ARTICLE

PDCD5 regulates iNKT cell terminal maturation and iNKT1 fate decision

Ke Wang[1], Xinwei Zhang[1], Yifan Wang[1], Gaowen Jin[1], Mingyang Li[1], Shusong Zhang[1], Jie Hao[1], Rong Jin[1], Xiaojun Huang[2], Hounan Wu[3], Jun Zhang[1], Yingyu Chen[1] and Qing Ge[1]

Invariant natural killer T1 (iNKT1) cells are characterized by the preferential expression of T-box transcription factor T-bet (encoded by *Tbx21*) and the production of cytokine IFN-γ, but the relationship between the developmental process and iNKT1 lineage diversification in the thymus remains elusive. We report in the present study a crucial role of programmed cell death 5 (PDCD5) in iNKT cell terminal maturation and iNKT1 fate determination. Mice with T cell-specific deletion of PDCD5 had decreased numbers of thymic and peripheral iNKT cells with a predominantly immature phenotype and defects in response to α-galactosylceramide. Loss of PDCD5 also selectively abolished the iNKT1 lineage by reducing T-bet expression in iNKT cells at an early thymic developmental stage (before CD44 upregulation). We further demonstrated that TOX2, one of the high mobility group proteins that was highly expressed in iNKT cells at stage 1 and could be stabilized by PDCD5, promoted the permissive histone H3K4me3 modification in the promoter region of *Tbx21*. These data indicate a pivotal and unique role of PDCD5/TOX2 in iNKT1 lineage determination. They also suggest that the fate of iNKT1 may be programmed at the developmental stage of iNKT cells in the thymus.

Cellular & Molecular Immunology (2019) 16:746–756; https://doi.org/10.1038/s41423-018-0059-2

INTRODUCTION

Type I invariant natural killer T cells (iNKT cells) are a critical and unique T-cell subset regulating both innate and adaptive immune responses. They are also involved in a variety of diseases, ranging from infection to cancer, allergy, and autoimmune diseases.[1] During development in the thymus, iNKT cells undergo positive selection upon recognizing CD1d/glycolipid complexes expressed by CD4 and CD8 double-positive (DP) thymocytes and engagement of the signaling lymphocytic activation molecule family (SLAMF) of surface receptors. The postselected iNKT cells pass through a four-stage developmental program from stage 0 (ST0) ($CD24^+CD44^-NK1.1^-$) to ST1 ($CD24^-CD44^-NK1.1^-$) to ST2 ($CD24^-CD44^+NK1.1^-$) and, eventually, to ST3 ($CD24^-CD44^+NK1.1^+$).[2–5] From ST1 to ST3, iNKT cells also differentiate into three effector lineages (iNKT1, iNKT2, and iNKT17 cells) based on their expression patterns of transcription factors and cytokines.[6–9] For instance, iNKT1 cells found within ST3 with a terminally differentiated phenotype are T-$bet^+PLZF^{lo}ROR\gamma t^-IFN\text{-}\gamma^+$, while iNKT2 cells found in ST1 and ST2 with an immature phenotype are GATA-$3^+PLZF^{hi}ROR\gamma t^-IL\text{-}4^+$. Recently, this linear stage-based maturation process of iNKT development has been challenged by the finding that the immature iNKT2 cells cannot give rise to the more mature iNKT1 cells.[8] These results also showed that a proportion of iNKT1 cells originated from CD4 and CD8 double-negative (DN) but not DP thymocytes.[10] Thus, more research is needed to demonstrate the relationship between the developmental maturation process and iNKT1 lineage diversification in the thymus.

The T-box transcription factor T-bet is essential in the lineage determination of iNKT1 and terminal maturation of iNKT cells.[11] Reduced T-bet expression is found in a variety of mutant mice that show decreased iNKT1 differentiation and impaired iNKT maturation, including interleukin-15 (IL-15),[12] TSC1/mTORC1,[13–15] vitamin D receptor,[16] mediator subunit Med1/TRAP220,[17] E and Id proteins,[18,19] Itk,[20,21] TRAF3,[22] Atg7,[23] Atg5,[24] and suppression of Wnt/β-catenin.[25] Although many of these molecules do not affect the earliest stages of iNKT development from DP thymocytes, how and at what stage they regulate T-bet expression and iNKT1 fate determination is not completely clear.[13]

Programmed cell death 5 (PDCD5) is a well-conserved protein that was first identified as an apoptosis-promoting molecule in a variety of cancer cells.[26] It was later found ubiquitously expressed in normal tissues, and deletion of *Pdcd5* resulted in embryonic lethality at mid-gestation.[27] As a p53-positive regulator, PDCD5 inhibits the interaction of p53 with E3-ubiquitin ligase Mdm2,[28,29] enhances the stability and acetylation activity of Tip60,[30] and promotes the cleavage and ubiquitin-dependent proteasomal degradation of histone deacetylase 3 (HDAC3),[31] thus facilitating genotoxic stress-triggered cancer cell apoptosis and TAJ/TROY-induced paraptosis-like cell death.[29,32–34]

In several types of autoimmune diseases and inflammatory processes, elevated PDCD5 can be detected in the serum or synovial fluid of patients.[35–37] Increased cell apoptosis cannot fully explain the connection between PDCD5 level and disease progress. As an altered iNKT cell pool or function has been reported in inflammatory diseases such as rheumatoid arthritis and liver diseases, we investigated whether PDCD5 could play a role in iNKT cell development and function.[38,39] Using PDCD5 conditional knockout mice (*Pdcd5*$^{fl/fl}$*Cd4-Cre*, or PDCD5KO), we

[1]Key Laboratory of Medical Immunology, Ministry of Health. Department of Immunology, School of Basic Medical Sciences, Peking University Health Science Center, 38 Xue Yuan Road, Beijing, China; [2]Peking University Institute of Hematology, People's Hospital, Beijing, China and [3]Peking University Medical and Health Analytical Center, Peking University Health Science Center, Beijing, China
Correspondence: Jun Zhang (junzhang@bjmu.edu.cn) or Yingyu Chen (yingyu_chen@bjmu.edu.cn) or Qing Ge (qingge@bjmu.edu.cn)

Received: 17 February 2018 Accepted: 30 May 2018
Published online: 19 June 2018

found that PDCD5 contributes to iNKT1 fate determination and iNKT terminal maturation by promoting the expression of T-bet and its target molecules. How and at what developmental stage PDCD5 regulates T-bet expression was then investigated.

MATERIALS AND METHODS

Mice

To specifically knock out *Pdcd5* in T and iNKT cells, we bred *Pdcd5*$^{fl/fl}$ mice with *Cd4-Cre* transgenic mice. C57BL/6 congenic mice (CD45.1$^+$ and CD45.2$^+$) were purchased from Peking University Health Science Center (Beijing, China). *Bcl-2* and *Cd4-Cre* transgenic mice were kindly provided by Professors Yu Zhang (Peking University Health Science Center, China) and Lilin Ye (Army Medical University, China), respectively. All the mice were on the C57BL/6 background and were used at 8–12 weeks of age. The animals were kept in a specific pathogen-free facility at Peking University Health Science Center (Beijing, China). The experimental procedures on the use and care of animals had been approved by the Ethics Committee of Peking University Health Science Center.

iNKT cell isolation

Single-cell suspensions of thymocytes were treated with anti-CD8 (3.155) monoclonal antibody and complement (guinea pig sera) to remove CD8$^+$ cells. After two cycles of killing and removal of dead cells by density centrifugation, iNKT cells were sorted using FACS Aria II (BD Biosciences, San Diego, CA, USA). At least 5–6 female mice were used for each purification, and 0.3–2.6 × 10^4 ST1 cells, 1.4–9.2 × 10^4 ST2 cells, and 0.8–3.0 × 10^4 ST3 cells were purified for western blotting.

Immunoprecipitation and western blotting

Cells were collected and lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40) with freshly added protease inhibitor cocktail (Roche, Basel, Switzerland). Appropriate antibodies were added to the lysates, which were rocked for 2 h at 4 °C, followed by the addition of 50 µl of a 50% slurry of Protein A-Sepharose (GE Healthcare, Pittsburgh, PA, USA) overnight at 4 °C. The immunoprecipitates were washed five times in washing buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM EDTA, 1% Nonidet P-40) and analyzed by western blotting. The protein bands were detected by chemiluminescence using the ECL detection reagent SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA) and exposed to ImageQuant LAS 500 (GE Healthcare). Protein bands of iNKT cells were detected by chemiluminescence using the ECL detection reagent SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). Primary antibodies against Flag, ubiquitin, and Ets1 were from Cell Signaling Technology (Danvers, MA, USA), those against green fluorescent protein (GFP), hemagglutinin (HA), and β-actin were from Proteintech (Rosemont, IL, USA), the antibody against PDCD5 was from Abcam (Cambridge, MA, UK), and the antibody against TOX2 was from Invitrogen (Carlsbad, CA, USA).

α-GalCer-induced liver injury

Mice were intravenously injected with 2 µg of α-galactosylceramide (α-GalCer). After 2 h, the levels of interferon-γ (IFN-γ) and IL-4 in the serum were measured by enzyme-linked immunosorbent assay (ELISA), and the percentages of IFN-γ$^+$, IL-4$^+$, and IL-17A$^+$ splenic iNKT cells after 3 h of brefeldin A (BFA; 3 µg/ml) blockade were measured by flow cytometry. After 24 h, the levels of alanine transaminase (ALT) and aspartate transaminase (AST) in serum were measured using an automatic analyzer (Mindray, Shenzhen, China), and the infiltration of neutrophils and the activation of T and B cells in liver were measured by flow cytometry. Liver samples were also fixed in 10% formalin, dehydrated, and embedded in paraffin. Sections with a thickness of 4 µm were stained with hematoxylin and eosin (H&E).

