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Cell-autonomous requirement for TCF1 and LEF1 in the development of Natural Killer T cells

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ABSTRACT

Natural killer T (NKT) cells develop from common CD4$^+$ CD8$^+$ thymocyte precursors. Transcriptional programs that regulate the development of NKT cells in the thymus development remain to be fully delineated. Here, we demonstrate a cell-intrinsic requirement for transcription factors TCF1 and LEF1 for the development of all subsets of NKT cells. Conditional deletion of TCF1 alone results in a substantial reduction in NKT cells. The remaining NKT cells are eliminated when TCF1 and LEF1 are both deleted. These data reveal an essential role for TCF1 and LEF1 in development of NKT cells.

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1. Introduction

Natural killer T (NKT) cells develop from CD4$^+$ CD8$^+$ double positive (DP) thymocytes that express a T-cell receptor α (TCRα) chain, Vα14-Jα18, paired with Vβ8, Vβ7 or Vβ2 in mice to generate a limited TCR repertoire that recognizes glycolipid antigens presented by the MHC class I-like molecule CD1d. TCR-dependent recognition of lipid antigens presented by CD1d expressed on DP thymocytes signals commitment to the NKT lineage (Bendelac, 1995; Gapin et al., 2001; Godfrey et al., 2010; Hu et al., 2011). Whereas the transcriptional programs that regulate development of conventional T cells from DP precursors have been defined, the transcriptional control of NKT cell generation remain to be delineated.

Several transcription factors are known to play an active role in the DP-to-NKT transition and the generation of NKT0 immature precursor cells that will subsequently mature into their effector phenotypes. Deletion of E protein HEB results in absence of NKT cells due to inability to generate Vα-Jα rearrangements (D'Cruz et al., 2010). HEB also affects expression of RORγt that together with Runx1 regulates the half-life of DP cells (Egawa et al., 2005). Likewise, c-Myb plays a role on the lifetime of DP cells and is also required for the expression of CD1d and signaling molecules SLAMF1, SLAMF6 and SAP (Hu et al., 2010). Bcl11b deletion blocks early NKT cell development at the DP-to-NKT transition (Albu et al., 2011). Finally, c-Myc controls proliferation during the DP-to-NKT transition (Dose et al., 2009). Together these observations point to a complex developmental program that leads to commitment to the NKT lineage.

Evolutionarily conserved transcription factor T cell factor (TCF)-1 has been shown to repress and activate gene expression (Schilham and Clevers, 1998; Klingel et al., 2012). In mammals, TCF1 is encoded by the *Tcf7* gene and is expressed exclusively in T cells. TCF1 expression is required for conventional T cell development in the thymus and mice with germline deletion of TCF1 have very few thymocytes that are impaired at multiple stages during development (Staal and Sen, 2008; Staal et al., 2008; Staal and Clevers, 2000, 2005). Recently, we showed that germline deletion of TCF1 leads to reduced lifetime of CD4$^+$ CD8$^+$ double positive (DP) thymocytes *in vivo* (Sharma et al., 2014). Reduction in lifetime results in failure to rearrange the distal TCR Vα14-Jα18 and express TCR proteins required for development of NKT cells. However, conditional deletion of the TCF1 gene with CD4-Cre has at least 30% DP thymocytes with undeleted TCF1 (Steinke et al., 2014). These TCF1 sufficient DP thymocytes permit selection of cells that rearrange TCRα to develop into NKT cells. Thus, the issue of cell-intrinsic requirement for TCF1 for NKT cell development remains unanswered. Furthermore, some

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functions of TCF1 during conventional T cell development have been shown to be redundant with a related transcription factor called lymphocyte enhancer-binding factor (LEF)-1 (Okamura et al., 1998; Yu et al., 2012). Thus, the cell intrinsic requirement for TCF1 and LEF1 in the generation and differentiation of NKT cells in the thymus remains to be fully defined.

This study shows that, whereas conditional deletion of TCF1 (TCF1-cKO) did not lead to a reduction in thymocyte numbers, TCF1 deficiency in NKT-precursor DP thymocytes substantially reduced the numbers of NKT cells. The few remaining NKT cells were NKT0 and NKT1 cells in TCF1-cKO thymus. Residual NKT cells were further eliminated in mice with conditional deletion of both transcription factors, TCF1 and LEF1, in DP thymocytes. These data show that cell autonomous expression of TCF1 and LEF1 expression are required for effective development of NKT cells at the earliest stages of development.

2. Materials and methods

2.1. Mice

Mice with single conditional deletion of LEF1 (LEF1-cKO), TCF1 (TCF1-cKO), and conditional deletion of both transcription factors (TCF1/LEF1-cDKO) are described elsewhere (Steinke et al., 2014). CD1d knockout (CD1d-KO) mice were obtained from the Jackson Laboratory and CD45.1+ C57BL/6.SJL mice were purchased from Taconic. All the mice used are on a C57BL/6 genetic background. CD45.1+2+ mice were generated by breeding C57BL/6.SJL mice with C57BL/6 mice. Age-matched (7–12 weeks old) littermate controls or C57BL/6 mice were used in all experiments. CD1d-KO mice used in experiment were 3–4 weeks of age. All mice were bred and maintained in the animal facility at the National Institute on Aging (NIA). The studies were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (NRC, 2010). The protocol was approved by the Animal Care and Use Committee of the NIA Intramural Research Program, NIH. This program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (File 401), registered by the United States Department of Agriculture (51-F-0016) and maintains an assurance with the Public Health Service (A4149-01).

