

# The Pten/PI3K pathway governs the homeostasis of V $\alpha$ 14iNKT cells

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**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 V $\alpha$ 14iNKT cells. A failure in the development of V $\alpha$ 14iNKT cells occurs in the *LckCrePten* thymus between stage 2 (CD44<sup>high</sup>NK1.1<sup>-</sup>) and stage 3 (CD44<sup>high</sup>NK1.1<sup>+</sup>), resulting in decreased numbers of peripheral V $\alpha$ 14iNKT cells. In vitro, Pten-deficient**

**V $\alpha$ 14iNKT cells show reduced proliferation and cytokine secretion in response to  $\alpha$ GalCer stimulation but enhanced inhibitory Ly49 receptor expression. Following interaction with dendritic cells (DCs) loaded with  $\alpha$ GalCer, Pten-deficient V $\alpha$ 14iNKT cells demonstrate activation of PI3K. Indeed, the effects of the Pten mutation require intact function of the PI3K subunits p110 $\gamma$  and p110 $\delta$ . In vivo, *LckCrePten* mice display reduced serum IFN $\gamma$  after  $\alpha$ GalCer administration.**

**Importantly, V $\alpha$ 14iNKT 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 V $\alpha$ 14iNKT cells. (Blood. 2007; 109:3316-3324)**

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## Introduction

V $\alpha$ 14iNKT cells, originally defined as NK1.1<sup>+</sup> T cells,<sup>1-3</sup> 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  $\alpha$  (TCR $\alpha$ ) chain, NK1.1, the IL-2/IL-15R $\beta$  chain (CD122), and Ly49. The TCR $\alpha$  chain expressed by V $\alpha$ 14iNKT cells is encoded by the V $\alpha$ 14 and J $\alpha$ 18 gene segments and is usually associated with a TCR $\beta$  chain containing the V $\beta$ 8 gene segment, a combination not expressed by conventional T cells.<sup>4</sup> V $\alpha$ 14iNKT cells constitute more than half of the NK1.1<sup>+</sup> T-cell population in the murine spleen and most NK1.1<sup>+</sup> T cells in the thymus and liver.<sup>5</sup> V $\alpha$ 14iNKT cells respond to glycolipid antigens such as the marine sponge-derived glycolipid  $\alpha$ -galactosylceramide ( $\alpha$ GalCer).  $\alpha$ GalCer 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, V $\alpha$ 14iNKT cells can be readily identified by staining with CD1d tetramers or dimers loaded with  $\alpha$ GalCer.<sup>6,7</sup>

During their development in the thymus, V $\alpha$ 14iNKT precursors at the CD4<sup>+</sup>CD8<sup>+</sup> (double positive [DP]) stage are positively selected by CD1d-mediated presentation of self-glycolipids (such as iGb3) by CD4<sup>+</sup>CD8<sup>+</sup> thymocytes.<sup>8,9</sup> V $\alpha$ 14iNKT precursors (CD44<sup>low</sup>NK1.1<sup>-</sup>; stage 1) then develop sequentially into immature NKT cells (CD44<sup>high</sup>NK1.1<sup>-</sup>; stage 2) and finally into mature V $\alpha$ 14iNKT cells (CD44<sup>high</sup>NK1.1<sup>+</sup>; stage 3).<sup>10,11</sup> V $\alpha$ 14iNKT 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.<sup>12-16</sup>

In response to TCR ligation, mature peripheral V $\alpha$ 14iNKT cells promptly produce large amounts of Th1 cytokines (IFN $\gamma$ , TNF) as well as Th2 cytokines (IL-4, IL-10, IL-13).<sup>17,18</sup> V $\alpha$ 14iNKT 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.<sup>5,19-21</sup> Indeed, mice deficient in V $\alpha$ 14iNKT cells show impaired tumor rejection.<sup>21</sup> This observation is consistent with the antitumor effect of  $\alpha$ GalCer administration on various tumor types, including melanomas and lung, colon, and hematopoietic cancers.<sup>22</sup> Despite the importance of V $\alpha$ 14iNKT cells, little is known about their intracellular signaling pathways or how these pathways might be regulated.

*PTEN* is a tumor suppressor gene<sup>23</sup> that is mutated in many human sporadic cancers and in hereditary tumor susceptibility disorders such as Cowden disease.<sup>24</sup> *PTEN* is a multifunctional phosphatase whose major substrate is phosphatidylinositol-3,4,5-triphosphate (PIP3),<sup>25</sup> 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: PI3K $\alpha$ ,  $\beta$ ,  $\delta$ , and  $\gamma$ .<sup>26</sup> 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.<sup>27</sup> 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.<sup>28</sup> Conversely, mice deficient for the PI3K subunit p110 $\gamma$  show impaired T-cell development and activation,<sup>29</sup> and T cells from animals with a kinase-inactivating mutation (D910A) of p110 $\delta$  have a defect in TCR-stimulated proliferation.<sup>30</sup> Analyses of these and other genetically modified mice have begun to provide insights into the pathways and molecules mediating intracellular signaling in V $\alpha$ 14iNKT 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 V $\alpha$ 14iNKT cells results in developmental failure leading to increased numbers of immature V $\alpha$ 14iNKT cells and decreased numbers of mature V $\alpha$ 14iNKT 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 V $\alpha$ 14iNKT cells and their antitumor surveillance function.