Bone marrow chimera

Recipient CD45.1$^+$ mice were subjected to lethal irradiation (900 rads). After 4 h, WT (CD45.2$^+$) and PDCD5KO (CD45.1$^+$ CD45.2$^+$) bone marrow cells were mixed in a 1:1 ratio, and a total of 5 × 10^6 cells were injected intravenously into recipient mice. Mixed bone marrow (BM) chimeric mice were analyzed 8 weeks after transplantation.

Retroviral production and transduction

For virus packaging, the mouse *Pdcd5* or *Tox2* gene was cloned and inserted into the retrovirus backbone pMSCV-ubc-EGFP. pMSCV-Flag-HA-PDCD5-ubc-EGFP or pMSCV-Myc-TOX2-ubc-EGFP and helper vector pCL-Eco were cotransfected into HEK 293T cells using lipofectamine 2000 (Invitrogen). The supernatant was harvested after 48 h. For retroviral transduction, DN32.D3 cells were suspended with retroviral supernatant in the presence of 4 µg/ml polybrene (Sigma-Aldrich, St Louis, MO, USA) and then spin-infected at 1500 × *g* for 2 h at 32 °C. Retroviral supernatants were then replaced with fresh culture medium after transduction. After overnight culture, cells were spin-infected again and cultured for an additional 48 h before being used for staining.

Statistical analysis

Unpaired Student's *t*-test (two-tailed) was used to evaluate the significance of the differences between two groups using GraphPad Prism 5 software. All data are shown as the mean ± SEM, and $P < 0.05$ was considered significant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$).

Additional experimental procedures are described in the supplemental Materials and Methods.

RESULTS

PDCD5-deficient mice have reduced numbers of iNKT cells

To investigate the functions of PDCD5 in iNKT cells, we bred *Pdcd5*-floxed (*Pdcd5*$^{fl/fl}$) mice with *Cd4-Cre* transgenic mice to generate the *Pdcd5*$^{fl/fl}$*Cd4-Cre* line (PDCD5KO), in which the *Pdcd5* gene was deleted in DP thymocytes. As shown in Fig. 1a, messenger RNA (mRNA) expression of *Pdcd5* was detected in wild-type (WT) DP thymocytes but was barely detectable in PDCD5KO DP thymocytes, indicating efficient deletion of the *Pdcd5* gene (Fig. 1a).

Compared with the *Pdcd5*$^{fl/fl}$*Cd4-Cre*$^-$ (WT) littermate controls, conventional αβT cell populations in PDCD5KO thymi were grossly normal (Fig. 1b, c). In contrast, the percentage and total numbers of iNKT cells in the thymus, spleen, and liver of PDCD5KO mice were nearly 7–23-fold lower than those of WT littermates (Fig. 1d, e). These results indicate that PDCD5 may play an important role in iNKT cell development.

PDCD5 regulates iNKT cells in a cell-intrinsic manner

To investigate whether the reduction of iNKT cells in PDCD5KO mice is caused by intrinsic or extrinsic mechanisms, we generated mixed BM chimeric mice by coinjecting PDCD5KO CD45.1$^+$CD45.2$^+$ and WT CD45.2$^+$ BM cells (1:1 ratio) into lethally irradiated CD45.1$^+$ WT mice. At 8 weeks after reconstitution, the KO (CD45.1$^+$) to WT (CD45.1$^-$) ratio of total thymocyte populations was maintained from 0.8 to 1 in the recipients (Fig. S1A). The ratios of PDCD5KO and WT iNKT cells, however, were 0.12, 0.24, and 0.07 in the thymus, spleen, and liver of WT recipients, respectively (Fig. 2a, b). Together with grossly normal conventional T-cell development in PDCD5KO thymus, these results demonstrate that defective iNKT cell generation is due to cell-intrinsic mechanisms.

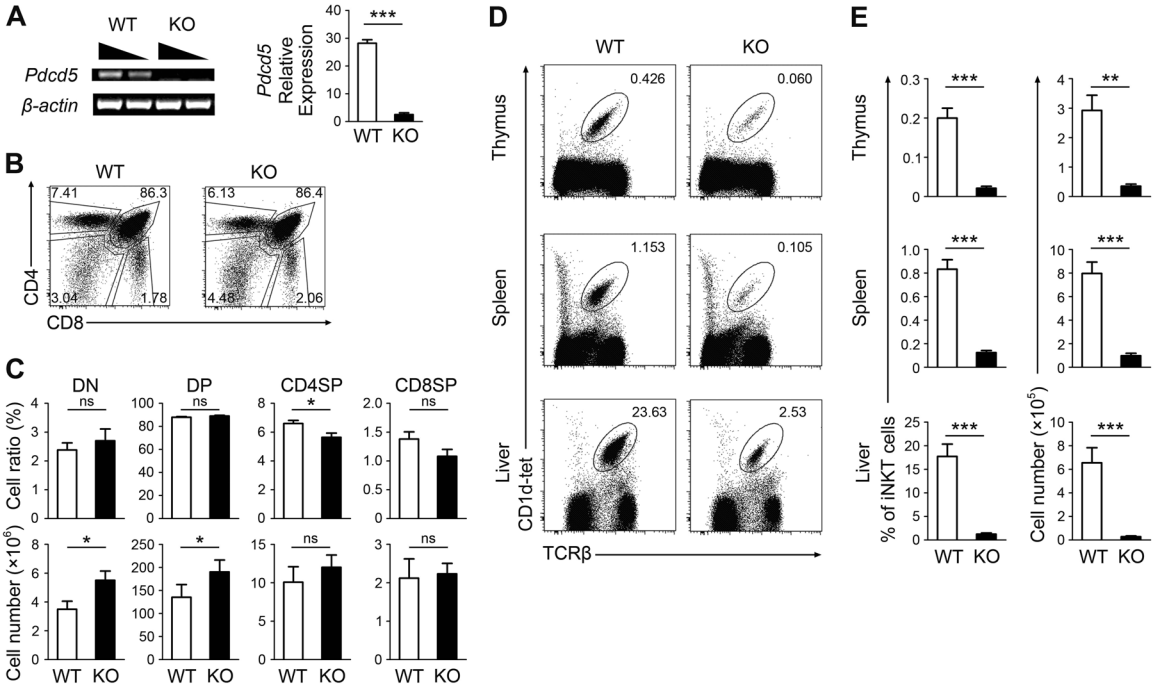

Fig. 1 Mice carrying a T cell-specific deletion of PDCD5 had reduced thymic and peripheral iNKT cells. **a** Semiquantitative RT-PCR (left panel) and quantitative PCR (right panel) analysis of *Pdcd5* mRNA in DP thymocytes from WT and PDCD5KO mice. *β-actin* was used as the housekeeping gene control. Data are representative of 3 independent experiments with 8 mice in each group. **b**, **c** Flow cytometry analysis of CD4 and CD8 expression in WT and PDCD5KO thymocytes. The frequency and number of CD4 and CD8 double-negative (DN), DP, CD4SP, or CD8SP thymocytes are shown (**c**). Data are representative of 3 independent experiments with 5 mice in the WT or PDCD5KO groups. **d**, **e** Flow cytometry analysis of iNKT cells (stained with PBS57-loaded CD1d tetramer (CD1d-tet) and TCRβ) in the thymus, spleen, and liver of WT and PDCD5KO mice. The percentage (left panels) and number (right panels) of iNKT cells in thymus, spleen, and liver were calculated (**e**). Data are representative of 3–4 independent experiments with 5–6 mice in each group. Student's *t*-test was used for statistical analysis. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, ns not significant

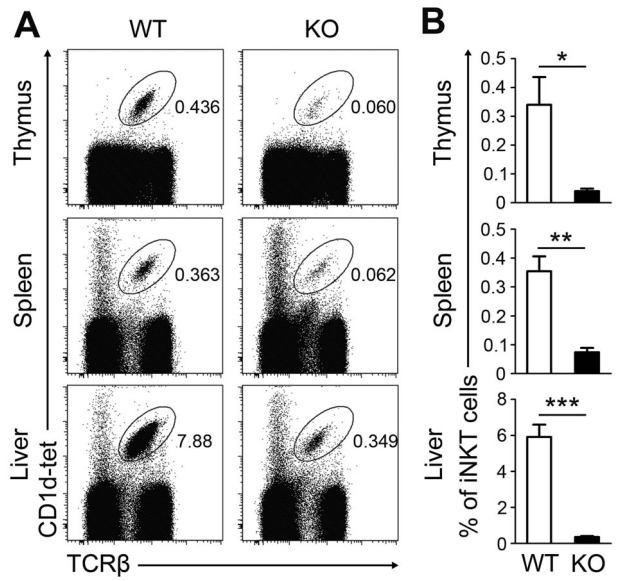

Fig. 2 PDCD5 regulated iNKT cell development in a cell-intrinsic manner. Flow cytometry analysis (**a**) and percentage (**b**) of iNKT cells in the thymus, spleen, and liver from mixed bone marrow chimeric mice are shown. Data are representative of four chimeric mice. Student's *t*-test was used for statistical analysis. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$*

Reduction of iNKT cells in PDCD5KO mice is not due to defects in positive selection

To determine the underlying cause of impaired iNKT development in PDCD5KO mice, we first examined whether PDCD5 was involved in regulating T cell receptor-α (TCRα) rearrangement and positive selection of iNKT cells. We measured DP thymocytes for their expression of Vα14-Jα18 transcripts, an indicator of the rearrangement of the Vα14 gene segment to the Jα18 gene segment (Fig. 3a). We also examined the protein and mRNA expression of CD1d, SLAMF6, SLAMF1, and SLAM-associated protein (SAP), which are known to be essential in the positive selection of iNKT cells (Fig. 3b, c). No significant differences were found between PDCD5KO and their WT littermates. The presentation of CD1d/glycolipid complexes by DP thymocytes was further examined by coculturing DP thymocytes with DN32.D3 iNKT hybridoma cells in the presence or absence of α-GalCer for 24 h. Again, comparable levels of IL-2 production were detected in the samples with WT and PDCD5KO thymocytes (Fig. S1B). Egr family members are one of the earliest transcription factors induced by TCR signaling during positive selection.[40–42] Compared with the WT controls, iNKT cells in PDCD5KO thymus showed similar or slightly higher expression of Egr2 (Fig. 3d). These results indicate that dysregulation of these iNKT selection-related molecules is not the cause of defective iNKT cell development in PDCD5KO mice.

