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Etoposide-induced Protein 2.4 Regulates The Development and IFN-γ Production of iNKT Cells*

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Abstract Invariant natural killer T (iNKT) cells are a subset of innate-like T cells, which play important regulatory roles in multiple diseases including infection, tumor and metabolic diseases. Revealing the cellular and molecular mechanisms that regulate the development, differentiation and function of iNKT cells is of great significance to elucidate the relationship between iNKT cells and diseases and to seek possible therapeutic approaches. Etoposide-induced protein 2.4 (Ei24) is an autophagy-associated protein which involved in the regulation of cell growth and apoptosis. However, whether Ei24 could regulate iNKT cell differentiation and functions remains unclear. Here, using Cre/loxP system to specifically delete Ei24 in T cells, we found that Ei24 was required for terminal maturation of iNKT cells in thymus, liver and spleen. iNKT1 and iNKT17, but not iNKT2 cells, were affected by Ei24 deficiency. Furthermore, we found that the production of IFN-γ, but not IL-4, was impaired in Ei24 deficient iNKT cells when lipid antigen α-GC was injected *in vivo*. These results demonstrate that Ei24 is required for the development and function of iNKT cells.

Key words Ei24, iNKT, development, IFN-γ
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Invariant natural killer T (iNKT) cells, also known as type Ⅰ or classical NKT cells, are innate-like T cells that express semi-invariant (Vα14-Jα18 paired with Vβ8.2, Vβ7 and Vβ2) T-cell receptor (TCR), which recognizes lipid antigens such as α-galactosylceramide (α-GC) presented by major histocompatibility complex class Ⅰ-like CD1d molecules on antigen-presenting cells (APCs) [1-5]. Upon activation, iNKT cells rapidly release abundant cytokines, including IL-4, IFN-γ, TNF-α, IL-2, IL-10, IL-13 and IL-17, and regulate functions of DCs, macrophages, B cells, NK cells, and conventional T cells in direct or indirect ways[6-10]. Plenty of studies have demonstrated that iNKT cells play important roles in regulating progression of diseases including autoimmune diseases, inflammatory diseases, infectious diseases, and tumors[11-12].

iNKT cells are derived from thymus and their development undergoes four stages: stage 0 ($CD24^+$ $CD44^-$ $NK1.1^-$), stage 1 ($CD24^-$ $CD44^-$ $NK1.1^-$), stage 2 ($CD24^-$ $CD44^+$ $NK1.1^-$), and stage 3 ($CD24^-$ $CD44^+$ $NK1.1^+$)[13]. Matured iNKT cells obtain the ability to produce IFN-γ[14]. Recent studies have shown distinct functional subsets of iNKT cells, including iNKT1, iNKT2 and iNKT17. The thymic iNKT cells in stage 3, which account for about 80% to 90% of total $CD24^-$ thymic iNKT cells, produce both IFN-γ and IL-4 and are referred to as iNKT1. These cells are $PLZF^{lo}$ $T\text{-}bet^+$. IL-17-producing iNKT (iNKT17) cells are rare and mostly confined to $PLZF^{int}$ $ROR\gamma t^+$, whereas iNKT2 cells predominantly produce IL-4 and are $PLZF^{hi}$ $GATA3^+$[15-17]. Different from iNKT1 cells, iNKT17 and iNKT2 cells have immature phenotype. Although some progress has been made in understanding the development and fate of iNKT

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cells, the molecular mechanisms are still poorly explored.

Etoposide-induced protein 2.4 (*Ei24*) is originally identified as a DNA damage response gene that induced cell death in a p53-dependent manner[18]. Meanwhile, Ei24 is an essential component of the autophagy pathway[19]. Overexpression of Ei24 inhibits cell growth and causes apoptosis/autophagy, whereas deletion of Ei24 results in suppression of apoptosis/autophagy in response to pro-apoptotic treatments[20-21]. Additionally, Ei24 has also been identified in early and late phases of autophagy in *C. elegans*[22-23]. It has been reported that autophagy plays essential roles during iNKT cell development[24-25]. Deletion of Atg5 or Atg7 causes decrease in the iNKT cell population. Considering the role of Ei24 in autophagy, we propose that Ei24 might regulate development and function of iNKT cell.

