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## **Conditional deletion of PTEN in CD45-expressing cells as a new model of T-cell lymphoblastic leukemia/lymphoma.**

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Running title: Conditional PTEN deletion in CD45 expressing cells

We declare no conflict of interests

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## **Abstract**

PTEN is one of the most frequently mutated tumor suppressor genes in human cancers. Loss of function alteration of PTEN results in increased activation of PI3K/Akt signaling, which is associated with increased proliferation, survival and neoplastic growth. Here, we have addressed the effects of conditional deletion of PTEN in hematopoietic cells. For this purpose, we have crossed PTEN conditional knock-out mice with a knock-in mouse expressing the CRE recombinase in the CD45 locus (CD45:Cre; PTEN fl/fl). CD45 is also known as leucocyte common antigen and is expressed in virtually all white cells as well as in hematopoietic stem cells. Using a reporter mice, we demonstrate that CD45:Cre mouse displays recombinase activity in both myeloid and lymphoid cells. However, CD45:Cre<sup>+/-</sup> PTEN<sup>fl/fl</sup> mice develop T-Acute lymphoblastic Leukemia and Lymphoma, but no other types of hematological malignancies such as myeloproliferative disorders or B-cell lymphomas. These results indicate that conditional deletion of PTEN in hematopoietic CD45-positive cells diverts neoplastic growth to the formation of T-cell malignancies.

## Introduction

PTEN (phosphatase and tensin homolog deleted on chromosome 10) encodes a dual lipid and protein phosphatase that plays a crucial role in the phosphatidylinositol-3 kinase/Akt/mammalian Target of Rapamycin (PI3K/Akt/mTOR) signaling pathway. PTEN dephosphorylates phosphatidylinositol-3,4,5-triphosphate (PIP3) to phosphatidylinositol-4,5-diphosphate (PIP2), thereby antagonizing the activation of the function of PI3K. <sup>12</sup>( Loss of PTEN function causes the activation of PI3K/Akt signaling that leads to increased cell growth and survival. <sup>345</sup>

PTEN is one of the most frequently mutated tumor suppressor genes in human cancers. The tumor suppressive function of PTEN was identified as the chromosome region 10q23 partially or completely deleted in multiple neoplasias. <sup>67</sup> Moreover, germline mutations of PTEN gene were identified in patients with Cowden disease. <sup>6</sup> Loss-of-function alterations of PTEN are frequently found in solid tumors such as glioblastomas, thyroid breast, colon prostate or endometrial carcinomas <sup>8</sup> and in hematological malignancies including both lymphoid <sup>9101112</sup> and myeloid neoplasms. <sup>131415</sup>

The PI3K/AKT/mTOR pathway has been implicated in human leukemogenesis. The role of PTEN in maintenance of hematological homeostasis has been evidenced by PTEN knock-out (KO) mouse models. Mice hemizygous for PTEN (PTEN<sup>+/-</sup>) develop multiple neoplasias, including both solid and hematological malignancies. <sup>161718</sup> Conditional deletion of both PTEN alleles in many cell types have been achieved by crossing conditional PTEN floxed mice <sup>19</sup> with different Cre expression systems. We have recently demonstrated tamoxifen-inducible expression of Cre recombinase in epithelial cells leads to the development of thyroid, endometrial and prostate neoplasias. <sup>20</sup> Conditional PTEN deletion in hematological system also results in the appearance of malignancies. Tamoxifen-inducible Cre under the control of Rosa26 promoter (R26CRE:ER) causes PTEN excision in a broad spectrum of cells, leading to development of multiple neoplasias including lymphomas. <sup>21</sup> Lck-mediated Cre expression allows conditional deletion of PTEN in double negative thymocytes, leading to development of T-cell lymphomas. <sup>22</sup> Finally, by using the polyinosine-polycytidine inducible Mx1-Cre mice (Yilmaz 2006) or the VE-cadherin-Cre mice, PTEN has been successfully deleted in hematopoietic stem cells (HSC). In both mice models, animals suffer from either acute myeloid leukemia (AML) or T-acute lymphoblastic leukemia/lymphoma (T-ALL).

To date, there is no existing mouse model to achieve specific PTEN deletion in hematopoietic cells without affection of other cell types. In the present work, we have addressed the effects of conditional deletion of PTEN in CD45 expressing cells. For these purpose, we have crossed PTEN conditional knock-out mice with a knock-in mouse expressing the CRE recombinase in the CD45 locus. The resulting mouse develops T-ALL, but no other types of hematological malignancies.

## Materials and methods

### Ethical statement

All procedures performed in this study followed the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Committee on Ethics of Animal Experiments of Universitat de Lleida/IRB Lleida (CEEAA).

### Mice

Mice were housed and maintained in a barrier facility, and pathogen-free procedures were used in all mouse rooms. Up to 15 mice were housed together in each cage and kept in a 12-hour light/dark cycle at 22°C with *ad libitum* access to autoclaved food and water.

Floxed homozygous PTEN (C;129S4-*Ptentm1Hwu*/J, hereafter called PTEN fl/fl) and reporter mT/mG (B6.129(Cg)-*Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo*/J) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). CD45:Cre mice were kindly donated by Dr. Alexander Medvinsky.<sup>23</sup>

CD45:Cre PTEN fl/fl mice were bred in a mixed background (C57BL6; 129S4) by crossing PTEN fl/fl and CD45:Cre mice; this followed by backcrossing CD45:Cre+/- PTEN fl/+ and PTEN fl/fl mice. Three weeks after birth, animals were weaned and tails were cut in order to genotype them. Isolation and PCR analysis of tail genomic DNA was performed as previously described.<sup>1924</sup>

During all the study, humane endpoints were used. Mice were monitored every day and euthanized when they showed lethargy, ruffled fur, haunched posture or anorexia. For this purpose, animals were anesthetized with isoflurane and sacrificed by cervical dislocation. Organs were collected and further processed for the different studies described below.