PDCD5 is critical for phenotypic maturation and functional maturation of iNKT cells

We next investigated whether the reduction of iNKT cells in PDCD5KO mice was due to a developmental blockage in the thymus. Compared with their WT counterparts, the percentages of ST0, ST1, and ST2 KO iNKT cells were approximately 3–10-fold higher while that of ST3 cells was 5-fold lower (Fig. 4a). The absolute numbers of ST0 and ST1 cells appeared normal while those of ST2 and ST3 cells were substantially reduced in PDCD5KO mice (Fig. 4a). In particular, the average numbers of ST3 cells were 24.5×10^4 and 0.69×10^4 in WT and PDCD5KO mice, respectively.

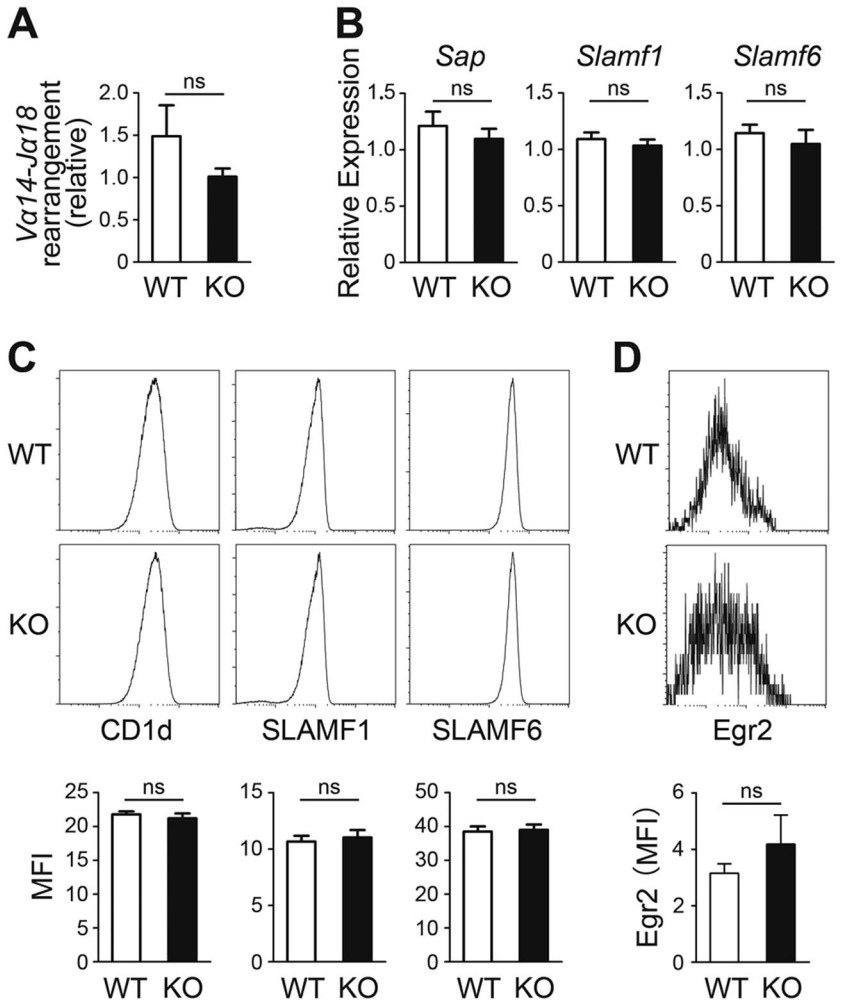

Fig. 3 PDCD5 did not affect thymic-positive selection. **a** Quantitative PCR analysis of invariant *Vα14-Jα18* TCR in WT and PDCD5KO DP thymocytes. Each group had five mice. **b** Quantitative PCR analysis of *Sap*, *Slamf1*, and *Slamf6* mRNA in DP thymocytes. Each group contained four mice. **c** Flow cytometry analysis of the expression of CD1d, SLAMF1, and SLAMF6 in WT and PDCD5KO DP thymocytes. Data are representative of 4–5 mice per group. **d** Flow cytometry analysis of Egr2 expression in stage 0 (ST0) iNKT cells from WT and PDCD5KO mice. Each group contained four mice. Student's *t*-test was used for statistical analysis; ns not significant

Decreased expression of Ly-6C, Ly-49C/I, granzyme B, and CD4 was also found in PDCD5KO iNKT cells in the thymus (Fig. 4b, c; Fig. S2A-B). Consistent with the reduction of NK1.1$^+$ cells in thymus, the percentage of NK1.1$^+$ cells in spleen and liver were also much lower in PDCD5KO than in WT mice (Fig. S2C), indicating that the terminal maturation of iNKT cells is impaired in PDCD5KO mice.

Upon activation, mature iNKT cells rapidly produce high amounts of effector cytokines. To determine whether impaired phenotypic maturation in PDCD5KO iNKT cells was associated with impaired functional maturation, the mice were stimulated with α-GalCer via intravenous injection. Compared with the serum samples obtained from WT mice, the ones from PDCD5KO mice had significantly lower levels of IFN-γ and IL-4 (Fig. 4d). Intracellular staining of cytokines further showed that IFN-γ$^+$IL-4$^+$ ($P = 0.0103$) and IFN-γ$^+$IL-4$^-$ ($P = 0.0581$) iNKT cells were reduced in PDCD5KO mice (Fig. 4e). The percentages of IFN-γ$^-$IL-4$^+$ and IL-17$^+$ iNKT cells were comparable in WT and PDCD5KO mice (Fig. 4e). It is known that cytokines produced by iNKT cells induce liver damage and infiltration/activation of neutrophils and lymphocytes. Compared with WT mice, PDCD5KO mice showed less severe liver damage, as measured by histology and ALT/AST levels (Fig. 4f, g). Reduced accumulation of CD11b$^+$Gr-1$^+$ neutrophils and activation (CD69$^+$) of T and B lymphocytes were observed in PDCD5KO liver (Fig. 4h, i; Fig. S2D). These results indicate that PDCD5KO iNKT cells cannot mount a fully competent response *in vivo*.

PDCD5 is critical for differentiation of iNKT1 cells

To explore the possible causes of reduced mature iNKT cells in PDCD5KO mice, we first measured iNKT cell proliferation and survival at various developmental stages. As shown in Fig. 5a, similar levels of cell proliferation were detected in WT and KO iNKT cells at early developmental stages (ST0 to ST2). In ST3 cells, however, a significant reduction in cell division was observed in WT but not KO samples, indicating that the few remaining ST3 cells in PDCD5KO mice failed to down-regulate cell proliferation.

We then determined whether there is a survival defect in PDCD5KO iNKT cells. As shown in Fig. 5b, comparable levels of cell apoptosis were found in ST0–ST2 iNKT cells in PDCD5KO and WT mice. However, the ratio of Annexin V$^+$ ST3 iNKT cells was 15.44% ± 2.33% in PDCD5KO mice compared to 9.66% ± 0.57% in WT mice (Fig. 5b). Thus, we examined the expression of the pro-survival molecules Bcl-2 and Bcl-xL, both of which were shown to play critical roles in promoting iNKT cell survival.[12,13,43] Compared with WT cells, the expression of Bcl-2 and Bcl-xL in PDCD5KO ST2 and ST3 iNKT cells was slightly but significantly elevated (Fig. 5c, d). The overexpression of a *Bcl2* transgene in PDCD5KO mice failed to