In this study, we showed that Ei24 was essential for the maturation of thymic iNKT cells, especially for the development of iNKT1 and iNKT17 subsets. Moreover, the deficiency of Ei24 in iNKT cells resulted in a remarkable decrease in IFN-γ production. Together, our findings indicate that Ei24 plays an important role in promoting the development and IFN-γ production of iNKT cells.

1 Materials and methods

1.1 Mice

All animal procedures were approved by the University of Science and Technology of China (USTC) Institutional Animal Care and Use Committee. All experiments were performed in accordance with the approved guidelines. To generate LCK^{cre} $Ei24^{fl/fl}$ mice, mice harboring LoxP sites flanking exon 3 of the *Ei24* gene were crossed with mice expressing Cre under the control of the *LCK* (lymphocyte protein tyrosine kinase) promoter (LCK^{cre}). The *LCK*-Cre-Flox strategy was used to delete the *Ei24* gene specifically in T cells.

1.2 Preparation of immune cells

LCK^{cre} $Ei24^{fl/fl}$ mice and $Ei24^{fl/fl}$ mice were sacrificed, thymus and spleen were minced and passed through 100 μm stainless steel meshes. Livers were minced and passed through 100 μm stainless steel meshes and centrifuged to remove the supernatant. The cell pellets were resuspended in 40% percoll and centrifuged. Red blood cells were lysed by erythrocyte lysis buffer (Solarbio, Beijing, China).

1.3 Cell enrichment

LCK^{cre} $Ei24^{fl/fl}$ mice and $Ei24^{fl/fl}$ mice were sacrificed and spleens were minced and passed through 100 μm stainless steel meshes. Cells were blocked with anti-mouse CD16/32 for 15 min, then stained with anti-CD4-PE for 30 min on ice. $CD4^+$ T cells were enriched with anti-PE microbeads (Miltenyi Biotec, Germany).

1.4 Western blot

$CD4^+$ T cells were harvested and lysed with sample buffer and boiled for 10 min. Proteins were separated by electrophoresis and detected by Western blot. The antibodies used were as follows: rabbit anti-Ei24 (Sigma, HPA047165), mouse anti-β-actin (Transgen, HC201), HRP-conjugated anti-rabbit immunoglobulin G (Jackson IR, 111-035-144) and HRP-conjugated anti-rabbit immunoglobulin G (Jackson IR, 111-035-146).

1.5 Activation of iNKT cells *in vivo*

LCK^{cre} $Ei24^{fl/fl}$ mice and $Ei24^{fl/fl}$ mice were injected intraperitoneally with α -GC (2 μg/mouse, Avanti Polar Lipids, Alabama), 4 h before tissue collection. Immune cells were harvested for flow cytometric analysis.

1.6 Flow cytometry

Cells were blocked with purified anti-mouse CD16/32 for 15 min on ice, then were stained with fluorochrome-conjugated monoclonal antibodies against CD44(IM7), NK1.1(PK136), CD24(M1/69), TCR- β(H57-597), CD1d-PBS57 tetramer. To detect intracellular transcription factor PLZF, T-bet, RORγt, and cytokine IL-4, IFN- γ, cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, Munich, Germany) and permeabilized with PBS buffer containing 0.1% saponin (Sigma-Aldrich, Munich, Germany) and 0.5% bovine serum albumin (BSA, Sigma-Aldrich, Munich, Germany). Then, cells were stained with antibodies against PLZF (9E12), T-bet (4B10), RORγt (AFKJS9), IFN- γ (XMG1.2), IL-4 (11B11) for 1 h on ice. All antibodies were purchased from BioLegend (USA). CD1d-PBS57 tetramer is provided by National Institute of Health (NIH) Tetramer Core Facility. Cells were acquired on a FACSVerse flow cytometer (BD Biosciences), and data were analyzed with FlowJo software (TreeStar).