### Genotyping of PTEN deletion

Genomic DNA was isolated from WT and KO spleens by standard procedures. Briefly, tissue was chopped and digested with 1mg/mL of proteinase K (Sigma, Saint Louis, MO) at 55°C for 4 hours. Samples were boiled 5 minutes and DNA was precipitated with 100% ethanol. Standard PCR was performed using three different primers: common forward P1 5'-ACTCAAGGCAGGGATGAGC-3', reverse P2 5'-AATCTAGGGCCTCTTGCC-3' and reverse P3 5'-

GCTTGATATCGAATTCCTGCAGC-3'. PCR products were resolved in agarose gels and visualized by ethidium bromide staining.

### **Western blot analysis**

Spleens were washed with PBS, frozen in liquid nitrogen and homogenized in ice with lysis buffer (2% sodium dodecyl sulfate, 125 mmol/L Tris-HCl, pH 6.8). Samples were boiled for 5 minutes at 95°C, sonicated for 30 seconds and centrifuged (10 minutes at 15000 rpm). Supernatants were recovered and protein concentrations were determined with a protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of proteins were subjected to SDS-PAGE and transferred to PVDF membranes (Millipore). Nonspecific binding was blocked by incubation with TBST (20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 0.1% Tween-20) plus 5% of non-fat milk. Membranes were incubated with primary antibodies overnight at 4°C followed by 1hour incubation with secondary antibody diluted 1/10000 in TBST. Signal was detected with ECL Advance (Amersham Pharmacia, Buckinghamshire, UK). Anti phospho-AKT and anti-PTEN rabbit antibodies were purchased from Cell Signaling (Beverly, MA); polyclonal rabbit anti-Cre was from Novagen (Billerica, MA); monoclonal mouse anti cyclin D1 and anti c-Myc were from Santa Cruz Biotechnology (Santa Cruz, Ca); monoclonal mouse anti-tubulin was from Sigma Aldrich (St Louis, MO).

### **Histopathology and Immunohistochemical analysis**

Animals were euthanized and organs were collected, formaline-fixed O/N at 4°C embedded in paraffin for histologic examination. Paraffin blocks were sectioned at a thickness of 3 µm, dried for 1h at 65° before pre-treatment procedure of deparaffinization, rehydration and epitope retrieval in the Pre-Treatment Module, PT-LINK (DAKO) at 95 °C for 20 min in 50x Tris/EDTA buffer, pH 9. Before staining the sections, endogenous peroxidase was blocked. The antibodies used were against CD3ε (clone M-20, Santa Cruz Biotechnology), PTEN (clone 6H2.1), Ki67 (clone TEC-3, DAKO, Denmark), CD20 (clone L26, DAKO, Denmark) and Myeloperoxidase (polyclonal, DAKO, Denmark). After incubation, the reaction was visualized with the EnVision FLEX Detection Kit (DAKO, Denmark) for CD20, C-KIT, Myeloperoxidase and PTEN, Polyclonal Rabbit Anti-Rat Immunoglobulins/Biotinylated and Streptavidin (DAKO, Denmark) for ki67 and Polyclonal Bovine Anti-Goat IgG/Biotinylated (Santa Cruz Biotechnology) and Streptavidin (DAKO, Denmark) for CD3ε using diaminobenzidine chromogen as a substrate. Sections were counterstained with hematoxylin. Appropriate negative controls were also tested. Representative images were taken with a Leica DMD 108 microscope.

Hematoxylin and Eosin (H&E) stained samples were histologically reviewed and evaluated by two pathologists, following uniform pre-established criteria

### **Cytological analysis**

Blood was collected from the submandibular vein and smears were immediately prepared and fixed in 100% ethanol. For diff-quick staining, slides were fixed with Diff-Quick fixative reagent and stained with Diff-Quick solution I (eosinophilic) and Diff-Quick solution II (basophilic) before being brought to distilled water, air dried and mounted.

For Papanicolaou stain, ethanol fixed slides were stained with hematoxylin and counterstained with Orange G and Eosin Azure (EA50), cleaned with 95% ethanol and mounted.

### **Proliferation analysis**

50 high power microscope fields were examined for mitotic figures and counted.

Ki-67 staining was used to assess the index of proliferation of splenic cells in KO and WT mice. Five representative fields of each tissue were photographed for each animal.

Proliferation was calculated as the percentage of Ki-67 positive nuclei to the total number of nuclei of each field (1000-2000 total nuclei were evaluated for each animal). Counting was performed using ImageJ software (Wayne Rasband, NIH, Bethesda, MD).

### **FACS**

Blood was collected from the submandibular vein in heparinized capillars. Bone marrow cells were obtained from the long bones (tibiae and femurs). After cleaning them from adherent soft tissue, the tip of each bone was removed and the marrow was harvested by inserting a syringe needle (30-gauge) into the end of the bone and flushing Dulbecco's Modified Eagle's Medium (DMEM; Gibco). Spleen was chopped and digested 30 minutes at 37°C in DMEM with 1mg/mL of collagenase D (Sigma, Saint Louis, MO). Splenocytes were filtered through a 70 µm-strainer (MARCA) and centrifuged. Red blood cells were removed from blood, bone marrow and spleen samples using lysis buffer (0, 15M NH<sub>4</sub>Cl, 0,01M KHCO<sub>3</sub>, 0,1 EDTA) FACS analysis of the bright cells was performed using the following antibodies: APC anti-mouse TCR and FITC anti-mouse B220, both obtained from EBioscience.