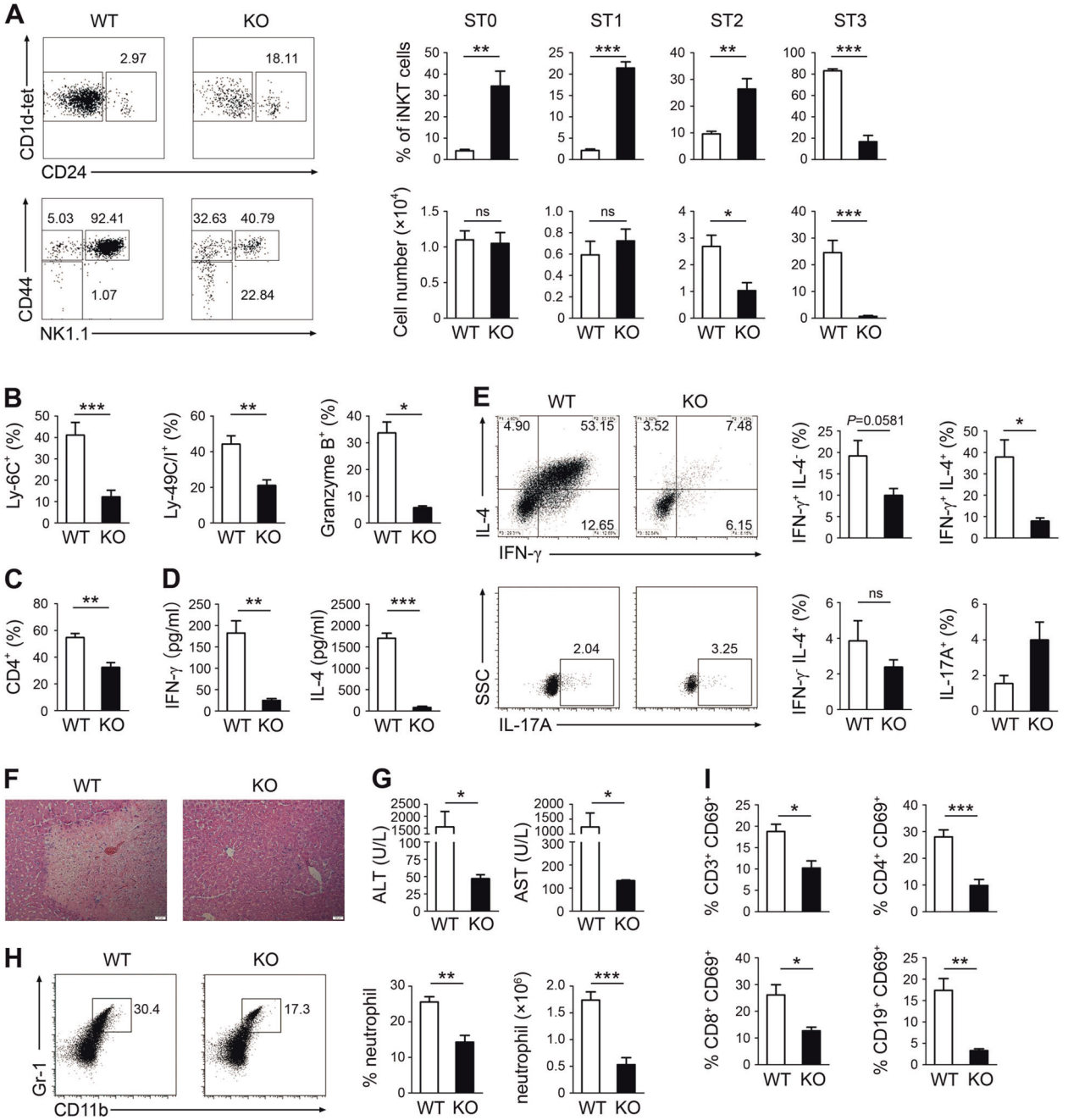

Fig. 4 Impaired phenotypic and functional maturation of thymic iNKT cells in PDCD5KO mice. **a** Thymic development of iNKT cells in WT and PDCD5KO mice was analyzed by first gating on the cells expressing CD1d-tet, TCRβ, CD24, CD44, and NK1.1. ST0 cells with the phenotype of CD1d-tet$^+$TCRβ$^+$CD24$^+$. The CD1d-tet$^+$TCRβ$^+$CD24$^-$ population was further analyzed for their expression of CD44 and NK1.1 (ST1, CD44$^-$NK1.1$^-$, ST2, CD44$^+$NK1.1$^-$, and ST3, CD44$^+$NK1.1$^+$). The frequency and number of each thymic iNKT cell population from WT and PDCD5KO mice were compared (right panels). Data are representative of three independent experiments with five mice in each group. **b** The percentages of Ly-6C$^+$, Ly-49C/I$^+$, and granzyme B$^+$ cells (measured by flow cytometry) in WT and PDCD5KO thymic iNKT cells. Data are representative of 2–5 independent experiments with 3–10 mice in each group. **c** Flow cytometry analysis of CD4 expression in WT and PDCD5KO thymic ST3 iNKT cells. Data are representative of 2 independent experiments with 4–5 mice in each group. **d** Analysis of serum IFN-γ and IL-4 levels by ELISA. WT and PDCD5KO mice were stimulated with α-GalCer (2 μg/mouse) via intravenous injection. The sera were collected 2 h later. Data are representative of 2 independent experiments with 3–4 mice in each group. **e** Flow cytometry analysis of IFN-γ$^+$, IL-4$^+$, and IL-17A$^+$ splenic iNKT cells. At 2 h after α-GalCer stimulation, the splenocytes from WT and PDCD5KO mice were harvested, cultured in the presence of BFA (3 μg/ml) for 3 h, and stained for intracellular cytokines. Data are representative of two independent experiments. **f** Representative H&E staining of liver sections obtained from α-GalCer-stimulated WT and PDCD5KO mice. The scale bar is 20 μm. **g** Quantification of serum ALT and AST obtained from α-GalCer-stimulated mice. Data are representative of 2 independent experiments with 3–4 mice in each group. **h** Flow cytometry analysis of neutrophils (Gr-1$^+$CD11b$^+$) infiltrated in the liver of α-GalCer-immunized mice. Data are representative of two independent experiments with five mice in each group. **i** The percentage of T- and B-cell activation (CD69$^+$) in the liver of α-GalCer-immunized mice. Data are representative of 2 independent experiments with 3–5 mice in each group. Student's *t*-test was used for statistical analysis. **P < 0.05, **P < 0.01, ***P < 0.001*

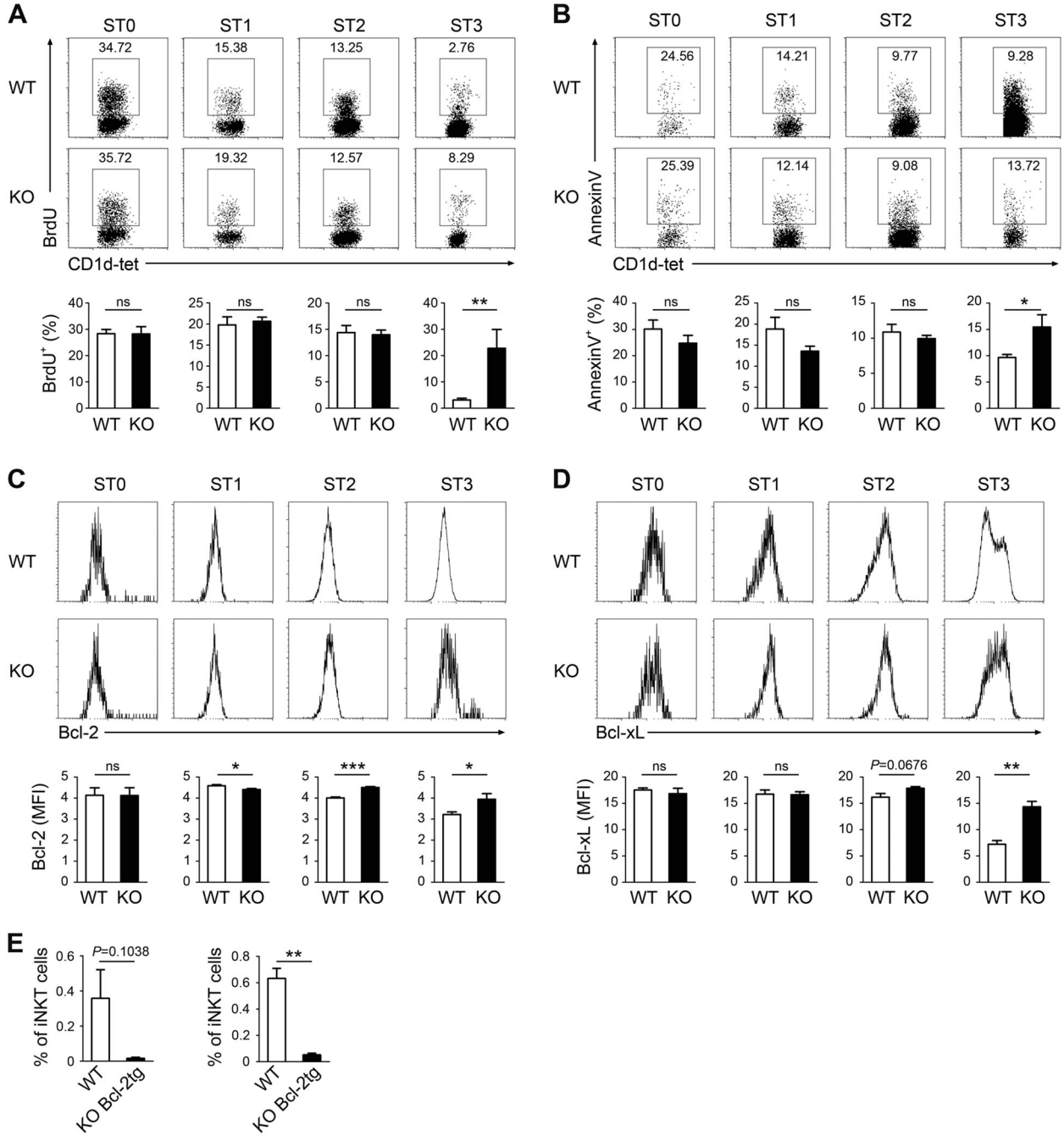

Fig. 5 PDCD5 did not affect cell proliferation and apoptosis at early developmental stages. **a** BrdU incorporation analysis of thymic iNKT cells from WT and PDCD5KO mice after 24 h of BrdU injection. Data are representative of 2–3 independent experiments with 4–7 mice in each group. **b** Flow cytometry analysis of Annexin V^+ cells in WT and PDCD5KO thymic iNKT cells. Data are representative of 2 independent experiments with 4–5 mice in each group. **c**, **d** Flow cytometry analysis of Bcl-2 (**c**) and Bcl-xL (**d**) expression in WT and PDCD5KO thymic iNKT cells. Data are representative of 2 independent experiments with 4–5 mice in each group. **e** Comparison of iNKT cell frequencies in the thymus (left panel) and spleen (right panel) of WT and *Pdcd5*-null *Bcl2*tg mice. Data are representative of three independent experiments with three mice in each group. Student's *t*-test was used for statistical analysis. **P < 0.05, **P < 0.01, ***P < 0.001*

restore the percentage and absolute number of iNKT cells in the thymus and periphery, suggesting that the reduced iNKT cells in *Pdcd5*-deficient mice may result from a developmental defect (Fig. 5e).

