhofer FM, Ribaudo RK, Yokoyama WM. MHC class I alloantigen specificity of Ly-49+ IL-2-activated natural killer cells. Nature. 1992;358:66-70.
48. Yu YY, George T, Dorfman JR, Roland J, Kumar V, Bennett M. The role of Ly49A and 5E6(Ly49C) molecules in hybrid resistance mediated by murine natural killer cells against normal T cell blasts. Immunity. 1996;4:67-76.
49. Webb LM, Vigorito E, Wymann MP, Hirsch E, Turner M. Cutting edge: T cell development requires the combined activities of the p110gamma and p110delta catalytic isoforms of phosphatidylinositol 3-kinase. J Immunol. 2005;175:2783-2787.
50. Okkenhaug K, Patton DT, Bilancio A, Garcon F, Rowan WC, Vanhaesebroeck B. The p110delta isoform of phosphoinositide 3-kinase controls clonal expansion and differentiation of Th cells. J Immunol. 2006;177:5122-5128.
51. Wang JW, Howson JM, Ghansah T, et al. Influence of SHIP on the NK repertoire and allogeneic bone marrow transplantation. Science. 2002;295: 2094-2097.
52. Ruegg C, Yilmaz A, Bieler G, Bamat J, Chaubert P, Lejeune FJ. Evidence for the involvement of endothelial cell integrin alphaVbeta3 in the disruption of the tumor vasculature induced by TNF and IFN-gamma. Nat Med. 1998;4:408-414.
53. Farber JM. Mig and IP-10: CXC chemokines that target lymphocytes. J Leukoc Biol. 1997;61:246-257.