For the reporter CD45:Cre mT/mG mice analysis, blood and BM were collected as described and green/red fluorescences were analysed.

Fluorescence emission was measured using FACSCalibur (BD Biosciences, San Jose, CA, USA)

### **CD45:Cre reporter assays**

For reporter assays of the cre activity, CD45:Cre<sup>+/-</sup> and mT/mG<sup>+/+</sup> mice were bred. The resulting offspring was weaned and genotyped. One month after birth, CreER<sup>+/-</sup> mT/mG<sup>+/-</sup> mice were sacrificed and organs were collected and fixed in 4% PFA for 4 hours at room temperature. Samples were cryoprotected in PBS with 30% sucrose overnight at 4°C and embedded in OCT (VWR International). 8-µm sections of frozen tissue were cut with cryostat and nuclei were counterstained with Hoescht and samples mounted with PBS:glycerol (1:1).

Tissue immunofluorescence was visualized and analyzed using a confocal microscope (Olympus, Tokyo, Japan). Confocal images were edited using FluoView software (Olympus).

### **Statistical analysis**

Experiments were performed at least three times and statistical significance was determined by Student's test with p-value ≤0.05 (\*); p-value ≤0.01 (\*\*) or p-value ≤0.001(\*\*\*).

## Results

### **Generation of conditional mouse model to delete PTEN in CD45-expressing cells**

To achieve conditional deletion of the two PTEN alleles in CD45 expressing cells, conditional PTEN knock-out mouse (PTEN<sup>fl/fl</sup>)<sup>19</sup> was crossed with a knock-in mouse expressing Cre recombinase into the CD45 locus (CD45:Cre)<sup>23</sup>

We first evaluated the effects of conditional deletion of the two PTEN alleles in mice lifespan (Fig.1A). Kaplan-Meier analysis of mice survival revealed that none of the mice (female n=15; male n=5) expressing Cre recombinase and the two floxed PTEN alleles (CD45:Cre<sup>+/-</sup> PTEN fl/fl, designed as KO) survived beyond 30 weeks of age. Mice lacking Cre expression but having two PTEN floxed alleles (CD45<sup>-/-</sup> PTEN fl/fl designed as wild type, WT) showed normal survival (females n=15; males n=4).

Necropsy of CD45:Cre<sup>+/-</sup> PTEN fl/fl mice revealed that 100% of male and female mice had severe splenomegaly, hepatomegaly and enlargement of other lymphoid organs such as the thymus and the lymph nodes (Fig. 1B). To demonstrate Cre mediated recombination of PTEN in lymphoid organs, we performed genotyping and western blot analysis of the spleen from one WT mouse and two KO littermates (Fig 1C). Genotyping demonstrated the presence of an amplified band corresponding to the recombined allele in KO mice. Accordingly, western blot analysis confirmed complete loss of PTEN expression and increased Akt phosphorylation in KO mice (Fig 1D).

### **Deletion of PTEN in CD45-expressing cells leads to development of diffuse peripheral lymphomas/leukemias**

As mentioned above, macroscopic evaluation evidenced a marked increase of lymphoid organs, thus suggesting that conditional deletion of PTEN in CD45 cells leads to development to lymphomas. To further demonstrate the presence of such malignancies, we performed histopathologic analysis of both lymphoid and non-lymphoid tissues. Histopathological examination of the spleen revealed loss of PTEN expression with a disruption of its normal architecture by a diffuse infiltration of lymphoid cells (Fig. 2A) that exhibited irregular nuclei, prominent nucleoli and high mitotic rates (Fig. 2B) as well as cytologic atypia and strong Ki-67 immunoreactivity (Fig. 2C). Western blot analysis revealed a marked increase of cyclin D1 and c-Myc expression in KO mice spleens (Fig. 2D), both of them targets of PTEN in

lymphomagenesis.

Histopathological analysis of other organs demonstrated atypical lymphoid cells lacking PTEN infiltrating in kidneys, liver, lungs, colon or ovary (Fig. 3A). Moreover, flow cytometer analysis and peripheral blood extension from WT and KO mice revealed a dramatic increase of lymphocyte population (Fig 3B, 3C). These results suggest that deletion of PTEN in CD45 expressing cells results in development of severe leukemia/lymphomas.

### **CD45:Cre<sup>+/-</sup> PTEN<sup>fl/fl</sup> mice develop T-cell but not B-cell lymphomas**

Next, we investigated the nature of lymphomas observed in CD45:Cre<sup>+/-</sup> PTEN<sup>fl/fl</sup> mice. For this purpose, we performed flow cytometry analysis using B220 and anti-TCR antibodies to differentiate B-lymphocytes from T-lymphocytes. Analysis of bone marrow, blood and spleen samples obtained from WT and KO mice revealed a marked decrease of B lymphocytes and an increased population of T-cells in KO mice (Fig 4A), suggesting that the malignancies developed by KO mice were compatible with T-cell lymphoma. To further demonstrate T-cell nature of lymphomas, we carried out immunohistochemical analysis of KO spleens, lymph nodes and bone marrow with anti-CD3 antibodies, which specifically recognize T cells. In WT mice, CD3<sup>+</sup> cells location was restricted to paracortical zone of lymph nodes and the periarteriolar lymphoid sheath of the spleen (Fig 4B). In contrast in KO mice, bone marrow, as well as the germinal centers of both lymph nodes and spleens, were invaded by CD3<sup>+</sup> cells. To address the presence of remaining myeloid cells in these tissues, we performed immunostaining of myeloperoxidase (MPO) in all of them. As expected, due to the increase in the lymphoid population, PTEN deletion caused a marked decrease of myeloperoxidase expressing cells. (Fig 4C)

Accordingly to the described results, lymphocytic infiltrates found in liver, lung, kidney or colon, were positive for CD3 staining (Fig 4D). Immunohistochemistry analysis of blood extensions also revealed an increase of CD3 expressing cells in peripheral blood (Fig4D). These results indicate that in all cases analyzed, KO mice developed T-cell lymphoma/leukemia, with no myeloid or B cell disease.