As IFN-γ-producing iNKT1 cells were mostly found in ST3 cells in the thymus and the functional defects in PDCD5KO iNKT cells were more pronounced in IFN-γ production, we investigated whether the reduction in PDCD5KO ST3 cells was associated with impaired iNKT1 cell differentiation. Compared with WT thymi, PDCD5KO thymi showed a significant reduction in the percentage of $PLZF^{lo}ROR\gamma t^-$ iNKT1 cells and increases in $PLZF^{hi}ROR\gamma t^-$ iNKT2 and $PLZF^{int}ROR\gamma t^+$ iNKT17 cells (Fig. 6a, b). The numbers of PDCD5KO iNKT1 and iNKT2 cells were lower than WT (Fig. 6b). iNKT17 cell numbers were similar between KO and WT thymi (Fig. 6b). We further determined the cytokine profile of these iNKT cells by in vitro stimulation of the thymocytes with PMA and ionomycin. PDCD5KO iNKT cells showed significantly less IFN-γ^+IL-4^- and IFN-γ^+IL-4^+ cells but similar IFN-γ^-IL-4^+ and

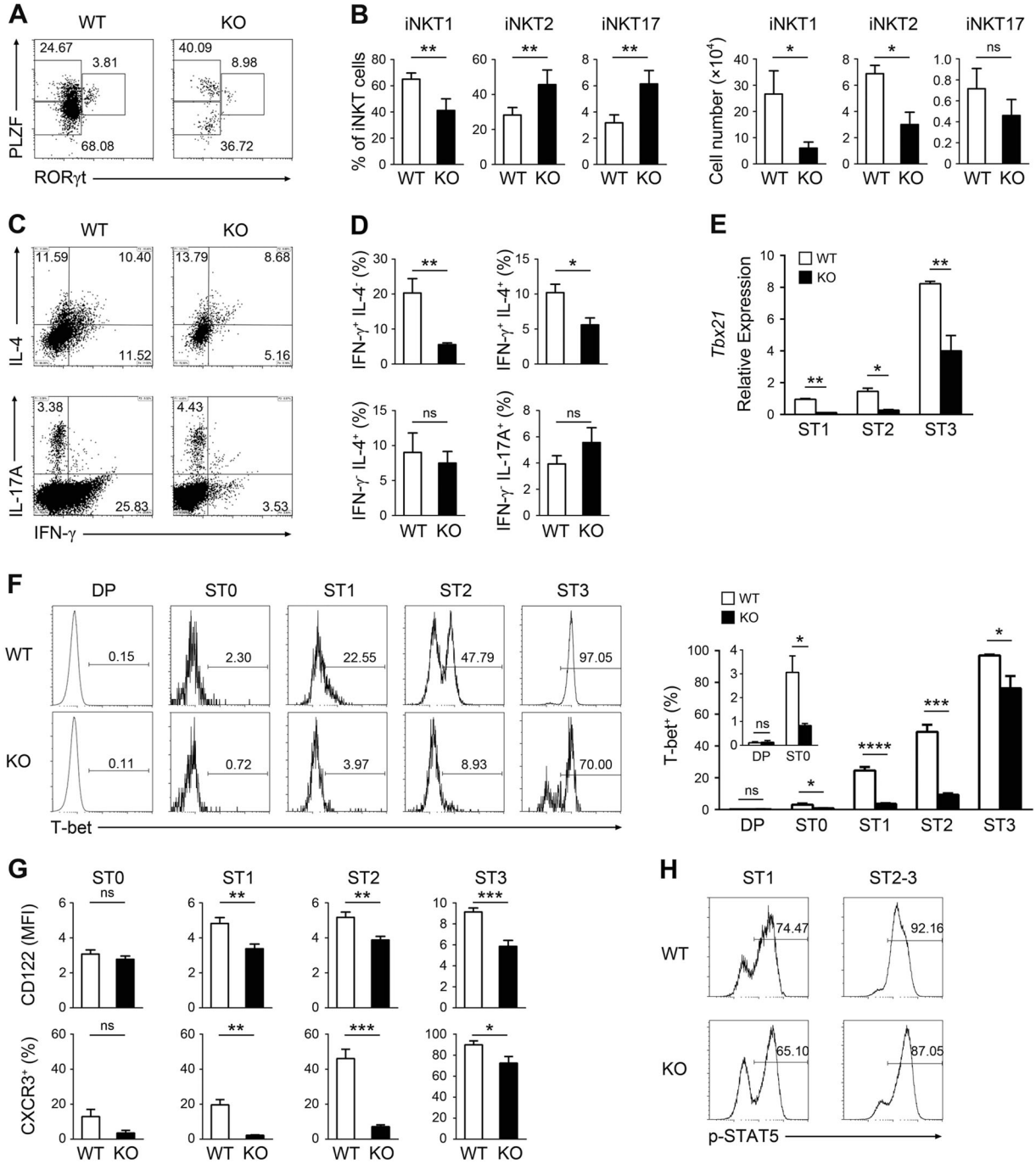

Fig. 6 Thymic iNKT1 cell development was impaired in PDCD5KO mice. **a**, **b** Flow cytometry comparison of iNKT1 (PLZF^{lo}RORγt^{-}), iNKT2 (PLZF^{hi}RORγt^{-}), and iNKT17 (PLZF^{int}RORγt^{+}) cells in thymic CD24^{-} iNKT cells obtained from WT and PDCD5KO mice. The percentages (left panel) and numbers (right panel) of iNKT1, iNKT2, and iNKT17 cells were calculated (**b**). Data are representative of 3–4 independent experiments with 4–9 mice per group. **c**, **d** Intracellular staining of IFN-γ, IL-4, and IL-17A in thymic CD24^{-} iNKT cells following 4 h of PMA (10 ng/ml) and ionomycin (100 ng/ml) stimulation and BFA (3 μg/ml) blockade. The percentages of IFN-γ^{+}IL-4^{-}, IFN-γ^{+}IL-4^{+}, IFN-γ^{-}IL-4^{+}, and IFN-γ^{-}IL-17A^{+} thymic CD24^{-} iNKT cells are shown (**d**). Data are representative of 2–3 independent experiments with 3–5 mice per group. **e** Quantitative PCR analysis of *Tbx21* mRNA in WT and PDCD5KO thymic iNKT cells. Data are representative of four independent experiments. **f** Flow cytometry analysis of T-bet expression in WT and PDCD5KO thymic iNKT cells and DP thymocytes. The percentages of T-bet^{+} cells among various cell populations were compared between WT and PDCD5KO mice (right panel). Data are representative of 3–4 independent experiments with 4–7 mice per group. **g** Comparison of the fluorescence intensity of CD122 staining (top panel) and percentage of CXCR3^{+} cells (bottom panel) between WT and PDCD5KO thymic iNKT cells. Data are representative of 3–4 independent experiments with 4–7 mice per group. **h** Flow cytometry analysis of p-STAT5 in WT and PDCD5KO thymic iNKT cells after 10 min of IL-15 stimulation. Data are representative of two independent experiments with three mice per group. Student's *t*-test was used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001