## Materials and methods

### Mice

*LckCrePten<sup>fllox/fllox</sup>*,<sup>28</sup> *Pten<sup>+/-</sup>*,<sup>31</sup> *PI3K p110 $\gamma$ <sup>-/-</sup>*,<sup>29</sup> *PI3K p110 $\delta$ <sup>D910A/D910A</sup>*,<sup>30</sup> *J $\alpha$ 18<sup>-/-</sup>*,<sup>21</sup> and *Akt<sup>PH-GFP<sup>tg/tg</sup></sup>* transgenic<sup>32</sup> mice (all C57BL/6J background, H-2<sup>b</sup>) have been previously described. Because *LckCrePten<sup>fllox/fllox</sup>* 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<sup>+/+</sup>* and *Pten<sup>fllox/fllox</sup>* mice<sup>28</sup> were indistinguishable in pilot experiments examining flow cytometry, cell proliferation, and cytokine production (data not shown), so that *Pten<sup>fllox/fllox</sup>* 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  $\alpha$ GalCer (KRN7000) was provided by the Kirin Brewery (Gumma, Japan). Antibodies to TCR $\beta$ -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 TCR $\beta$ -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.<sup>33</sup> For routine staining of V $\alpha$ 14iNKT cells, MNCs were first preincubated with CD16/32 mAb to prevent nonspecific binding of antibodies to Fc $\gamma$ R. The cells were then incubated with CD1d dimmer-Ig ( $\alpha$ GalCer-CD1d)<sup>7</sup> followed by anti-mouse IgG1-biotin, PE-Cy5-streptavidin, and anti-TCR $\beta$ -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-TCR $\beta$ -APC, CD1d dimmer-Ig ( $\alpha$ GalCer-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 TCR $\beta$ <sup>+</sup>NK1.1<sup>+</sup>HSA<sup>low</sup> thymocytes of 8-week-old *Pten<sup>fllox/fllox</sup>* and *LckCrePten<sup>fllox/fllox</sup>* mice were amplified by polymerase chain reaction (PCR) as described.<sup>34</sup> Sense primer (5'-GTGAAAGTGGCCCAACATAAGG-3') and antisense primer (5'-CTCCACCAATGAACAAACAGT-3') were used to detect the WT and floxed *Pten* alleles; sense primer (5'-GGCCTAGGACTCACTAGATAGC-3') and antisense primer (5'-CTCCACCAATGAACAAACAGT-3') were used to detect the deleted *Pten $\Delta$*  allele. Amplified fragments of 514 bp (*Pten<sup>fllox</sup>* allele) and 705 bp (*Pten $\Delta$*  allele) were obtained.

Total lysates (10  $\mu$ g) of sorted TCR $\beta$ <sup>+</sup>NK1.1<sup>+</sup>HSA<sup>low</sup> thymocytes from 8-week-old *Pten<sup>fllox/fllox</sup>* and *LckCrePten<sup>fllox/fllox</sup>* 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<sup>fllox/fllox</sup>* or *Pten<sup>fllox/fllox</sup>* mice, and NK1.1<sup>+</sup>TCR $\beta$ <sup>+</sup>HSA<sup>low</sup> NKT cells were purified by cell sorting.  $\alpha$ GalCer-loaded dendritic cells (DCs) were prepared from spleens as described.<sup>35</sup> 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  $\alpha$ GalCer. Nonadherent CD11c<sup>+</sup> cells were plated at 2  $\times$  10<sup>4</sup> per well in a 96-well plate and  $\gamma$ -irradiated (30 Gy). These cells were used as the  $\alpha$ GalCer-loaded DCs. Purified NKT cells (1  $\times$  10<sup>4</sup> per well) were incubated with  $\alpha$ GalCer-loaded DCs in 96-well plates in RPMI 1640 medium containing 10% FCS. The  $\alpha$ GalCer-stimulated NKT cells were harvested on day 2, and their proliferation was assayed by pulsing for 12 hours with 0.5  $\mu$ Ci (0.0185 MBq) [<sup>3</sup>H]-thymidine (Amersham, Arlington Heights, IL) per well and measuring [<sup>3</sup>H]-thymidine incorporation by standard methods. Culture supernatants were assayed for the production of IFN $\gamma$  and IL-4 by ELISA (Pierce Endogen, Rockford, IL).

For in vivo activation of NKT cells,  $\alpha$ GalCer (2  $\mu$ g) was intraperitoneally injected into 8-week-old *Pten<sup>fllox/fllox</sup>*, *Pten<sup>+/-</sup>*, and *LckCrePten<sup>fllox/fllox</sup>* mice. Serum IL-4 and IFN $\gamma$  levels were examined by enzyme-linked immunosorbent assay (ELISA) at 2 hours and 12 hours after  $\alpha$ GalCer injection, respectively.

### NKT cell maturation in FTOC

Fetal thymus organ culture (FTOC) was performed as described<sup>11</sup> with some modifications. Fetal thymus lobes from embryonic day 14.5 *J $\alpha$ 18<sup>-/-</sup>* 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  $\beta$ -mercaptoethanol (all from Invitrogen, Carlsbad, CA). Purified CD44<sup>high</sup>NK1.1<sup>-</sup> $\alpha$ GalCer-CD1d<sup>+</sup>TCR $\beta$ <sup>+</sup> immature V $\alpha$ 14iNKT cells (1  $\times$  10<sup>5</sup>) isolated from the thymus of a 3-week-old *LckCrePten<sup>fllox/fllox</sup>* or *Pten<sup>fllox/fllox</sup>* mouse were microinjected into each thymic lobe of a *J $\alpha$ 18<sup>-/-</sup>* 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. *J $\alpha$ 18<sup>-/-</sup>* mice (8 weeks old) were intravenously inoculated with B16F10 cells (4  $\times$  10<sup>5</sup>). After 3 hours, these mice were intravenously injected with sorted  $\alpha$ GalCer-CD1d<sup>+</sup>TCR $\beta$ <sup>+</sup> thymocytes (1  $\times$  10<sup>6</sup>) from 8-week-old *Pten<sup>fllox/fllox</sup>*, *LckCrePten<sup>fllox/+</sup>*, or *LckCrePten<sup>fllox/fllox</sup>* mice. After 30 minutes, 2  $\mu$ g  $\alpha$ GalCer was intraperitoneally injected into the mice. On days 4 and 8 after cell transfer, the mice received an additional 2  $\mu$ g  $\alpha$ GalCer. 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  $\pm$  SEM.

### Confocal video microscopy

TCR $\beta$ <sup>+</sup>NK1.1<sup>+</sup>HSA<sup>low</sup> NKT cells ( $1 \times 10^4$ ) purified from thymocytes of 8-week-old *AktPH-GFP<sup>tg</sup>* mice and DCs ( $1 \times 10^5$ ) either loaded or not with  $\alpha$ GalCer 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<sup>32</sup> 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 $\times$ /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.