### **CD45:Cre mouse display recombinase activity in myeloid and lymphoid lineages**

CD45 is expressed in all nucleated hematopoietic cells, including both lymphoid and

myeloid lineages. However, all CD45:Cre<sup>+/-</sup> PTEN fl/fl mice analyzed showed T-ALL while none of them seemed to develop myeloid malignancies. Thus, we wondered whether CD45:Cre recombinase activity was causing recombination (and therefore PTEN ablation) in both cell lineages. To test CD45:Cre recombinase activity, we crossed CD45:Cre mice with a double fluorescent reporter mouse that expresses membrane-targeted tandem dimer Tomato (mT) prior to Cre mediated excision, and membrane-targeted green fluorescent protein (mG) after excision (mT/mG mouse).<sup>24</sup> One month after birth, the resulting offspring (CD45:Cre<sup>+/-</sup> mT/mG<sup>+/-</sup>) was analyzed for the presence of red and green fluorescence by flow cytometry and confocal microscopy. First, we analyzed the percentage of myeloid cells and lymphocytes displaying red or green fluorescence in peripheral blood samples. Flow cytometric analysis revealed the presence of both lymphocytes and myeloid cells expressing green fluorescence (Fig 5A), suggesting that Cre activity was able to induce recombination in both cell populations. These results were further confirmed by confocal microscopy analysis of peripheral blood and bone marrow extensions, as well as spleen and lymph node sections. In all those tissues, we observed the presence of green lymphocytes and granulocytes (Fig 5B). The evidences indicate that, as expected, the expression and activity of Cre recombinase under the control of CD45 promoter was able to cause recombination in all blood lineages, although the efficiency was higher in lymphoid cells than in myeloid populations.

## Discussion

In the present report, we have investigated the effects of conditional deletion of PTEN in hematopoietic cells. To date, there is no existing mouse model to achieve deletion of PTEN exclusively in hematopoietic cells, but not in other cell types. For this purpose, we have crossed a previously described knock-in mouse expressing Cre recombinase in CD45 locus<sup>23</sup> with conditional PTEN floxed mouse. Such combination resulted in a mouse model that specifically deletes PTEN in hematopoietic system, but not in other cell type, resulting in the development of T-ALL but no other types of solid or hematological malignancies.

CD45 encodes a receptor type protein with phosphatase activity that is expressed in all nucleated hematopoietic cells, including hematopoietic stem cells. and mature lymphoid and myeloid cells. For this reason, CD45 has traditionally been designated as the Leucocyte Common Antigen (LCA) and commonly used to distinguish hematopoietic cells by either flow cytometry or immunohistochemistry. Here, we have demonstrated that conditional deletion of PTEN in CD45 expressing cells leads to development of T-ALL, with no evidence of other hematological malignancies. These results raise several questions. First question is, why conditional PTEN deletion in all CD45 cells only develops lymphoid neoplasms but no other types of hematological malignancies? Second, why all lymphoid neoplasms correspond to T-cell malignancies but none of the mice analyzed showed evidence of B-cell neoplasias?

Regarding the first question, we considered the possibility that PTEN was not deleted in myeloid cells because Cre recombinase was not efficiently expressed in this lineage. However, using mT/mG reporter mice, we have demonstrated Cre recombination in both myeloid and lymphoid cells. Although these results suggest that loss of PTEN in hematopoietic cells favors the formation of lymphoid malignancies, we cannot rule out the possibility that the differences observed are affected by differences in the efficiency of recombination, which is lower in the myeloid lineage. Two previous studies have demonstrated that PTEN expression is required to maintain HSCs and participates in lineage choice and leukemogenesis.<sup>2526</sup> The use of Mx1-Cre mice for PTEN ablation leads to a rapid formation of myeloproliferative disease (MPD) that mainly progresses to acute myeloid leukemia (AML)<sup>2526</sup> On the other hand, the use of the VE-cadherin-cre mice for PTEN excision has been reported to cause T-ALL in 74% of the animals analyzed.<sup>27</sup> It is important to note that in the first model, PTEN is silenced in adult mice, while in the second one it occurs at the fetal level. In this sense, our model is closer to the VE-cadherin-cre mice, as CD45 is expressed already in the fetal state of

hematopoiesis and we observe T-ALL but not any myeloid leukemia. These facts suggest that the outcome of the mutation can be affected not only by the tissue where it happens, but also the moment when it occurs. Another point to take into account is the fact that neither Mx1-Cre nor VE-cadherin-cre mice delete PTEN exclusively in hematopoietic cells. We report the first study where PTEN has been knocked in all the hematopoietic system since its very beginning without affecting other cell types and avoiding stimulation of the system with inducers as IFN, which has been reported to modulate hematopoiesis.<sup>28</sup>

Regarding the second question, is worth to mention that the vast majority of lymphoid neoplasms are of B-cell origin (85-90%), with most of the remainder being T cell tumors.<sup>29</sup> Nonetheless, immunostaining analysis of KO mice revealed that 100% of mice displayed T-ALL, but none of them had signs of B-ALL. Previous studies have demonstrated that, while conditional deletion of PTEN in mouse T cells results in lethal T cell lymphomas<sup>22</sup>, animals lacking PTEN in B cells showed altered B lymphocyte differentiation, but no evidence of malignancy.<sup>30</sup> Furthermore, reduction of B cells has also been found by Zhang and collaborators<sup>26</sup> after conditional deletion of PTEN in HSCs using the inducible Mx1-Cre mouse. In agreement to these mouse models for conditional deletion of PTEN, our model suggests that PTEN ablation diverts the formation of lymphoid malignancies to T lineages. Accordingly, PTEN +/- mice, with a single copy of PTEN in all the cell of the organisms, develop T-cell lymphomas, but no B cell neoplasias.<sup>161718</sup>