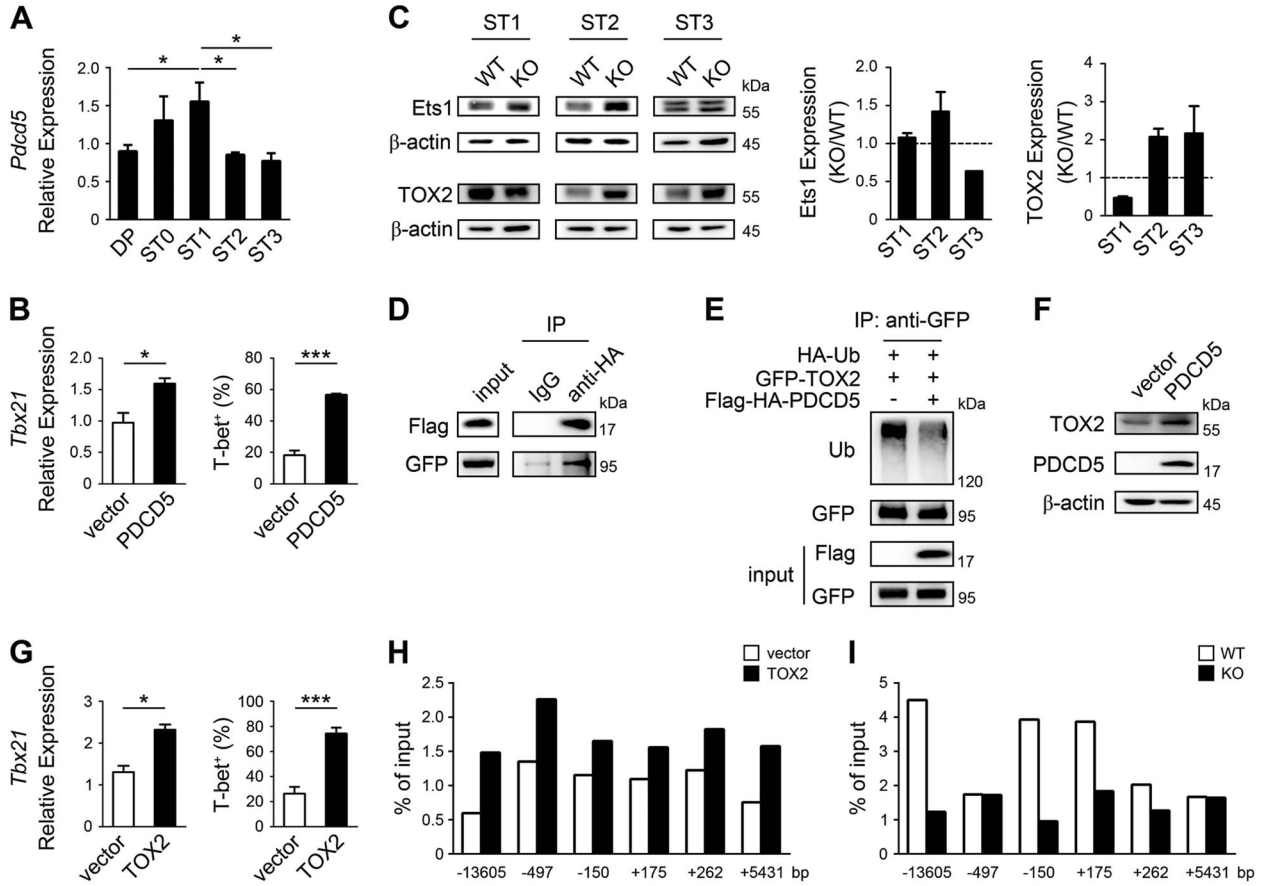

Fig. 7 Epigenetic regulation of T-bet expression by PDCD5/TOX2. **a** Quantitative PCR analysis of *Pdcd5* mRNA in DP thymocytes and various thymic iNKT cell populations obtained from WT and PDCD5KO mice. Data are representative of 2–4 independent experiments. **b** The analysis of *Tbx21* mRNA (by quantitative PCR, left panel) and the T-bet$^+$ cell percentage (by flow cytometry, right panel) in vector- or PDCD5-transduced DN32.D3 cells. Data are representative of three independent experiments. **c** Western blotting of Ets1 and TOX2 expression in WT and PDCD5KO thymic iNKT cells (left panel). The ratio of KO-to-WT Ets1 and TOX2 protein expression is shown in the right panels. The expression levels were normalized to β-actin. Data are representative of 2–4 independent experiments. **d** 293T cells were cotransfected with Flag-HA-PDCD5 and GFP-TOX2 plasmids. The interaction of PDCD5 and TOX2 was measured by immunoprecipitation and western blotting. Data are representative of two independent experiments. **e** 293T cells were cotransfected with HA-Ub, GFP-TOX2, and Flag-HA-PDCD5 or control vector. After 24 h, the cells were treated with 10 μM MG132 for another 6 h. Ubiquitination of TOX2 was measured by immunoprecipitation and western blotting. Data are representative of two independent experiments. **f** Western blotting of TOX2 expression in vector- or PDCD5-transduced DN32.D3 cells. **g** Analysis of *Tbx21* mRNA (by quantitative PCR, left panel) and the T-bet$^+$ cell percentage (by flow cytometry, right panel) in vector- or TOX2-transduced DN32.D3 cells. Data are representative of 2–3 independent experiments. **h** ChIP-qPCR of the H3K4me3 modification in the promoter region of *Tbx21* in vector- or TOX2-transduced DN32.D3 cells. Data are representative of two independent experiments. **i** ChIP-qPCR of the H3K4me3 modification in the promoter region of *Tbx21* in thymic iNKT cells from WT and PDCD5KO mice. Data are representative of two independent experiments. Student's *t*-test was used for statistical analysis. *P < 0.05, ***P < 0.001

IFN-γ$^-$IL-17A$^+$ cells when compared to WT (Fig. 6c, d). These data demonstrate that the differentiation of iNKT1 cells is defective in PDCD5KO mice.

A recent finding suggests that a proportion of IFN-γ$^+$ DN iNKT cells originate from the DN stage before CD4 and CD8 expression.[10] Thus, we examined whether the few remaining IFN-γ$^+$ iNKT cells in *Pdcd5$^{fl/fl}$Cd4*$^-$Cre mice were DN cells developed directly from the DN stage without *Pdcd5* deletion. As shown in Fig. S3A, the percentage of CD4$^+$ cells in CD24$^-$IFN-γ$^+$ iNKT cells was slightly increased in PDCD5KO than in WT mice, while the percentage of IFN-γ$^+$ cells in CD24$^-$CD4$^+$ or CD24$^-$CD4$^-$ iNKT cells was substantially reduced in PDCD5KO than in WT mice. The IL-7 receptor was highly expressed in IFN-γ$^+$ iNKT cells of DN-stage origin.[10] We also measured CD127 (IL-7 receptor α) expression and found comparable levels of CD127 in WT and PDCD5KO ST3 iNKT cells (Fig. S3B). Therefore, although the existence of IFN-γ$^+$ cells of DN-stage origin cannot be excluded among the few remaining IFN-γ$^+$ iNKT cells in PDCD5KO mice, these results again indicate that the differentiation of iNKT1 cells of DP-stage origin is impaired in PDCD5KO mice.

PDCD5 regulates T-bet expression in iNKT cells

T-bet (encoded by *Tbx21*) is the master transcription factor regulating iNKT1 differentiation and iNKT cell maturation.[8] As shown in Fig. 6e, the transcription of *Tbx21* in WT iNKT cells was low in ST1 and ST2 cells but significantly upregulated in ST3 cells. PDCD5KO iNKT cells, however, showed lower levels of T-bet mRNA at all stages. Notably, the upregulation of *Tbx21* transcription could still be detected in KO ST3 cells (Fig. 6e). The levels of T-bet protein were also lower in PDCD5KO iNKT cells at all stages, as measured by flow cytometry, yet an upregulation of T-bet could still be observed in the few remaining ST3 cells in PDCD5KO mice, albeit at a lower level (Fig. 6f). We further found that the ratio of CD4$^+$ cells in T-bet$^+$ ST3 iNKT cells was more than 50% in WT mice and less than 40% in PDCD5KO mice. The ratio of T-bet$^+$ cells in CD4$^-$ ST3 iNKT cells was more than 90% in WT mice and 30–60%

in PDCD5KO mice (Fig. S4A). Taken together, these results suggest that PDCD5 is involved in regulating T-bet expression in iNKT cells at early developmental stages, even though a few remaining cells that manage to reach ST3 show an upregulation of T-bet.

The expression of CD122 (IL-15Rβ) and CXCR3 in iNKT cells is regulated by T-bet and shows maximal elevations in ST3 cells. CD122 is also involved in the further upregulation of T-bet in ST3 cells.[44] Compared with WT iNKT cells, PDCD5KO iNKT cells at ST1–ST3 showed significant reductions in CD122 and CXCR3 expression (Fig. 6g; Fig. S4B-C). The expressions of CD122 and CXCR3 in ST0 iNKT cells was very low but similar between PDCD5KO and WT mice. Consistent with the upregulation of T-bet in PDCD5KO ST3 cells, an elevation of CD122 and CXCR3 was also observed in these cells (Fig. 6g; Fig. S4B-C). We further examined IL-15/CD122 responsiveness by measuring the phosphorylation of signal transducer and activator of transcription 5 (p-STAT5). Compared with WT cells, the percentage of p-STAT5^{+} cells in PDCD5KO iNKT cells was slightly reduced (Fig. 6h). Taken together with the data for T-bet, these results suggest that PDCD5 is required for T-bet/CD122/CXCR3 expression in iNKT cells at early developmental stages.

Epigenetic regulation of Tbx21 transcription by PDCD5/TOX2

To investigate how PDCD5 regulates T-bet expression in iNKT cells, we first measured the expression levels of PDCD5 at various developmental stages. Compared with DP thymocytes, *Pdcd5* transcription was upregulated in ST0 and ST1 iNKT cells and then downregulated in ST2 and ST3 cells (Fig. 7a). We next determined whether T-bet expression could be altered by PDCD5. Compared with the controls, higher levels of T-bet mRNA and protein were found in DN32.D3 cells transduced with PDCD5-expressing retrovirus (Fig. 7b). These results suggest that T-bet expression can be regulated by PDCD5.