1.7 Statistical analyses

Error bars represent SEM. Statistical analyses

were performed using student's *t*-test (GraphPad Software). *P < 0.05, **P < 0.01, and ***P < 0.001 were considered statistically significant.

2 Results

2.1 Ei24 is crucial for iNKT cell development in thymus

Ei24 has been shown to negatively regulate cell growth[26]. To investigate whether Ei24 plays a similar role in iNKT cell development, we used Cre/loxP system to generate mice with T cells-specific knockout of Ei24. Homozygous loss of Ei24 is embryonically lethal[27]. In *LCK*^{cre} *Ei24*$^{fl/fl}$ mice, Ei24 protein in CD4$^+$ T cells was almost fully deleted (Figure 1a). We analyzed iNKT cells from thymus of *LCK*^{cre} *Ei24*$^{fl/fl}$ mice and *Ei24*$^{fl/fl}$ mice, and found a significantly decrease in frequency and absolute cell number in the absence of Ei24 (Figure 1b). Next, we investigated which stage was affected by the deficiency of Ei24 during the development of iNKT cells. *LCK*^{cre} *Ei24*$^{fl/fl}$ mice had a lower frequency and absolute number of stage 3 iNKT cells (CD24$^-$ NK1.1^{hi} CD44^{hi}) than littermate controls, whereas there was no difference in stage 1 iNKT cells (CD24$^-$ NK1.1^{lo} CD44^{lo}) and stage 2 iNKT cells (CD24$^-$ NK1.1^{lo} CD44^{hi}) (Figure 1c, 1d). Taken together, these data suggest that Ei24 regulates maturation of iNKT cells in thymus.

2.2 Ei24 is required for the development of iNKT1 and iNKT17 cells

iNKT cells can be divided into three different subsets: iNKT1, iNKT2 and iNKT17[28]. Next, we investigated if Ei24 controlled the development of iNKT cell subsets. We found the absolute number of iNKT1 and iNKT17 cells were lower in *LCK*^{cre} *Ei24*$^{fl/fl}$ mice, and no difference in the absolute number of iNKT2 cells was observed (Figure 2a, 2b). These data demonstrate that Ei24 is important for iNKT1 and iNKT17 cells development.