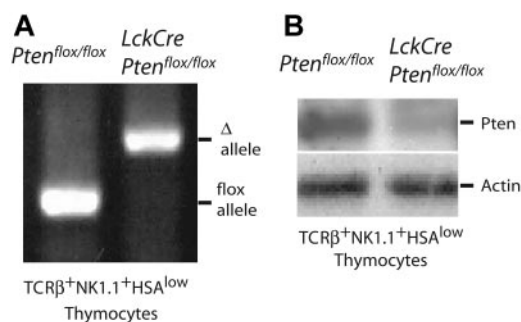
## Results

### Pten deficiency in NKT cells of *LckCrePten<sup>flox/flox</sup>* mice

We previously reported a conditional *Pten* knockout mouse in which the *Pten* gene was deleted by *Lck*, a T cell–specific promoter (*LckCrePten<sup>flox/flox</sup>* mice).<sup>28</sup> Genomic PCR examination of DNA from purified TCR $\beta$ <sup>+</sup>NK1.1<sup>+</sup>HSA<sup>low</sup> thymocytes from 8-week-old *Pten<sup>flox/flox</sup>* and *LckCrePten<sup>flox/flox</sup>* 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<sup>+</sup>TCR $\beta$ <sup>+</sup> NKT population in the thymus, spleen, and liver of 7- to 8-week-old *LckCrePten<sup>flox/flox</sup>* and *Pten<sup>flox/flox</sup>* mice. *LckCrePten<sup>flox/flox</sup>* mice showed reductions not only in the percentages of NK1.1<sup>+</sup>TCR $\beta$ <sup>+</sup> 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<sup>flox/flox</sup>* mice. (A) Genomic DNA from TCR $\beta$ <sup>+</sup>NK1.1<sup>+</sup>HSA<sup>low</sup> thymocytes of the indicated genotype was analyzed by PCR. In *LckCrePten<sup>flox/flox</sup>* mice, most TCR $\beta$ <sup>+</sup>NK1.1<sup>+</sup>HSA<sup>low</sup> thymocytes contained the deleted *Pten* allele ( $\Delta$ ) 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 V $\alpha$ 14iNKT cells, a major subset of NKT cells, using the reagent CD1d dimer–Ig ( $\alpha$ GalCer-CD1d).<sup>7</sup> The thymi of WT and *LckCrePten<sup>flox/flox</sup>* mice contained equal numbers of  $\alpha$ GalCer-CD1d<sup>+</sup>TCR $\beta$ <sup>+</sup>NKT cells (V $\alpha$ 14iNKT cells) (Figure 2C-D, left panels), indicating that *Pten* deficiency has no effect on total V $\alpha$ 14iNKT cell numbers. We then determined CD44/NK1.1 profiles<sup>10,11</sup> to follow the development of V $\alpha$ 14iNKT cells in thymus. While about 80% of thymic V $\alpha$ 14iNKT cells in *Pten<sup>flox/flox</sup>* mice were mature and had reached stage 3 (CD44<sup>high</sup>NK1.1<sup>+</sup>), only 9% of V $\alpha$ 14iNKT cells in *LckCrePten<sup>flox/flox</sup>* mice were at stage 3 and most had arrested at stage 2 (immature CD44<sup>high</sup>NK1.1<sup>-</sup> 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<sup>low</sup>NK1.1<sup>-</sup>) 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, V $\alpha$ 14iNKT cells in *LckCrePten<sup>flox/flox</sup>* 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 V $\alpha$ 14iNKT cells because no increases were observed in the percentage of NK1.1<sup>-</sup> V $\alpha$ 14iNKT cells in mutant spleen and liver (Figure S1B).

V $\alpha$ 14iNKT 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.<sup>8,12</sup> Thus, normal CD1d expression on DP thymocytes and thymic/peripheral DCs is required for proper NKT cell development.<sup>8,9</sup> Flow cytometric analysis revealed no differences in CD1d levels expressed by DP thymocytes or thymic or splenic DCs from *Pten<sup>flox/flox</sup>* and *LckCrePten<sup>flox/flox</sup>* mice (Figure S1C). These data indicate that the impaired development of *Pten*-deficient V $\alpha$ 14iNKT cells is not due to altered CD1d expression by antigen-presenting cells.

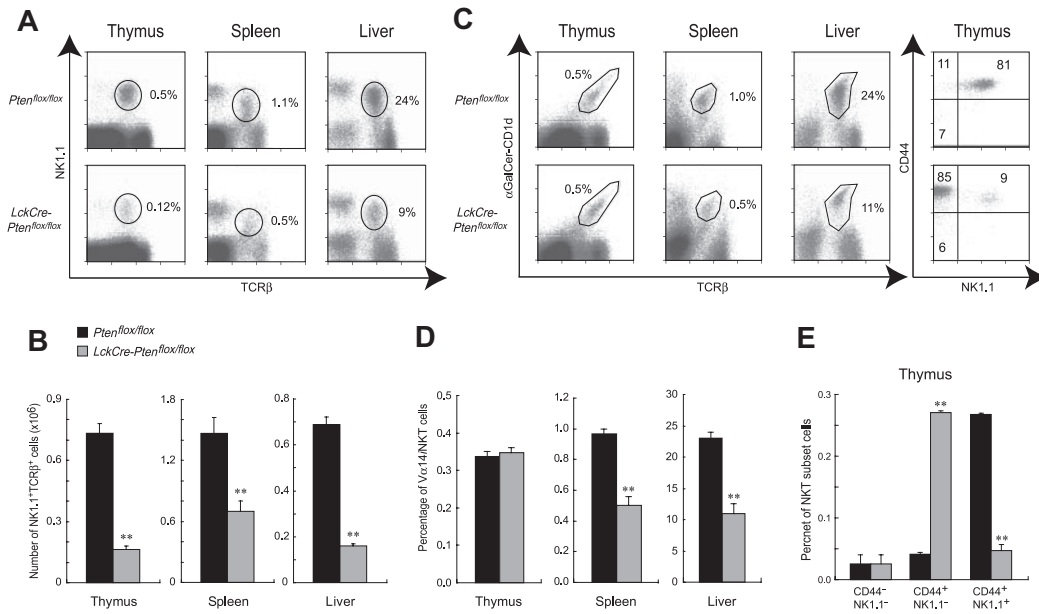
### The developmental failure of V $\alpha$ 14iNKT cells in *LckCrePten<sup>flox/flox</sup>* mice is cell autonomous