In summary, we describe a new mouse model in which PTEN has been specifically deleted in CD45 positive hematopoietic cells. Such deletion resulted in the development of T-ALL, but no other types of hematological malignancies such as AML or B-cell lymphomas, indicating that loss of PTEN in hematopoietic cells diverts the formation of hematological neoplasias to the formation of T cell malignancies. As PTEN is a frequently mutated gene in human blood malignancies, understand how it regulates hematopoiesis and the tumorigenic process in mouse models is a valuable tool that will help to improve the understanding of the human disease and the designing of new therapies.

## Author contributions

C.M, N.E, X.M and X.D conceived and designed the experiments

C.M., N.E. and M.A.D. performed the experiments

M.S., and J.Y. provided technical support

X.D, A.M, F.V. and X.M. analyzed the data

C.M. and X.D. wrote the paper

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## FIGURE LEGENDS.

### **Figure 1. CD45:Cre<sup>+/-</sup> PTEN fl/fl mice develop a lethal disease that affects lymphoid organs.**

- (A) Kaplan-Meier curve of WT and KO males and females analyzed for 60 weeks. Note that survival decrease dramatically after 20 weeks and none of the KO animals survived longer than 30 weeks.
- (B) Comparison of representative spleen, liver, thymus and lymph nodes from WT and KO mice.
- (C) Genotyping analysis by standard PCR of the recombined (delta) allele in spleen from KO but not from WT mice.
- (D) Western blot from WT and KO mice showing cre recombinase expression, in concordance with complete PTEN deletion and increase of Akt phosphorylation. Membranes were reprobed with tubulin to ensure protein loading.

### **Figure 2. Histopathological analysis reveals that PTEN deletion in CD45 expressing cells leads to development of lymphomas.**

- A) Representative images showing H&E staining (top panels) and PTEN immunohistochemistry (bottom panels) of spleen from WT and KO mice.
- B) Quantification of mitotic activity in spleens from WT and KO mice. Results are expressed as number of mitotic figures observed per 50 high-power microscope fields.
- C) Representative images and quantification of Ki-67 immunostaining in WT (left) and KO (right) mice.
- D) PTEN deletion in spleen from KO mice increases expression of cyclin D1 and c-Myc, as showed by western blot. Membranes were reprobed with tubulin to ensure protein loading.

### **Figure 3. PTEN deletion in CD45:Cre mice causes diffuse peripheral lymphomas/leukemia.**

- A) Representative images showing H&E staining (top panels) and PTEN immunohistochemistry (bottom panels) from WT and KO mice. Note the

presence of PTEN negative infiltrates in different organs including lung, liver, colon, kidney and ovary.

- B) Flow cytometry analysis of blood samples from WT and KO mice. KO shows and increased percentage of lymphocytes in total blood.
- C) Representative images of papanicolau stained blood extensions obtained from WT and KO mice.

**Figure 4. CD45:Cre<sup>+/-</sup> PTEN fl/fl mice develop T cell but not B cell lymphomas**

- A) Flow cytometry analysis of blood, bone marrow and spleen from WT and KO mice. Deletion of PTEN in CD45 cells leads to a dramatic increase in the amount of T lymphocytes, with a decrease in B cell population.
- B) Representative images of CD3 immunohistochemistry from spleen, lymph nodes and bone marrow from WT and KO mice.
- C) Representative images of myeloperoxidase immunohistochemistry from spleen, lymph nodes and bone marrow from WT and KO mice.
- D) Representative images of CD3 immunohistochemistry in lung, liver, kidney and colon from CD45:Cre<sup>+/-</sup> PTEN fl/fl (KO) mice.
- E) Representative images showing CD3 immunostaining of blood extension obtained from WT and KO mice.

**Figure 5. CD45:Cre<sup>+/-</sup> mice show recombination in all blood cell lineages.**

- A) Flow cytometry analysis of peripheral blood from CD45:Cre mT/mG mice shows green and red fluorescence in both lymphocytes and granulocytes from CD45:Cre<sup>+/-</sup> mT/mG +/- animals.
- B) Representative confocal images corresponding to blood, bone marrow, spleen and lymph nodes from CD45:Cre<sup>+/-</sup> mT/mG +/- mice showing recombination y all tissues. Note the presence of recombined (green) lymphocytes (white arrows) and granulocytes (yellow arrows).