2.2. Flow cytometry

Single-cell suspensions were prepared from thymus and spleen as per standard protocols. Hepatic lymphocytes were isolated from livers that were homogenized, filtered through nylon mesh and washed in PBS with 1% FBS. Cells were then resuspended in 44% Percoll (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), underlaid with 66% Percoll, and centrifuged for 20 min at 2000 rpm. Cells at the interface were collected, washed, and counted. Cells were stained and acquired on a FACSCantoII (Becton Dickinson) and analyzed with FlowJo (Treestar). Dead cells were excluded using the Fixable Viability Dye eFluor®506 (eBioscience). The following antibodies and their isotype controls conjugated to FITC, PE, PerCP-Cy5.5, PE-Cy7, APC, APC-Cy7 or Pacific Blue (from BD Biosciences, eBioscience or BioLegend) were used for staining: anti-CD4 (GK1.5), anti-CD8α (53-6.7), anti-TCRβ (H57-597), anti-NK1.1 (PK136), anti-CD24 (M1/69). PE- or APC- conjugated mouse CD1d tetramers loaded with glycolipid PBS-57 (CD1d-tet) and an unloaded tetramer comprised of only the glycolipid PBS57 were obtained from the tetramer facility of the US National Institutes of Health. In brief, cells were incubated with FC block and stained with antibodies, and then fixed with 2% paraformaldehyde. For PLZF, T-bet, TCF-1 and LEF-1 intracellular staining, cells were permeabilized and stained accordingly with anti-PLZF (D-9) (Santa Cruz Biotechnology, Inc.) plus FITC anti-mouse (BD Biosciences), anti-T-bet (eBio4B10) (eBioscience), anti-TCF-1 (C63D9) and/or anti-LEF-1 (C18A7) (both from Cell Signaling) followed by goat anti-rabbit-Alexa647 or Alexa488 (Invitrogen), using the Foxp3 Staining Buffer kit (eBioscience).

2.3. Bone marrow chimeras

For BM transplantation experiments, the CD45.1+ recipient mice were lethally irradiated with 950 rads 24 h before receiving BM transfers. BM cells were harvested from the femurs and tibias of two donor mice and depleted of mature T cells, B cells, and MHC class II-positive lymphocytes by using a cocktail of antibodies containing anti-CD4, anti-CD8, anti-CD19 (1D3), and anti-MHC class II (M5/114) followed by complement-mediated lysis. For the BM mixed chimera experiments, BM cells from two different types of donor mice were mixed at 1:1 ratio. Each recipient mouse received 9×10^6 cells in 250 μl of PBS through i.v. injection. For the other BM chimera experiments, each recipient mouse received 2×10^6 whole bone marrow cells from a single donor in 400 μl of PBS through i.v. injection. In all experiments, CD45 congenic markers were used to distinguish cells derived from the different sources. All BM chimeras were reconstituted for at least 7 weeks before analysis.

2.4. Statistics

Statistical significance was determined by the Student's *t*-test.

3. Results

3.1. TCF1 controls the development of NKT cells

To study the role of TCF1 in NKT cell generation we analyzed the expression of TCF1 in NKT cells from C57BL/6 control mice. We found TCF1 was highly expressed in all NKT cells with highest level of expression in NKT2 and NKT17 subsets (Fig. 1A). To determine a role for TCF1 in NKT cell generation, we analyzed thymocytes from mice with conditional deletion of TCF1 in thymocytes, $Tcf7^{flox/flox}$ CD4-Cre$^+$ (TCF1-cKO) mice (Steinke et al., 2014). Conditional deletion of TCF1 did not substantially alter the number of thymocytes compared to control mice (Fig. 1B). By contrast, TCF1 deficiency resulted in greater than 80% reduction in NKT cell numbers from TCF1-cKO compared to control mice (Fig. 1B). Detailed analysis showed that significantly fewer mature NKT1, NKT2 and NKT17 cells developed in the thymus of TCF1-cKO mice compared to control mice (Fig. 1C). Finally, analysis of NKT cells in spleen and liver also showed a substantial reduction in all subsets of NKT cells in the peripheral organs (Fig. 1D). These data show that when TCF1 is conditionally deleted in precursor DP thymocytes, NKT cell development was substantially blocked. We conclude that TCF1 plays an important role in specifying NKT cell lineage.

3.2. TCF1 and LEF1 control the earliest stages of NKT cell development

To determine if the residual NKT cells that develop in TCF1-cKO mice result from a redundant role of LEF1 in NKT cell development, we analyzed mice with conditional deletion of both transcription factors in DP thymocytes in TCF1/LEF1-cDKO mice (Steinke et al., 2014). First, we noted that LEF1 was expressed in all NKT cells with highest level of expression in NKT2 and NKT17 cells (Fig. 2A). Total number of thymocytes has been shown to be substantially comparable in mice with conditional deletion of TCF1 and/or LEF1 using *Cd4*-Cre compared to control mice (Steinke et al., 2014). However, single deficiency of either LEF1 or TCF1 both showed lower