The *Pten* gene is deleted in both NKT cells and T cells in *LckCrePten<sup>flox/flox</sup>* mice, and DP T cells are an important stromal component for normal NKT cell development in the thymus.<sup>8,9,12</sup> To rule out the possibility that it was the *Pten* mutation in DP T cells that caused the decrease in NKT cells in *LckCrePten<sup>flox/flox</sup>* mice, purified CD44<sup>high</sup>NK1.1<sup>-</sup> $\alpha$ GalCer-CD1d<sup>+</sup>TCR $\beta$ <sup>+</sup> immature V $\alpha$ 14iNKT cells (stage 2) from *Pten<sup>flox/flox</sup>* and *LckCrePten<sup>flox/flox</sup>* mice were transferred into *Ja18<sup>-/-</sup>* fetal thymus (which lacks all V $\alpha$ 14iNKT cells) and cultured for 9 days using the FTOC method.<sup>11</sup> As shown in Figure 3, more than 60% of immature *Pten<sup>flox/flox</sup>* V $\alpha$ 14iNKT cells developed into mature V $\alpha$ 14iNKT cells (CD44<sup>high</sup>NK1.1<sup>+</sup> $\alpha$ GalCer-CD1d<sup>+</sup>TCR $\beta$ <sup>+</sup>; stage 3) in this environment. In contrast, almost all transferred immature *LckCrePten<sup>flox/flox</sup>* V $\alpha$ 14iNKT cells remained at stage 2. This result clearly indicates that the reduced number of V $\alpha$ 14iNKT cells in *LckCrePten<sup>flox/flox</sup>* 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 V $\alpha$ 14iNKT cells by determining their proliferation and cytokine

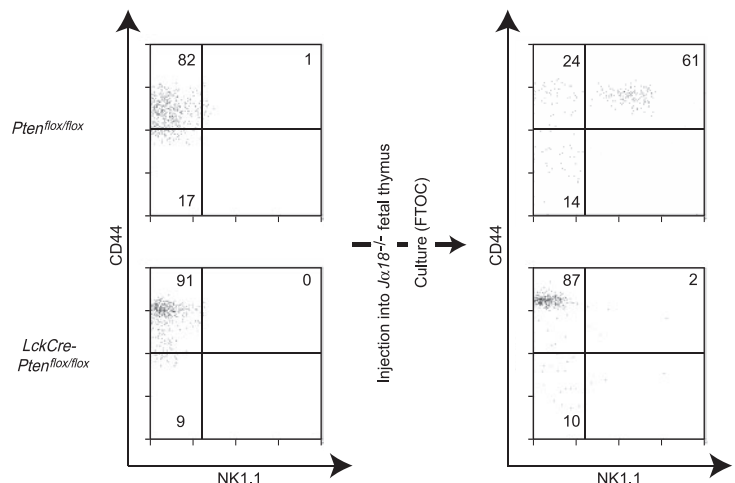




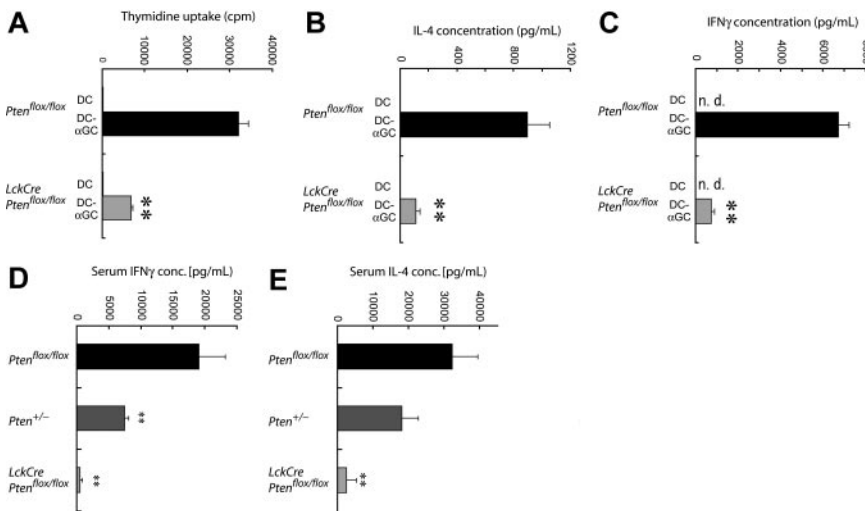
**Figure 2. Altered NKT cell populations in *LckCrePten*<sup>flox/flox</sup> mice.** (A–B) Decreased numbers of NK1.1<sup>+</sup>TCRβ<sup>+</sup> cells in *LckCrePten*<sup>flox/flox</sup> mice. (A) MNCs were isolated from the thymus, spleen, and liver of mice of the indicated genotypes, and NK1.1<sup>+</sup>TCRβ<sup>+</sup> cells were analyzed by flow cytometry. Numbers within the panels indicate the relative percentages of NK1.1<sup>+</sup>TCRβ<sup>+</sup> cells in total lymphocyte populations. (B) Absolute numbers of the NK1.1<sup>+</sup>TCRβ<sup>+</sup> cells in panel A. (C–E) Altered maturation and numbers of V $\alpha$ 14/NKT cells. (C, left panel, and D) The percentage of V $\alpha$ 14/NKT cells in total MNCs is normal in the thymus of *LckCrePten*<sup>flox/flox</sup> mice but decreased in spleen and liver. (C, right panel, and E) Stage 2 (CD44<sup>+</sup>NK1.1<sup>-</sup>) V $\alpha$ 14/NKT cells were increased, but stage 3 (CD44<sup>+</sup>NK1.1<sup>+</sup>) cells were decreased in the thymus of *LckCrePten*<sup>flox/flox</sup> mice. The percentages of NKT subsets in  $\alpha$ GalCer-CD1d<sup>+</sup>TCRβ<sup>+</sup>-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  $\pm$  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 V $\alpha$ 14iNKT fraction was found to be slightly activated prior to any exogenous stimulation. We therefore purified NK1.1<sup>+</sup>TCRβ<sup>+</sup> cells from *Pten*<sup>flox/flox</sup> and *LckCrePten*<sup>flox/flox</sup> thymi and activated these cells using DCs that had been loaded in vitro with  $\alpha$ GalCer. In response to this activation, NK1.1<sup>+</sup>TCRβ<sup>+</sup> mature NKT cells from *Pten*<sup>flox/flox</sup> thymus showed high levels of proliferation (Figure 4A) as well as vigorous production of IL-4 (Figure 4B) and IFN $\gamma$  (Figure 4C). In contrast, NK1.1<sup>+</sup>TCRβ<sup>+</sup> mature NKT cells from *LckCrePten*<sup>flox/flox</sup> 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 V $\alpha$ 14iNKT cells in response to  $\alpha$ GalCer in vivo, we intraperitoneally injected  $\alpha$ GalCer into *Pten*<sup>flox/flox</sup>, *Pten*<sup>+/-</sup>, and *LckCrePten*<sup>flox/flox</sup> mice. Administration of  $\alpha$ GalCer to WT mice led to a rapid increase in serum IL-4 and IFN $\gamma$  levels within 2 hours and 12 hours, respectively (Figure 4D–E). It is known that V $\alpha$ 14iNKT cells are solely responsible for the production of these cytokines at these time points.<sup>22</sup> Accordingly, in both *Pten*<sup>+/-</sup> and *LckCrePten*<sup>flox/flox</sup> mice that received intraperitoneal injection of  $\alpha$ GalCer, serum levels of IFN $\gamma$  were severely decreased.  $\alpha$ GalCer-treated *LckCrePten*<sup>flox/flox</sup> mice also showed a significant decrease in serum IL-4 whereas *Pten*<sup>+/-</sup> mice showed only a slight (insignificant) reduction. These data indicate that, as well as reducing their numbers, mutation of Pten functionally impairs mature V $\alpha$ 14iNKT cells both in vitro and in vivo.