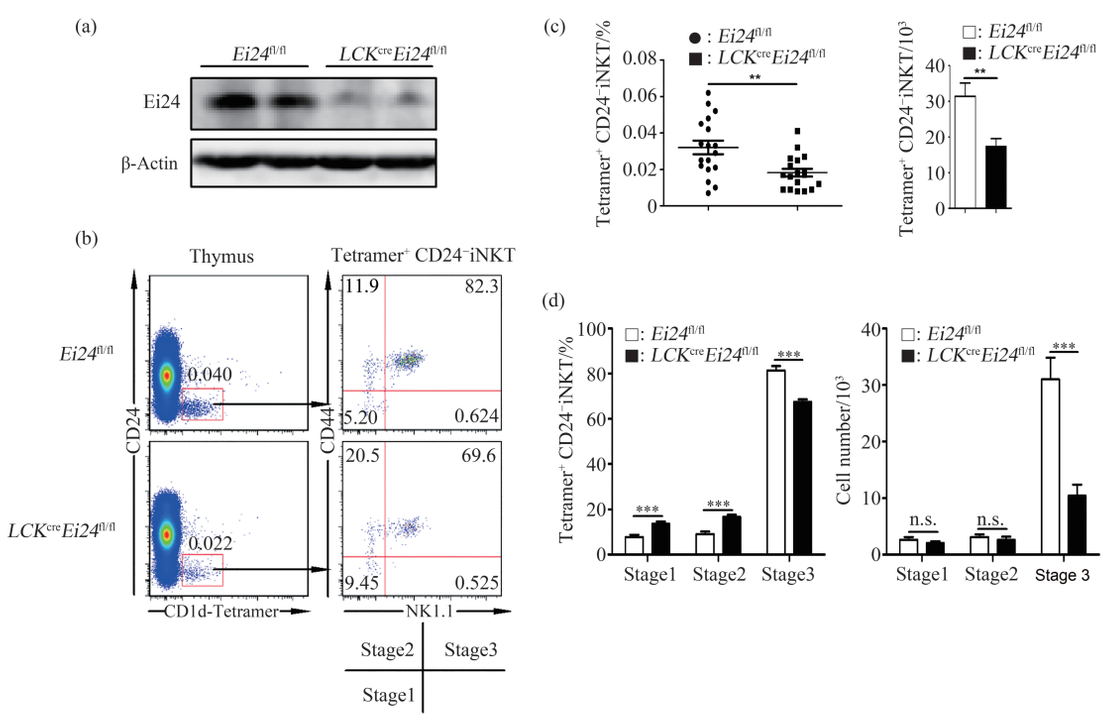

Fig. 1　Development of iNKT cells is dependent on Ei24 expression

（a） Western blot analysis of Ei24 protein from enriched CD4$^+$ T cells in spleen from *LCK*^{cre} *Ei24*$^{fl/fl}$ mice and *Ei24*$^{fl/fl}$ mice. β-Actin served as the control. （b） Frequencies of stage 1—3 iNKT cells in the thymus from *LCK*^{cre} *Ei24*$^{fl/fl}$ mice and *Ei24*$^{fl/fl}$ mice. In the FACS plots， numbers adjacent to outlines indicate the percentages of cells in the gate. The frequency （c） and absolute number （d） of iNKT cells at stage 1—3 among CD24$^-$ CD1d-tetramer$^+$ iNKT cells in indicated mice. Data are mean ± SEM of nineteen mice per group. Statistical analysis was performed using a student's *t*-test （GraphPad Software）. **P < 0.01； ***P < 0.001.

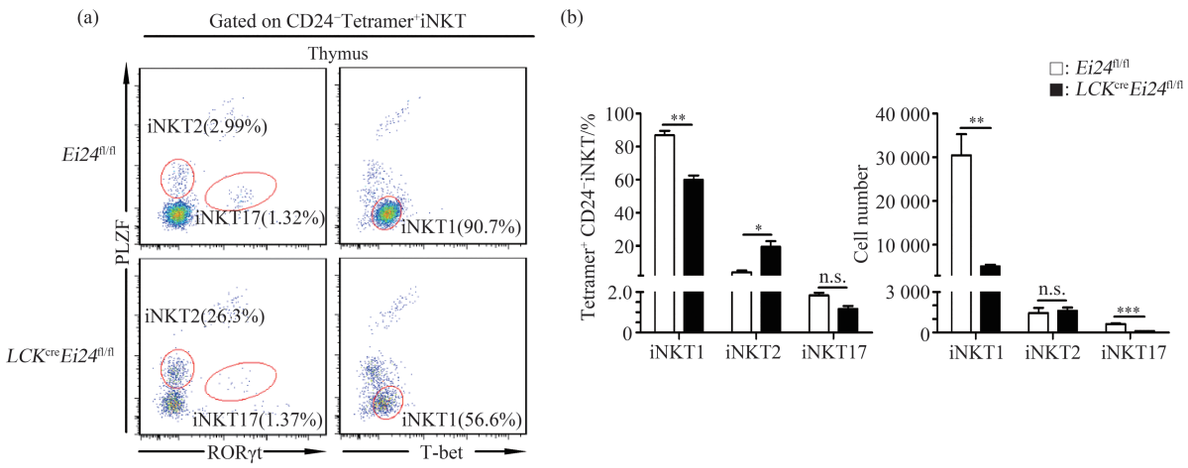

Fig. 2　Ei24 is required for the development of iNKT1 and iNKT17 cells

（a）Representative FACS plots of iNKT1，iNKT2 and iNKT17 cells in the thymus，compared between *LCK*^{cre} *Ei24*$^{fl/fl}$ mice and Ei24$^{fl/fl}$ mice. In the FACS plots，numbers adjacent to outlines indicate the percentages of cells in the gate. （b）The frequency （left） and absolute number （right） of iNKT subsets among CD24^{-} CD1d-tetramer^{+} iNKT cells in indicated mice. Data are mean ± SEM of nine mice per group. Statistical analysis was performed using a student's *t*-test （GraphPad Software）. $^{*}P < 0.05$； $^{**}P < 0.01$； $^{***}P < 0.001$.