FIGURE 1

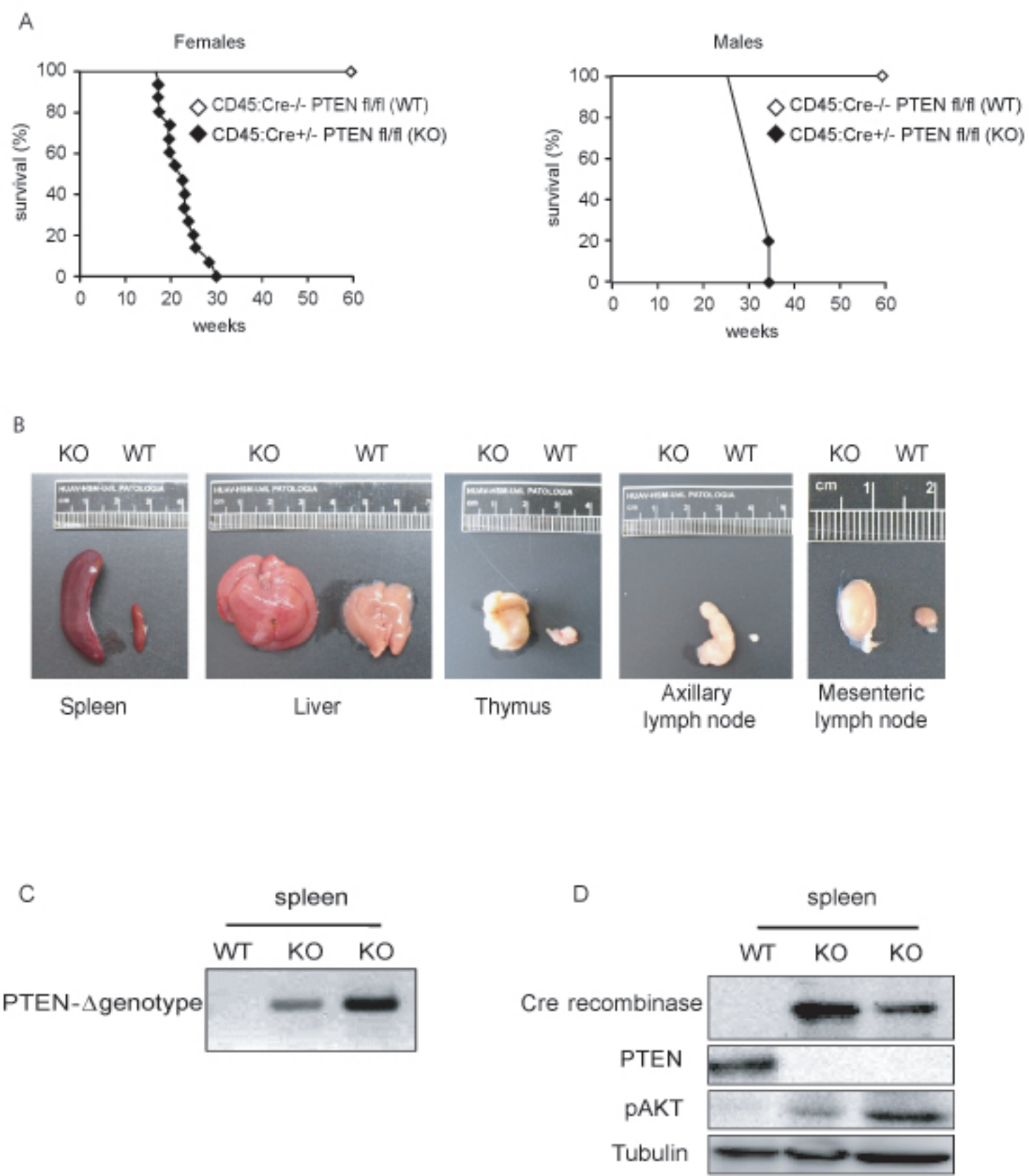
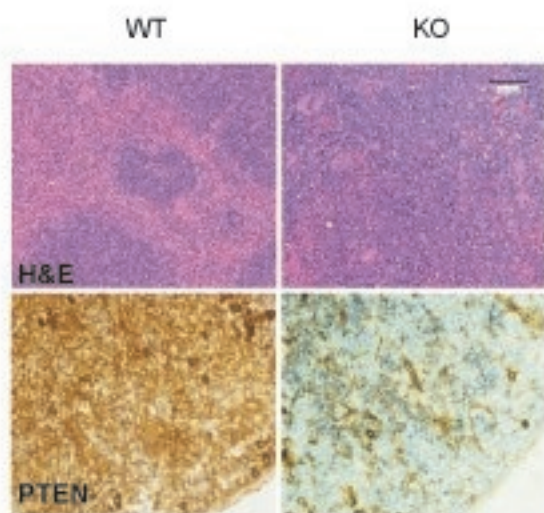
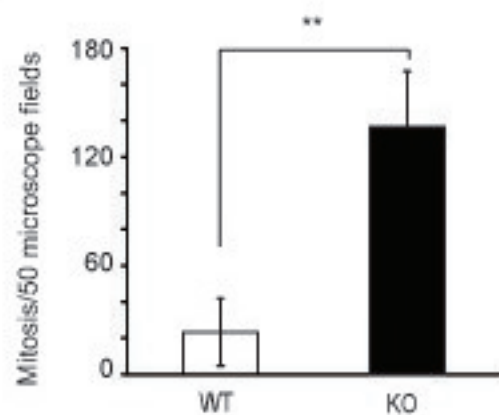