**Figure 3. Failure of Pten-deficient immature V $\alpha$ 14iNKT cells to mature in vitro.** CD44<sup>high</sup>NK1.1<sup>-</sup> (stage 2, immature) V $\alpha$ 14iNKT cells were purified from the thymi of *Pten*<sup>flox/flox</sup> or *LckCrePten*<sup>flox/flox</sup> mice and injected into FTOCs derived from a *J $\alpha$ 18*<sup>-/-</sup> embryo. (Left) Flow cytometric analysis of cell populations injected. (Right) At 9 days after injection, V $\alpha$ 14iNKT cells in the FTOC were isolated and analyzed by flow cytometry to detect stage 3 (mature) cells. Mature V $\alpha$ 14iNKT 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<sup>+</sup>TCRβ<sup>+</sup> cells were purified from the thymus of *Pten<sup>flx/flx</sup>* or *LckCrePten<sup>flx/flx</sup>* mice and cocultured with DCs loaded with vehicle (DC) or αGalCer (DC-αGC). After 48 hours, cells were pulsed with [<sup>3</sup>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 Vα14iNKT cells to αGalCer administration *in vivo*. αGalCer was intraperitoneally injected into *Pten<sup>flx/flx</sup>*, *Pten<sup>+/-</sup>*, or *LckCrePten<sup>flx/flx</sup>* 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.

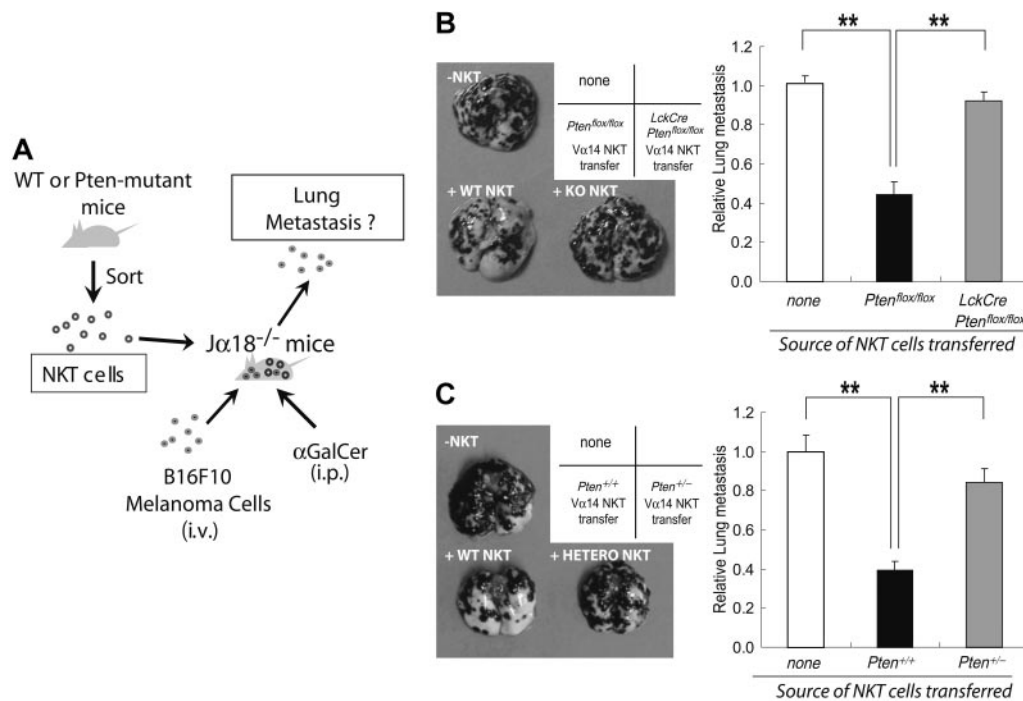
### *Pten*-deficient Vα14iNKT cells show defective antitumor immunity