2.3　Ei24 deficiency results in decreased splenic and hepatic iNKT cells

During the development in thymus, immature stage 2 iNKT cells can leave the thymus and colonize in peripheral organs. A final maturation step that occurs either in the thymus or in the peripheral organs is accompanied by the expression of NK1.1[13,29]. In agreement with the data in the thymus, iNKT cell frequencies and numbers were also decreased in the liver and spleen of *LCK*^{cre} *Ei24*$^{fl/fl}$ mice. However, there was no difference of iNKT cells in the lymph node (Figure 3). These data demonstrate impaired maturation of splenic and hepatic iNKT cells in *LCK*^{cre} *Ei24*$^{fl/fl}$ mice.

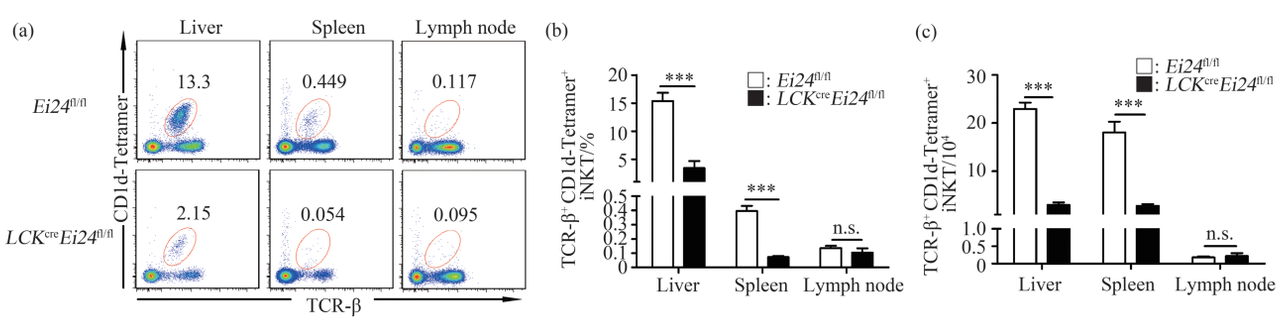

Fig. 3　Deletion of Ei24 results in diminished splenic and hepatic iNKT cell numbers

（a）Representative FACS plots of iNKT cells in the spleen，liver and lymph node，compared between *LCK*^{cre} *Ei24*$^{fl/fl}$ mice and *Ei24*$^{fl/fl}$ mice. In the FACS plots，numbers adjacent to outlines indicate the percentages of cells in the gate. The frequency （b）and absolute number （c）of iNKT cells in the spleen，liver and lymph node of indicated mice. Data are mean ± SEM of eight to nine mice per group. Statistical analysis was performed using a student's *t*-test （GraphPad Software）. $^{***}P < 0.001$.

2.4　Ei24 deficiency results in impaired IFN−γ production in splenic and hepatic iNKT cells

Next, we studied whether deletion of Ei24 affected peripheral iNKT cell functions. Remarkably, the majority of the splenic and hepatic iNKT cells are iNKT1 cells (Figure 4a). Upon activation by α-GC, hepatic iNKT cells from *LCK*^{cre} *Ei24*$^{fl/fl}$ mice showed

significantly impaired IFN-γ production compared to *Ei24*$^{fl/fl}$ mice. However, Ei24 deficiency showed no influence on IL-4 production in hepatic iNKT cells (Figure 4b, 4d). Consistently, we found similar reduction of IFN-γ in splenic iNKT cells in the absence of Ei24 (Figure 4c, 4e). These results indicate that Ei24 promotes IFN-γ production in iNKT cells.