The transcription factors that positively regulate T-bet expression include Ets1 and thymocyte selection-associated high mobility group-box 1 and 2 (TOX1 and TOX2) in T cells and/or NK cells.[45–48] To examine how PDCD5 regulates T-bet, we performed western blotting and found that the expression level of Ets1 was not altered while that of TOX2 was reduced in ST1 iNKT cells in PDCD5KO mice (Fig. 7c, the number of ST0 cells was too few to perform western blotting). In contrast, ST3 iNKT cells in WT mice downregulated TOX2 to a minimum level while those in PDCD5KO mice maintained a relatively high level of TOX2 (Fig. 7c). We further performed a coimmunoprecipitation experiment and found an interaction of PDCD5 with TOX2 but not with Ets1 (Fig. 7d and data not shown). Similar to the role of PDCD5 in promoting the stability of p53 by inhibiting its interaction with E3-ubiquitin ligase,[28,29] we also found that the ubiquitination of TOX2 was suppressed by PDCD5 (Fig. 7e). Furthermore, higher levels of TOX2 protein were found in PDCD5-transduced DN32.D3 cells than the controls (Fig. 7f). These data suggest that in ST1 iNKT cells, the high level of TOX2 is at least partially due to the high level of PDCD5 expression, which prevents the proteasomal degradation of TOX2.

We next investigated whether TOX2 could regulate T-bet expression in iNKT cells. The transduction of TOX2-expressing retrovirus led to an upregulation of T-bet mRNA and protein in DN32.D3 cells (Fig. 7g). However, we did not observe positive bindings of TOX2 to the promoter region of *Tbx21* by chromatin immunoprecipitation (ChIP)-quantitative PCR (Fig. S5A). The promoter region of *Il2rb* was also examined, and no binding of TOX2 was detected (Fig. S5B). In T effector cells, histone modifications have been shown to be essential in the regulation of *Tbx21* transcription.[49,50] With a highly conserved high mobility group DNA binding domain, TOX2 may function in a similar way to other high mobility group proteins to alter chromatin structure and the accessibility of transcription factors to DNA.[51] Thus, we examined the profile of permissive histone three lysine four trimethylation (H3K4me3) in DN32.D3 cells in the presence or absence of TOX2. As shown in Fig. 7h, TOX2 increased the H3K4me3 modification in the promoter region of *Tbx21*. Consistent with this result *in vitro*, the H3K4me3 modification in the promoter region of *Tbx21* was significantly reduced in PDCD5KO than in WT iNKT cells (Fig. 7i). Taken together, these data indicate that TOX2 promotes T-bet expression by epigenetic modulation of the histone H3K4 methylation status at the *Tbx21* gene locus.

DISCUSSION

The development and maturation of iNKT cells require many different signaling pathways that are activated at the right stage and at appropriate levels. In the current study, we demonstrate that the epigenetic regulation of T-bet expression by PDCD5/TOX2 at early developmental stages of thymic iNKT cells is crucial for lineage determination of iNKT1 cells and terminal maturation of ST3 iNKT cells.

The master transcription factor for iNKT1 differentiation and ST3 iNKT cell terminal maturation is T-bet.[11,52] A significant upregulation of T-bet mRNA and protein can be detected in thymic ST3 iNKT cells.[11,13] The loss of PDCD5 leads to reduced T-bet expression but does not block T-bet upregulation in the few remaining ST3 cells. This finding suggests that the substantial changes in T-bet expression during ST2-to-ST3 transition may not be sufficient to determine the fate of iNKT1 and promote the maturation of iNKT cells. Notably, a small percentage of ST0 iNKT cells already express a low level of T-bet (Fig. 6f),[11,13] likely induced by TCR/TRAF3 signaling[53] and promoted by PDCD5/TOX2. Thus, the reduced T-bet expression at early developmental stages (ST0/ST1) may account for the decreased numbers of ST3 cells in PDCD5KO mice. A similar phenomena can be observed in a variety of mutant mice that also have defects in terminal maturation or iNKT1 differentiation, including IL-15$^{-/-}$ mice, ET-2 knockin mice, or mice with T cell-specific deletion of *Tsc1* and *Traf3*.[12,13,19,22] In these mice, reduced T-bet expression is also detected in thymic ST1 or ST2 iNKT cells. However, ST3 cells in mutant mice still show an upregulation of T-bet mRNA or protein, even though the degree of upregulation in some cases is less than that in wild-type controls. Taken together, these findings indicate that T-bet induction in ST0/ST1 cells is critical for ST3 cell maturation and iNKT1 fate decisions. They also suggest that a second and different signal is required for the upregulation of T-bet in ST3 cells, which may further promote cell survival and IFN-γ production.

The data from PDCD5KO mice also demonstrate that early T-bet expression in ST1 and probably also in ST0 cells (ST0 cell numbers were too small to generate clear and consistent results from western blotting) depends on the PDCD5/TOX2 pathway. First, the expression of PDCD5 and TOX2 is highest in ST1 cells, consistent with the developmental stage in which T-bet is induced. Second, T-bet expression can be promoted by overexpression of PDCD5 and TOX2, respectively. Third, the interaction of PDCD5 with TOX2 prevents the ubiquitination and proteasomal degradation of TOX2. Fourth, TOX2 enhances a permissive H3K4me3 modification in the promoter region of *Tbx21*. Thus, *Pdcd5*-deficient iNKT cells at an early developmental stage exhibit reduced TOX2 activity and the H3K4me3 mark at the *Tbx21* locus, leading to impaired T-bet expression and iNKT1 differentiation and maturation. Notably, the level of TOX2 is decreased in WT ST2 and ST3 iNKT cells compared with ST1 cells, further suggesting that factors other than TOX2 may regulate the final upregulation of T-bet during the ST2-to-ST3 transition. Thus, even though the expression of TOX2 at later stages (ST2 and ST3) of PDCD5KO iNKT cells remains high and may play a role in maintaining the accessibility of the *Tbx21* locus, it may not be sufficient to fully upregulate T-bet expression and facilitate the ST2-to-ST3 transition or iNKT1 development.

The epigenetic regulation of genes organizes gene loci and alters the accessibility of transcription factors and polymerases to gene promoters and enhancers. In particular, histone H3K4me3 modification at transcription start sites is associated with open access and active transcription. Three pairs of SET/MLL family members, SETd1A/SETd1B, MLL1/MLL2, and MLL3/MLL4, are H3K4 methyltransferases that are critical for mammalian hematopoiesis.[54] Whether TOX2 modulates the recruitment of one of these enzymes or their partners awaits further investigation.

Thus, we demonstrate that early induction of T-bet before CD44 upregulation is crucial for iNKT1 fate determination and iNKT cell maturation in the thymus. PDCD5 promotes the early transcription of T-bet by stabilizing TOX2 and subsequently promoting the permissive H3K4me3 modification in the promoter region of the *Tbx21* locus.

ACKNOWLEDGEMENTS

The authors wish to thank Yu Zhang (Peking University Health Science Center, China) and Lilin Ye (Army Medical University, China) for kindly providing *Bcl2* and *Cd4*$^{-}$*Cre* transgenic mice, Li Bai (University of Science and Technology of China, China) for the DN32.D3 cell line, and Zhongjun Dong (Tsinghua University, China) for the pMSCV-ubc-EGFP and pCL-Eco plasmids. The authors also thank Zhongjun Dong, Xiaoyu Hu (Tsinghua University, China), Yu Zhang, Yanhui Yin, Dan Lv, Chao Zhong, and Wenling Han (Peking University Health Science Center, China) for critical comments, helpful discussions, and critical reagents. The α-galactosylceramide and unloaded and PBS57-loaded CD1d tetramers conjugated to phycoerythrin or allophycocyanin were supplied by the National Institutes of Health tetramer facility. This work was supported by grants from the National Key Research and Development Program of China, 2017YFA0104500 (Q.G.), the National Natural Science Foundation of China, 81471525 and 31671244 (Q.G.), 31470843 (J.Z.),31370898 (Y.C.) the Foundation for Innovative Research Groups of the National Natural Science Foundation of China, 81621001 (Q.G.), Program for New Century Excellent Talents in University, NCET-13-0018 (J.Z.) and the Fundamental Research Funds for the Central Universities.

AUTHOR CONTRIBUTIONS

Q.G. and K.W. designed the research, analyzed data, and wrote the manuscript. K.W. performed the research. X.Z., Y.W., S.Z., G.J., and M.L. performed the research. J.H., R.J., and X.H. contributed reagents and technical support. H.W. helped with the flow cytometry. Y.C. and J.Z. edited the manuscript. All authors reviewed the manuscript.

ADDITIONAL INFORMATION

The online version of this article (https://doi.org/10.1038/s41423-018-0059-2) contains supplementary material.