Because Vα14iNKT cells were abnormal in number and function in *LckCrePten<sup>flx/flx</sup>* mice, we investigated whether these animals had a defect in Vα14iNKT-mediated antitumor immunity *in vivo*. First, we purified αGalCer-CD1d<sup>+</sup>TCRβ<sup>+</sup> (Vα14iNKT) cells from *Pten<sup>flx/flx</sup>* mice and adoptively transferred them into αGalCer-treated *Ja18<sup>-/-</sup>* (Vα14iNKT cell-deficient) recipients that had been inoculated with B16F10 melanoma cells (Figure 5A). The presence of WT Vα14iNKT cells markedly inhibited the metastasis of B16F10 cells to the lungs (about 60% reduction; Figure 5B-C), consistent with previous observations.<sup>36</sup> In contrast, only a minor

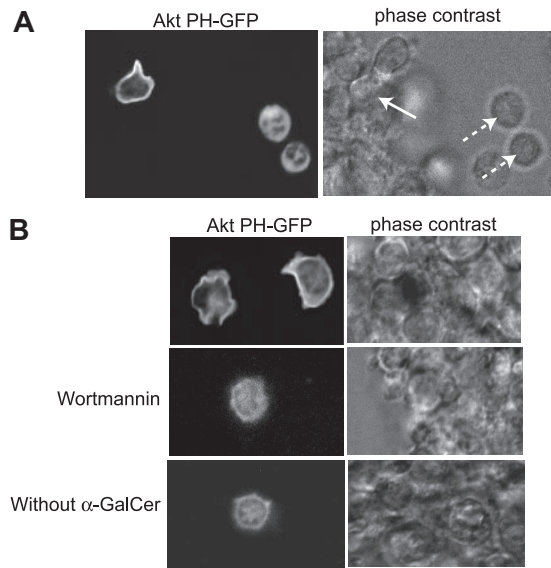
inhibition of B16F10 metastasis was achieved using Vα14iNKT cells from αGalCer-treated *Pten<sup>+/-</sup>* mice (16% reduction; Figure 5C) or αGalCer-treated *LckCrePten<sup>flx/flx</sup>* mice (8% reduction; Figure 5B). These findings show that mutation of *Pten* in Vα14iNKT cells impairs antitumor immunity.

### PI3K signaling is responsible for the phenotypes of *Pten*-deficient Vα14iNKT cells

To determine whether PI3K was activated in Vα14iNKT cells that interacted with αGalCer-loaded DCs, we analyzed levels of the PI3K product PIP3. Purified Vα14iNKT cells expressing the fusion bioprobe AktPH-GFP<sup>32</sup> were cultured with αGalCer-loaded DCs in



**Figure 5. Defective antitumor surveillance by *Pten*-deficient Vα14iNKT cells.** (A) Scheme describing the experimental protocol used to evaluate antitumor surveillance by NKT cells *in vivo*. Vα14iNKT cells were purified from mice of the indicated genotypes and adoptively transferred into *Ja18<sup>-/-</sup>* 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<sup>flx/flx</sup>* or *LckCrePten<sup>flx/flx</sup>* Vα14iNKT cells (B) or after the transfer of *Pten<sup>+/-</sup>* or *Pten<sup>+/-</sup>* Vα14iNKT 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 Vα14iNKT cells. \*\**P* < .01.



**Figure 6. PI3K is activated in  $V\alpha 14$ /NKT cells that interact with  $\alpha$ GalCer-loaded DCs.** Localization of PIP3 to the NKT cell membrane.  $TCR\beta^+NK1.1^+HSA^{low}$  NKT cells were purified from thymocytes of 8-week-old *AktPH-GFP<sup>tg</sup>* mice (WT for *Pten*) expressing the PIP3-binding AktPH-GFP bioprobe I (left). These  $V\alpha 14$ /NKT cells were incubated with  $\alpha$ GalCer-loaded DCs, and PIP3 localization was determined by confocal fluorescence (left) and phase contrast (right) microscopy. (A)  $V\alpha 14$ /NKT cells that made contact with  $\alpha$ GalCer-loaded DCs showed membrane localization of PIP3 (solid arrow), while PIP3 remained in the cytoplasm of  $V\alpha 14$ /NKT cells that did not make contact with DCs (dotted arrows). (B) AktPH-GFP bioprobe localization to the plasma membrane of  $V\alpha 14$ /NKT cells was inhibited by wortmannin or by contact with DCs lacking  $\alpha$ GalCer.

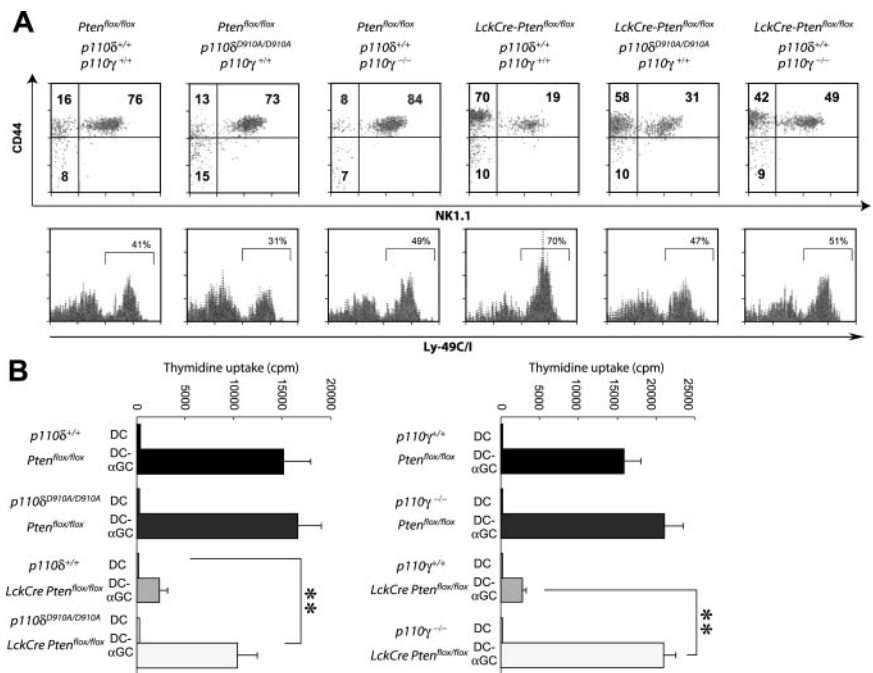
fibronectin-coated glass-base dishes. As shown in Figure 6A, the AktPH-GFP bioprobe was recruited to the plasma membrane of WT  $V\alpha 14$ /NKT cells that interacted with  $\alpha$ GalCer-loaded DCs (solid arrow) but remained in the cytoplasm of WT  $V\alpha 14$ /NKT cells that did not make contact with the DCs (dotted arrows). In addition, the bioprobe was not recruited to the plasma membrane of WT  $V\alpha 14$ /NKT cells that interacted with  $\alpha$ GalCer-loaded DCs in the presence of wortmannin (a pan-PI3K inhibitor) or in  $V\alpha 14$ /NKT