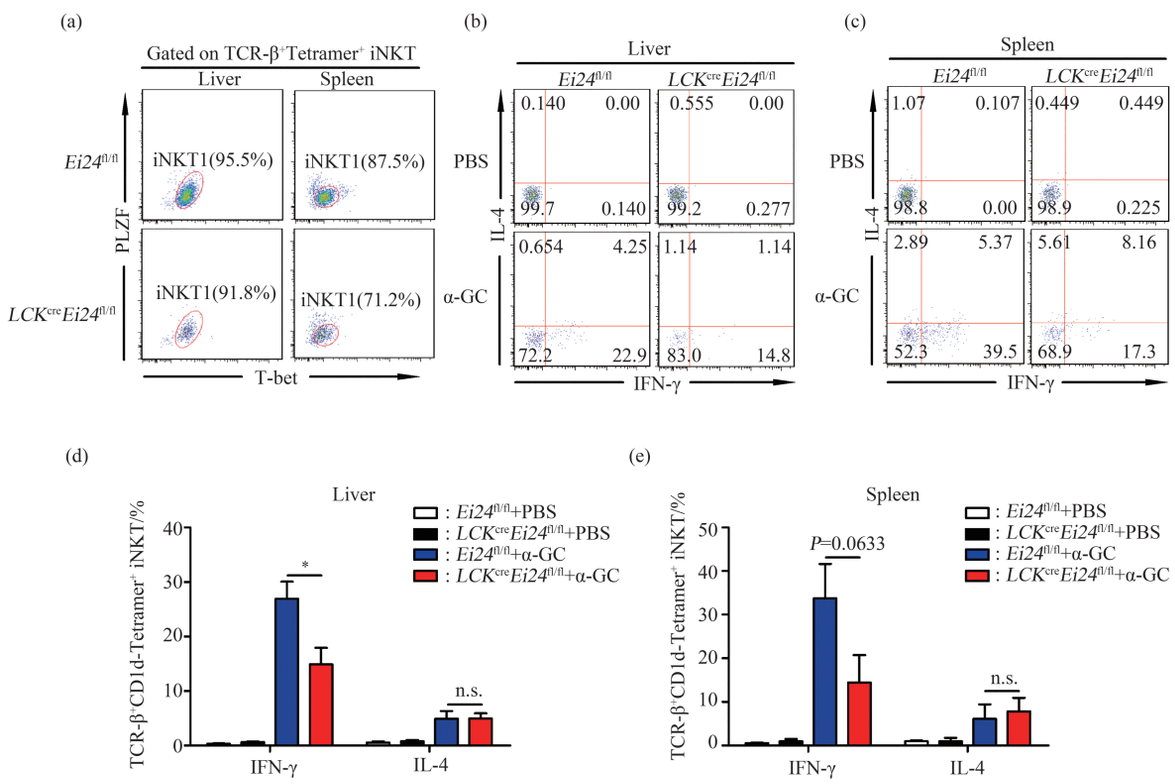

Fig. 4　Ei24 deficiency results in impaired IFN−γ production in iNKT cells

（a—c）Representative FACS plots of iNKT1 cells among hepatic and splenic iNKT cells （a），intracellular staining of IFN-γ and IL-4 in hepatic （b）and splenic （c）iNKT cells 4 h after i.p. administration of α-GC，from *LCK*^{cre} *Ei24*$^{fl/fl}$ mice and *Ei24*$^{fl/fl}$ mice as indicated. （d，e）Frequencies of IFN-γ$^+$ iNKT cells and IL-4$^+$ cells iNKT cells from the liver （d） and spleen （e） of *LCK*^{cre} *Ei24*$^{fl/fl}$ mice and *Ei24*$^{fl/fl}$ mice with or without α-GC injection. Data are mean ± SEM of six mice per group. Statistical analysis was performed using a student's *t*-test （GraphPad Software）. *$P < 0.05$.