FIGURE 2

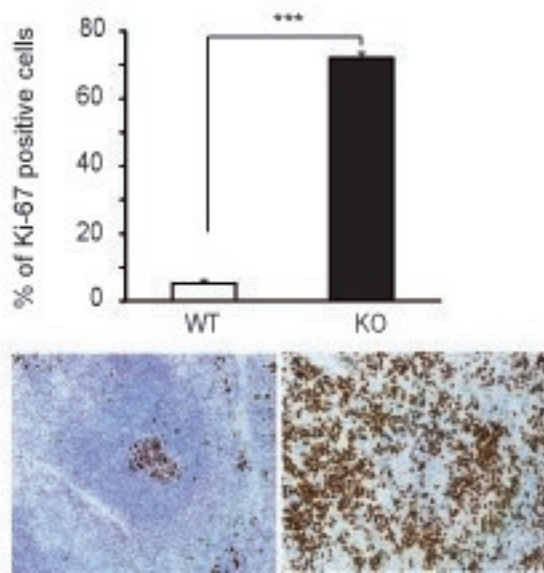
A



B



C



D

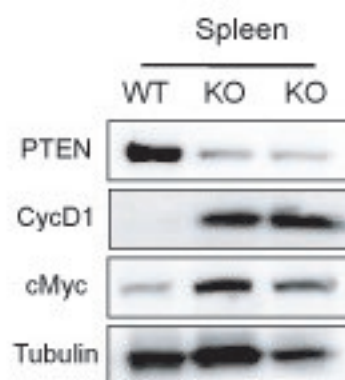
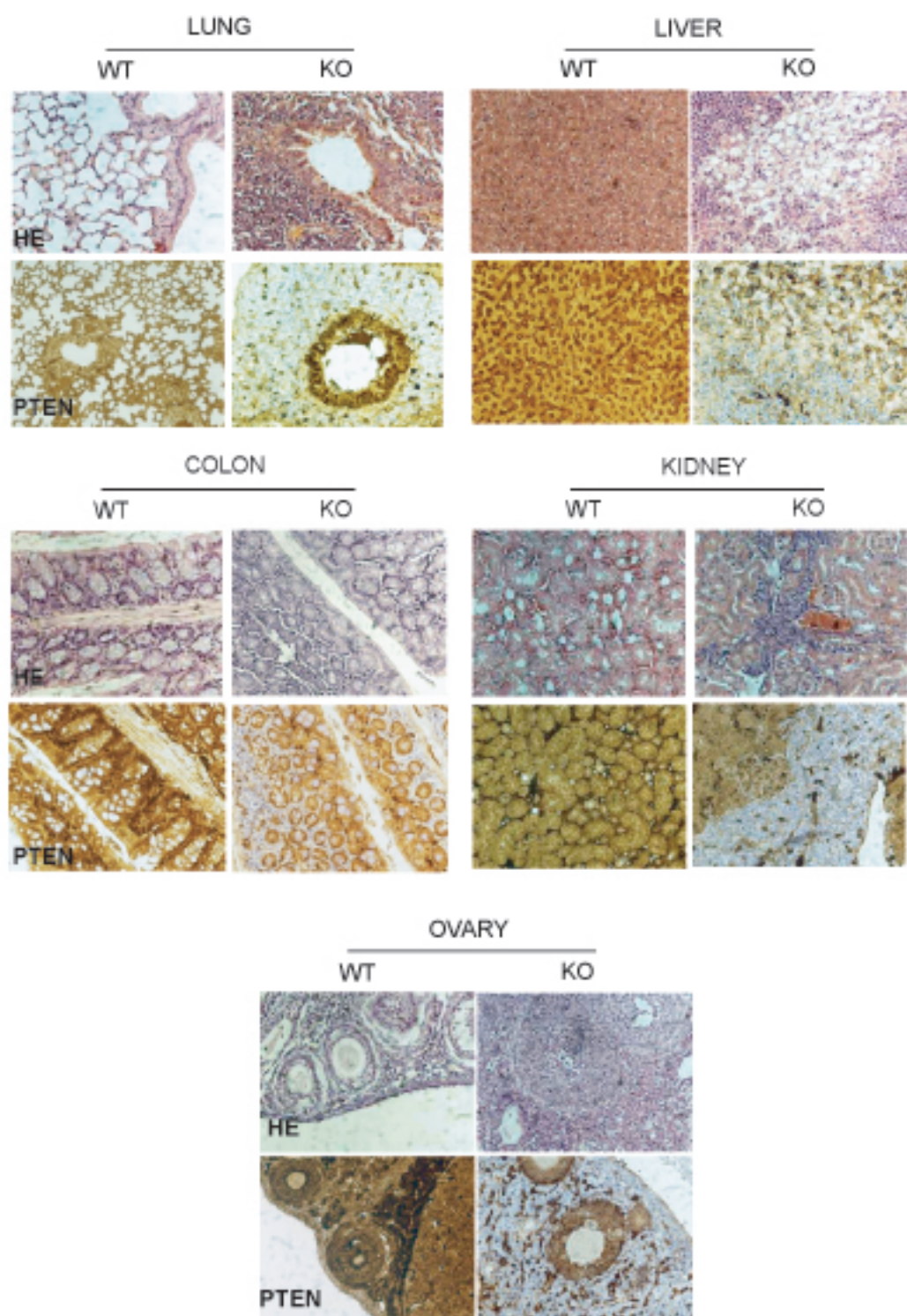
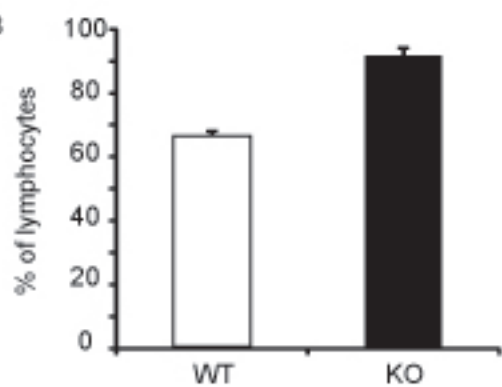