cells that contacted DCs lacking  $\alpha$ GalCer (Figure 6B). These findings suggest that PI3K is activated in  $V\alpha 14$ /NKT cells when these cells are stimulated by interaction with  $\alpha$ GalCer-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.<sup>26,28</sup> Curiously, *Pten* deficiency in  $V\alpha 14$ /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 *p110 $\delta$*  is highly expressed in hematopoietic cells and is the major catalytic subunit of class IA PI3Ks in T-lineage cells.<sup>30</sup> The PI3K subunit *p110 $\gamma$* , 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.<sup>29</sup> To determine whether PI3K signaling was responsible for the defects of *Pten*-deficient  $V\alpha 14$ /NKT cells, we examined double-mutant mice lacking *Pten* and PI3K *p110 $\gamma$* <sup>29</sup> (*LckCrePten<sup>lox/lox</sup>/p110 $\gamma$ <sup>-/-</sup>*) or *Pten* and PI3K *p110 $\delta$* <sup>30</sup> (*LckCrePten<sup>lox/lox</sup>/p110 $\delta$ <sup>D910A/D910A</sup>*). The mutation of *p110 $\gamma$*  partially rescued  $V\alpha 14$ /NKT cell numbers in *LckCrePten<sup>lox/lox</sup>* mice (Figure 7A, upper panel) and completely rescued the proliferation defect of *Pten*-deficient  $V\alpha 14$ /NKT cells (Figure 7B). A slightly weaker but still significant effect was seen upon mutation of *p110 $\delta$* . Moreover, numbers of peripheral *Pten*-deficient  $V\alpha 14$ /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  $V\alpha 14$ /NKT cells.

**Enhanced expression of inhibitory Ly49 receptors by *Pten*-deficient  $V\alpha 14$ /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,<sup>37</sup> 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  $V\alpha 14$ /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  $\alpha$ GalCer- $CD1d^+TCR\beta^+NK1.1^+$  cells in the top panel were analyzed by flow cytometry to determine relative expression levels of Ly49C/I. (B) Impaired proliferation. The  $NK1.1^+TCR\beta^+$  cells from panel A were stimulated by incubation with  $\alpha$ GalCer-loaded DCs, and [<sup>3</sup>H]-thymidine incorporation was measured. Data shown are the mean  $\pm$  SEM for 3 mice per group. \*\**P* < .01. For both panels, the defects of *Pten*-deficient  $V\alpha 14$ /NKT cells were partially rescued by mutation of a PI3K subunit (*p110 $\gamma$*  or *p110 $\delta$* ).



Ly49–MHC class I interaction,<sup>38</sup> and mice transgenic for Ly49A show impaired NKT cell maturation and selection.<sup>39</sup> 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 V $\alpha$ 14iNKT cells. Specifically, we determined the expression of Ly49C/I (which recognizes MHC class I, H-2K<sup>b</sup>, H-2D<sup>d</sup>) and Ly49A (which recognizes MHC class I, H-2D<sup>d</sup>) by thymic V $\alpha$ 14iNKT cells of *LckCrePten<sup>fllox/fllox</sup>* mice and the double mutants. As expected, the proportion of  $\alpha$ GalCer-CD1d<sup>+</sup>TCR $\beta$ <sup>+</sup>NK1.1<sup>+</sup> thymocytes showing strong expression of Ly49C/I (Figure 7A, bottom) and Ly49A (Figure S2) was increased in *LckCrePten<sup>fllox/fllox</sup>* mice compared with *Pten<sup>fllox/fllox</sup>* mice. At least for Ly49C/I, this increased expression was mitigated by the presence of the *p110 $\delta$ <sup>D910A/D910</sup>* or *p110 $\gamma$ <sup>-/-</sup>* mutation. There were no differences between *LckCrePten<sup>fllox/fllox</sup>* mice and *Pten<sup>fllox/fllox</sup>* mice in the H2-K<sup>b</sup> 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 V $\alpha$ 14iNKT cells. Taken together, our results suggest that the reduced number and functions of Pten-deficient V $\alpha$ 14iNKT cells may be linked to their increased expression of inhibitory Ly49 receptors.

## Discussion

Many studies in mouse models have demonstrated that V $\alpha$ 14iNKT cells are activated in a broad range of disorders, including autoimmune diseases, microbial infections, graft rejection, and cancer progression/metastasis.<sup>5,19-21</sup> 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 V $\alpha$ 14iNKT cells impairs the development and functions of these cells and that these defects depend on PI3K activity. An in vivo consequence of the defective V $\alpha$ 14iNKT compartment is enhanced tumor metastasis.