3　Discussion

Our data demonstrated a critical role of Ei24 in regulating maturation and function of iNKT cells. In the absence of Ei24, thymic, splenic, and hepatic iNKT cell numbers were significantly reduced (Figure 1 and 3). In thymus, only stage 3 but not stage 1 or stage 2 iNKT cells were influenced by deletion of Ei24 (Figure 1d). In spleen and liver, most of the iNKT cells are at stage 3. Therefore, Ei24 controls the terminal maturation of iNKT cells. Ei24 has been reported to mediates autophagy signaling pathways[30]. Autophagy regulates cell differentiation, proliferation, and survival in multiple immune cells, including iNKT cells. It has been reported that, in the absence of autophagy, the number of iNKT cells is reduced in the thymus. Autophagy defect results in the accumulation of mitochondrial superoxide species, leading to apoptotic cell death. Moreover, autophagy is especially required for the Th1 response of iNKT cells, the production of IFN-γ in iNKT cells is reduced in the absence of autophagy[24-25]. Here, deficiency of Ei24 inhibited development and function of iNKT cells in a similar manner as deficiency of autophagy. It is possible that Ei24 may regulate iNKT cell development through modulating autophagy, detailed mechanism study is required.

Moreover, we identified Ei24 as a key player in controlling differentiation of iNKT1 and iNKT17 rather than iNKT2 cell lineages in the thymus (Figure 2). However, we did not observe impaired expression of T-bet and RORγt in these two iNKT subsets when Ei24 was deleted. The mechanisms how Ei24 regulate differentiation of iNKT1 and iNKT17 remain unclear.

iNKT cells play important roles in regulating immune responses. Majority iNKT cells in mice are iNKT1 cells. Upon activation by lipid antigens, iNKT1 cells can produce large amounts of Th1 and Th2 cytokines, such as IFN-γ and IL-4. IFN-γ is a key player in body defense against tumor cells and bacteria, whereas IL-4 helps to suppress inflammation and maintain homeostasis. Th1 or Th2 biased cytokine production of iNKT cells has been related to progression of many diseases[10,31-33]. In our studies, deletion of Ei24 inhibited IFN-γ production in iNKT cells but did not influenced IL-4 production (Figure 4). These results suggested a functional polarization of iNKT cells toward Th2 response in the absence of Ei24. Taken together, our findings suggest that Ei24 is required for the development and Th1 function of iNKT cells.

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依托泊苷诱导蛋白2.4调控iNKT细胞的发育和IFN-γ应答*

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摘要　iNKT细胞是一类特殊的固有样T淋巴细胞，在感染、肿瘤、自身性免疫疾病和代谢类疾病中都发挥重要的调控作用. 揭示调控iNKT细胞发育、分化和功能的细胞分子机制，对于阐释iNKT细胞与疾病的关系以及寻求可能的治疗途径都具有重要的意义. 依托泊苷诱导蛋白2.4（Etoposide-induced protein 2.4，Ei24）可调控细胞生长、凋亡和自噬等多种生物学功能，但其对iNKT细胞的发育和功能的影响仍不清楚. 本研究利用Cre/loxP重组酶系统成功构建T细胞中Ei24特异性敲除小鼠. 敲除Ei24后，iNKT细胞在胸腺中的发育受到明显抑制，肝脏和脾脏等组织中的iNKT比例和数目明显减少. 进一步研究发现，Ei24主要影响iNKT1和iNKT17 2个亚群，对iNKT2的调控作用相对较小. 当腹腔注射iNKT细胞特异性活化抗原α-GC后，敲除Ei24后iNKT细胞的IFN-γ应答更低，但不影响iNKT细胞的IL-4应答. 以上结果表明，Ei24可以调控iNKT细胞的发育与功能.

关键词　依托泊苷诱导蛋白2.4，iNKT细胞，发育，γ干扰素

中图分类号　R392.12，Q28　　**DOI**：10.16476/j.pibb.2019.0087

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