FIGURE 3

A



B



C

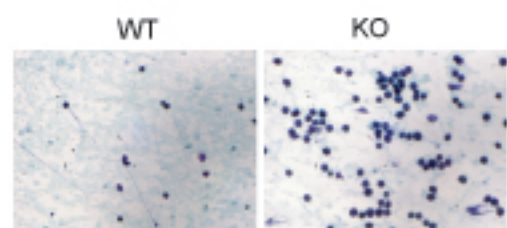


FIGURE 4

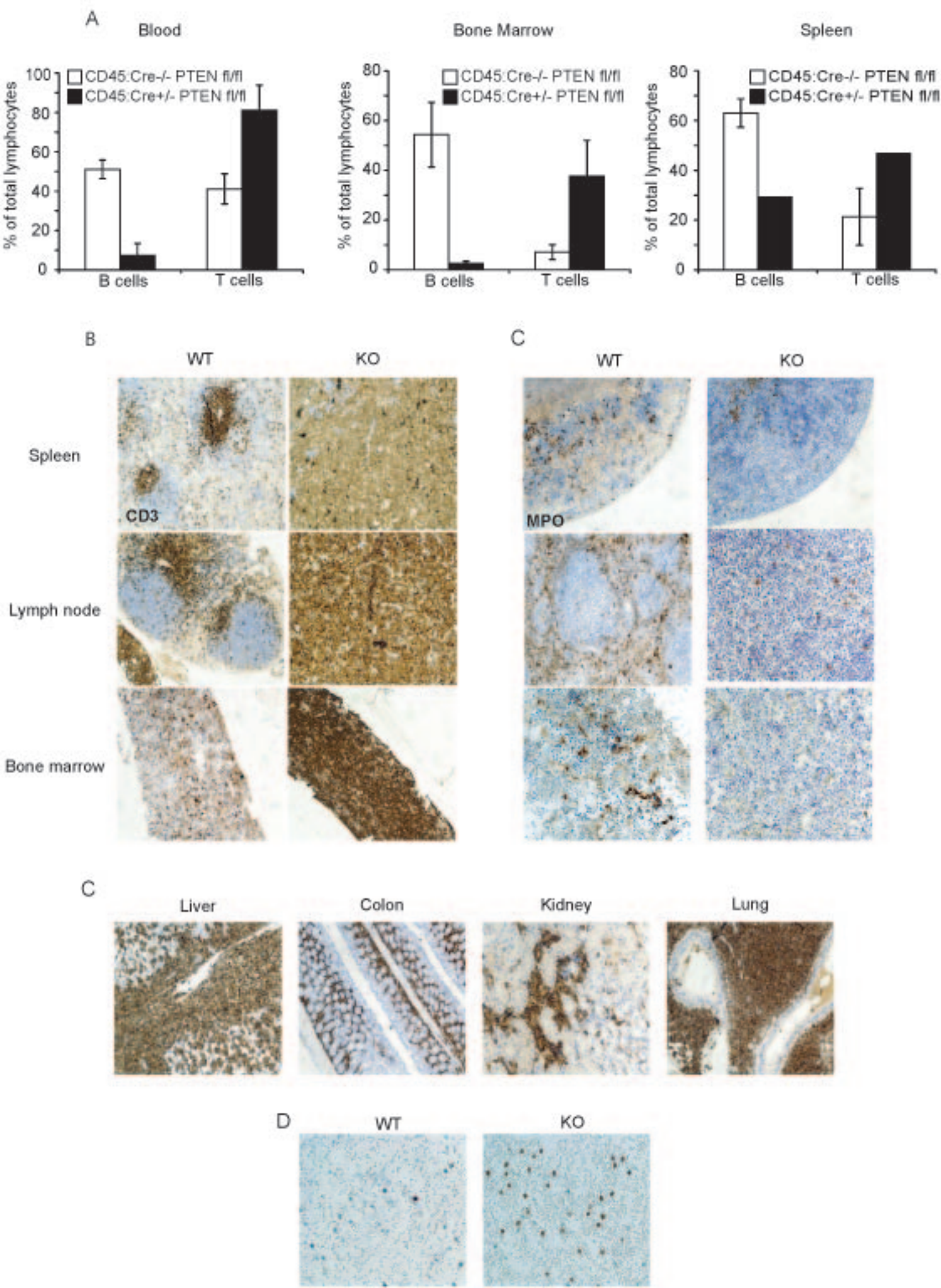
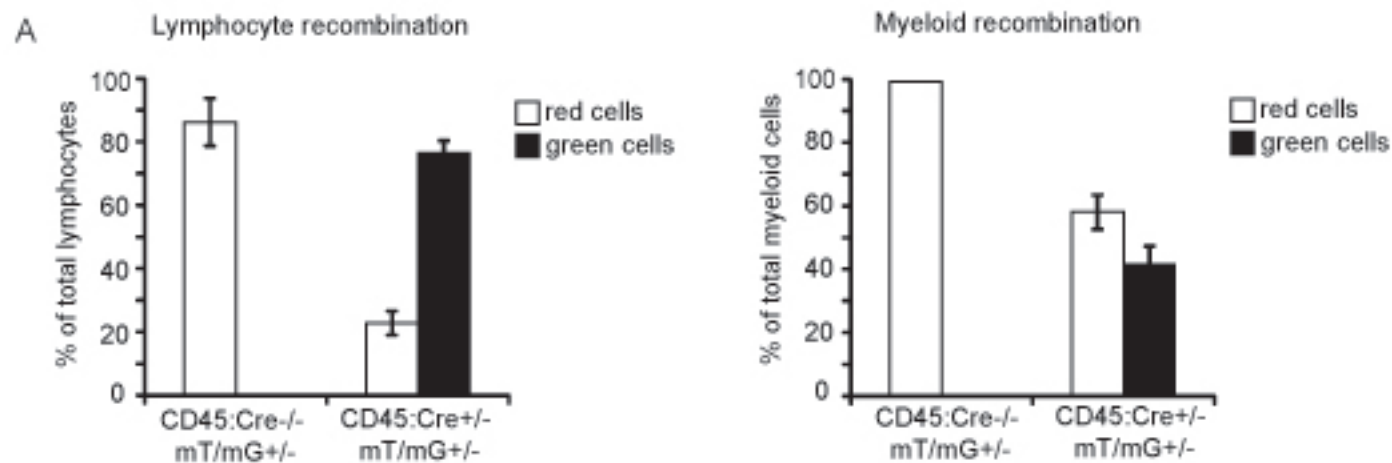


FIGURE 5

**B**

Blood

Bone Marrow

Spleen

Lymph Node