The study of genetically modified mice has begun to yield important insights into the mechanisms controlling the maturation of V $\alpha$ 14iNKT cells. To date, investigations of diverse gene-deficient mice have revealed 3 general categories of V $\alpha$ 14iNKT cell abnormalities.<sup>40</sup> 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) V $\alpha$ 14iNKT cells. In the second category (group 2), the mutant animals exhibit a block in V $\alpha$ 14iNKT 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 V $\alpha$ 14iNKT 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 V $\alpha$ 14iNKT cells from DP precursors.<sup>40,41</sup> Mice lacking T-bet<sup>42</sup> or Itk<sup>43</sup> 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 V $\alpha$ 14iNKT cells. Mice deficient for IL-15 or transgenic for mIkB $\alpha$  fall into group 3 because these molecules are important for the survival of mature NKT cells.<sup>40,44</sup> While the phenotypes observed in the V $\alpha$ 14iNKT cells of T-bet- or Itk-deficient mice are strikingly similar to those we observed for V $\alpha$ 14iNKT cells of *LckCrePten<sup>fllox/fllox</sup>* 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  $\beta$  subunit (CD122),<sup>42</sup> the proliferative response to IL-15 by mature NKT cells of *LckCrePten<sup>fllox/fllox</sup>* mice was normal (Figure S3). Second, Itk-deficient V $\alpha$ 14iNKT cells show reduced expression of the inhibitory NK receptors Ly49C/I and intact IFN $\gamma$  secretion,<sup>43</sup> but Pten-deficient V $\alpha$ 14iNKT cells exhibit enhanced Ly49C/I and Ly49A expression and reduced IFN $\gamma$  secretion. In addition, because Itk is activated by Pten deficiency,<sup>45</sup> reduction of Itk signaling is unlikely to have occurred in our *LckCrePten<sup>fllox/fllox</sup>* mice. For these reasons, we believe that the phenotypes observed in V $\alpha$ 14iNKT cells of *LckCrePten<sup>fllox/fllox</sup>* 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 V $\alpha$ 14iNKT cells.

Ly49 is a multigene receptor family expressed mainly by NK cells and stage 3 mature V $\alpha$ 14iNKT cells.<sup>44</sup> 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.<sup>46</sup> It has been clearly shown that the interaction between Ly49A and H-2D<sup>d</sup> and between Ly49C and H-2K<sup>b</sup> or H-2D<sup>d</sup> negatively regulates NK effector functions such as cytotoxicity and bone marrow graft rejection.<sup>47,48</sup> Similarly, the appropriate inhibitory Ly49–MHC class I interactions can negatively regulate the maturation, proliferation, and functions of NKT cells.<sup>37,38,39</sup> In our study, we found that most  $\alpha$ GalCer-CD1d<sup>+</sup>TCR $\beta$ <sup>+</sup>NK1.1<sup>+</sup> thymocytes in *LckCrePten<sup>fllox/fllox</sup>* 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 V $\alpha$ 14iNKT cells can plausibly be explained by their enhanced expression of inhibitory Ly49 receptors.

Several lines of evidence suggest that developing V $\alpha$ 14iNKT cells undergo negative selection if they encounter high-avidity antigen or abundant self-antigen. First, the addition of  $\alpha$ GalCer to early-stage FTOC prevents the development of V $\alpha$ 14iNKT cells in a dose-dependent manner.<sup>14,16</sup> Second, overexpression of CD1d on DCs or thymocytes in transgenic mice results in a reduced number of V $\alpha$ 14iNKT cells that exhibit both altered V $\beta$  repertoire usage and reduced sensitivity to antigen.<sup>12,14</sup> Third, in mice doubly transgenic for the activatory Ly49D receptor and its ligand H2-D<sup>d</sup>, V $\alpha$ 14iNKT 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<sup>d</sup> transgenic mice resulted in a partial rescue of NKT cell development.<sup>15</sup> A possible interpretation of these findings is that V $\alpha$ 14iNKT 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 V $\alpha$ 14iNKT cell development. Pten-deficient V $\alpha$ 14iNKT cells exposed to endogenous glycolipid antigens may transmit supraphysiological levels of signaling which, in turn, may drive enhanced negative selection of V $\alpha$ 14iNKT cells. The deficit of V $\alpha$ 14iNKT cells in the peripheral organs and thymus may then be partially restored by repopulation of the mature V $\alpha$ 14iNKT compartment with cells

showing increased expression of inhibitory Ly49 receptors, altered V $\beta$  usage, and reduced sensitivity to antigen.<sup>14</sup>

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.<sup>49</sup> In addition, T-cell responses induced by TCR stimulation are much weaker in p110 $\delta^{-/-}$ <sup>50</sup> and p110 $\gamma^{-/-}$ <sup>29</sup> 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 p110 $\gamma$ . These results suggest that TCR signaling may directly activate both PI3K $\delta$  and PI3K $\gamma$  in V $\alpha$ 14iNKT cells. Alternatively, inhibitory Ly49 receptors may directly affect both PI3K $\delta$  and PI3K $\gamma$ , 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.<sup>51</sup>

The molecular mechanisms underlying the impaired function of V $\alpha$ 14iNKT 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<sup>flox/flox</sup>* V $\alpha$ 14iNKT 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  $\alpha$ GalCer has antitumor effects against cancer cells of various origins and that this protection depends on an initial burst of IFN $\gamma$  production by NKT cells and subsequent ongoing IFN $\gamma$  production by NK cells.<sup>36</sup> IFN $\gamma$  itself is not directly toxic to tumor cells, but the IFN $\gamma$  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, IFN $\gamma$  has potent antiangiogenic activity<sup>52</sup> and induces other leukocytes to produce IFN $\gamma$ -inducible factors such as IFN-inducible protein 10 (IP-10) and monokine induced by IFN $\gamma$ .<sup>53</sup> These cytokines may indirectly inhibit tumor neovascularization and thus induce tumor hypoxia. We have demonstrated that Pten-deficient V $\alpha$ 14iNKT cells fail to produce IFN $\gamma$  both in vitro and in vivo, most likely accounting for the impaired antitumor surveillance observed in *LckCrePten<sup>flox/flox</sup>* mice.

Our study is the first to show that engagement of the TCRs of murine V $\alpha$ 14iNKT cells by  $\alpha$ GalCer-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 V $\alpha$ 14iNKT cells leads to a failure in their development. The few mature V $\alpha$ 14iNKT cells that survive are tolerant to  $\alpha$ GalCer, resulting in defective antitumor surveillance and accelerated metastasis. Mice heterozygous for the Pten mutation (*Pten<sup>+/-</sup>*) showed a significant reduction in serum IFN $\gamma$  production after administration of  $\alpha$ GalCer in vivo. In addition, adoptive transfer of *Pten<sup>+/-</sup>* V $\alpha$ 14iNKT cells into  $\alpha$ GalCer-treated, B16F10 melanoma cell-inoculated *Ja18<sup>-/-</sup>* 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.

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