Competing interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

REFERENCES

1. Georgiev, H., Ravens, I., Benarafa, C., Forster, R. & Bernhardt, G. Distinct gene expression patterns correlate with developmental and functional traits of iNKT subsets. *Nat. Commun.* **7**, 13116 (2016).
2. Bendelac, A., Savage, P. B. & Teyton, L. The biology of NKT cells. *Annu. Rev. Immunol.* **25**, 297–336 (2007).
3. Godfrey, D. I., Stankovic, S. & Baxter, A. G. Raising the NKT cell family. *Nat. Immunol.* **11**, 197–206 (2010).
4. Kronenberg, M. Toward an understanding of NKT cell biology: progress and paradoxes. *Annu. Rev. Immunol.* **23**, 877–900 (2005).
5. Ma, C. S., Nichols, K. E. & Tangye, S. G. Regulation of cellular and humoral immune responses by the SLAM and SAP families of molecules. *Annu. Rev. Immunol.* **25**, 337–379 (2007).
6. Brennan, P. J., Brigl, M. & Brenner, M. B. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat. Rev. Immunol.* **13**, 101–117 (2013).
7. Constantinides, M. G. & Bendelac, A. Transcriptional regulation of the NKT cell lineage. *Curr. Opin. Immunol.* **25**, 161–167 (2013).
8. Lee, Y. J., Holzapfel, K. L., Zhu, J., Jameson, S. C. & Hogquist, K. A. Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells. *Nat. Immunol.* **14**, 1146–1154 (2013).
9. Das, R., Sant'Angelo, D. B. & Nichols, K. E. Transcriptional control of invariant NKT cell development. *Immunol. Rev.* **238**, 195–215 (2010).
10. Dashtsoodol, N. et al. Alternative pathway for the development of Valpha14(+) NKT cells directly from CD4(-)CD8(-) thymocytes that bypasses the CD4(+)CD8(+) stage. *Nat. Immunol.* **18**, 274–282 (2017).
11. Townsend, M. J. et al. T-bet regulates the terminal maturation and homeostasis of NK and Valpha14i NKT cells. *Immunity* **20**, 477–494 (2004).
12. Gordy, L. E. et al. IL-15 regulates homeostasis and terminal maturation of NKT cells. *J. Immunol.* **187**, 6335–6345 (2011).
13. Wu, J. et al. iNKT cells require TSC1 for terminal maturation and effector lineage fate decisions. *J. Clin. Invest.* **124**, 1685–1698 (2014).
14. Zhang, L. et al. Mammalian target of rapamycin complex 1 orchestrates invariant NKT cell differentiation and effector function. *J. Immunol.* **193**, 1759–1765 (2014).
15. Wei, J., Yang, K. & Chi, H. Cutting edge: Discrete functions of mTOR signaling in invariant NKT cell development and NKT17 fate decision. *J. Immunol.* **193**, 4297–4301 (2014).
16. Yu, S. & Cantorna, M. T. The vitamin D receptor is required for iNKT cell development. *Proc. Natl. Acad. Sci. USA* **105**, 5207–5212 (2008).
17. Yue, X., Izcue, A. & Borggrefe, T. Essential role of Mediator subunit Med1 in invariant natural killer T-cell development. *Proc. Natl. Acad. Sci. USA* **108**, 17105–17110 (2011).
18. D'Cruz, L. M., Stradner, M. H., Yang, C. Y. & Goldrath, A. W. E and Id proteins influence invariant NKT cell sublineage differentiation and proliferation. *J. Immunol.* **192**, 2227–2236 (2014).
19. Hu, T. et al. Increased level of E protein activity during invariant NKT development promotes differentiation of invariant NKT2 and invariant NKT17 subsets. *J. Immunol.* **191**, 5065–5073 (2013).
20. Felices, M. & Berg, L. J. The Tec kinases Itk and Rlk regulate NKT cell maturation, cytokine production, and survival. *J. Immunol.* **180**, 3007–3018 (2008).
21. Qi, Q. et al. A unique role for ITK in survival of invariant NKT cells associated with the p53-dependent pathway in mice. *J. Immunol.* **188**, 3611–3619 (2012).
22. Yi, Z., Stunz, L. L. & Bishop, G. A. TNF receptor associated factor 3 plays a key role in development and function of invariant natural killer T cells. *J. Exp. Med.* **210**, 1079–1086 (2013).
23. Salio, M. et al. Essential role for autophagy during invariant NKT cell development. *Proc. Natl. Acad. Sci. USA* **111**, E5678–E5687 (2014).
24. Pei, B. et al. Invariant NKT cells require autophagy to coordinate proliferation and survival signals during differentiation. *J. Immunol.* **194**, 5872–5884 (2015).
25. Pyaram, K., Sen, J. M. & Chang, C. H. Temporal regulation of Wnt/beta-catenin signaling is important for invariant NKT cell development and terminal maturation. *Mol. Immunol.* **85**, 47–56 (2017).
26. Li, G., Ma, D. & Chen, Y. Cellular functions of programmed cell death 5. *Biochim. Biophys. Acta.* **1863**, 572–580 (2016).
27. Li, G. et al. Deletion of Pdcd5 in mice led to the deficiency of placenta development and embryonic lethality. *Cell Death Dis.* **8**, e2811 (2017).
28. Xu, L. et al. PDCD5 interacts with p53 and functions as a positive regulator in the p53 pathway. *Apoptosis* **17**, 1235–1245 (2012).
29. Essers, P. B. et al. The von Hippel-Lindau tumor suppressor regulates programmed cell death 5-mediated degradation of Mdm2. *Oncogene* **34**, 771–779 (2015).
30. Xu, L. et al. PDCD5 interacts with Tip60 and functions as a cooperator in acetyltransferase activity and DNA damage-induced apoptosis. *Neoplasia* **11**, 345–354 (2009).
31. Choi, H. K. et al. Programmed cell death 5 mediates HDAC3 decay to promote genotoxic stress response. *Nat. Commun.* **6**, 7390 (2015).
32. Xu, H. Y. et al. Transfection of PDCD5 effect on the biological behavior of tumor cells and sensitized gastric cancer cells to cisplatin-induced apoptosis. *Dig. Dis. Sci.* **57**, 1847–1856 (2012).
33. Yin, A., Jiang, Y., Zhang, X., Zhao, J. & Luo, H. Transfection of PDCD5 sensitizes colorectal cancer cells to cisplatin-induced apoptosis in vitro and in vivo. *Eur. J. Pharmacol.* **649**, 120–126 (2010).
34. Wang, Y. et al. An alternative form of paraptosis-like cell death, triggered by TAJ/TROY and enhanced by PDCD5 overexpression. *J. Cell Sci.* **117**(Pt 8), 1525–1532 (2004).
35. Wang, J., Guan, Z. & Ge, Z. Plasma and synovial fluid programmed cell death 5 (PDCD5) levels are inversely associated with TNF-alpha and disease activity in patients with rheumatoid arthritis. *Biomarkers* **18**, 155–159 (2013).
36. Wang, J. F. et al. Programmed cell death 5 correlates with disease activity and interleukin-17 in serum and synovial fluid of rheumatoid arthritis patients. *Chin. Med J. (Engl.).* **126**, 296–299 (2013).
37. Chen, Y. et al. Serum programmed cell death protein 5 (PDCD5) levels is upregulated in liver diseases. *J. Immunoass. Immunochem.* **34**, 294–304 (2013).

38. Mansour, S. et al. Structural and functional changes of the invariant NKT clonal repertoire in early rheumatoid arthritis. *J. Immunol.* **195**, 5582–5591 (2015).
39. Bandyopadhyay, K., Marrero, I. & Kumar, V. NKT cell subsets as key participants in liver physiology and pathology. *Cell Mol. Immunol.* **13**, 337–346 (2016).
40. Lawson, V. J., Weston, K. & Maurice, D. Early growth response 2 regulates the survival of thymocytes during positive selection. *Eur. J. Immunol.* **40**, 232–241 (2010).
41. Lazarevic, V. et al. The gene encoding early growth response 2, a target of the transcription factor NFAT, is required for the development and maturation of natural killer T cells. *Nat. Immunol.* **10**, 306–313 (2009).
42. Hu, T., Gimferrer, I., Simmons, A., Wiest, D. & Alberola-Ila, J. The Ras/MAPK pathway is required for generation of iNKT cells. *PLoS One* **6**, e19890 (2011).
43. Thapa, P., Romero Arocha, S., Chung, J. Y., Sant'Angelo, D. B. & Shapiro, V. S. Histone deacetylase 3 is required for iNKT cell development. *Sci. Rep.* **7**, 5784 (2017).
44. Matsuda, J. L. et al. Homeostasis of V alpha 14i NKT cells. *Nat. Immunol.* **3**, 966–974 (2002).
45. Yu, X. & Li, Z. TOX gene: a novel target for human cancer gene therapy. *Am. J. Cancer Res.* **5**, 3516–3524 (2015).
46. Yun, S. et al. TOX regulates the differentiation of human natural killer cells from hematopoietic stem cells in vitro. *Immunol. Lett.* **136**, 29–36 (2011).
47. Vong, Q. P. et al. TOX2 regulates human natural killer cell development by controlling T-BET expression. *Blood* **124**, 3905–3913 (2014).
48. Aliahmad, P. & Kaye, J. Development of all CD4 T lineages requires nuclear factor TOX. *J. Exp. Med.* **205**, 245–256 (2008).
49. Bending, D. et al. Epigenetic changes at Il12rb2 and Tbx21 in relation to plasticity behavior of Th17 cells. *J. Immunol.* **186**, 3373–3382 (2011).
50. Wei, G. et al. Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4+T cells. *Immunity* **30**, 155–167 (2009).
51. Reeves, R. Nuclear functions of the HMG proteins. *Biochim. Biophys. Acta* **1799**, 3–14 (2010).
52. Ranson, T. et al. IL-15 availability conditions homeostasis of peripheral natural killer T cells. *Proc. Natl. Acad. Sci. USA* **100**, 2663–2668 (2003).
53. Yi, Z., Wallis, A. M. & Bishop, G. A. Roles of TRAF3 in T cells: many surprises. *Cell Cycle* **14**, 1156–1163 (2015).
54. Yang, W. & Ernst, P. SET/MLL family proteins in hematopoiesis and leukemia. *Int J. Hematol.* **105**, 7–16 (2017).