The Multiple Roles of Id2 and Id3 in Invariant NKT Cell Development and NKT Lymphoma Formation in Mice

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Immunology in the Graduate School of Duke University

2014
ABSTRACT

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Abstract

Invariant NKT (iNKT) cells represent a unique group of αβ T cells that have been classified based on their exclusive usage of the invariant Vα14-Jα18 TCRα-chain and their innate-like effector function. Thus far, the transcriptional programs that control Vα14-Jα18 TCRα rearrangements and the population size of iNKT cells remain incompletely defined.

E protein transcription factors have been shown to play multiple roles in T cell development including lineage commitment, receptor gene recombination, proliferation and lineage choice. Inhibitor of DNA-binding (Id) proteins are the natural inhibitors of E protein transcription factors. The goal of this dissertation is to examine E protein functions in the development of iNKT cells in the mouse after combined deletion of genes encoding E protein inhibitors Id2 and Id3.

We revealed important roles of Id proteins and E proteins in regulating iNKT cell development. Deletion of Id2 and Id3 in T cell progenitors resulted in a partial block at the pre-TCR selection checkpoint and a dramatic increase in numbers of iNKT cells. This increase in iNKT cells is accompanied with a biased rearrangement involving Vα14 to Jα18 recombination at the double-positive stage and enhanced proliferation of iNKT cells. We further demonstrate that a 50% reduction of E proteins can cause a dramatic lineage shift from iNKT cells to innate-like γδ T cells in Id2/3 double-deficient mice. Collectively,
these findings suggest that Id2- and Id3-mediated inhibition of E proteins controls iNKT development by restricting lineage choice and population expansion.

Our study also uncovered a novel role of Id proteins in development of NKT lymphoma. Id deficient NKT cells gradually progresses into NKT lymphoma, a rare form of tumor with no clearly defined etiology. Id and E proteins have been demonstrated to be involved in multiple lymphoma and cancer subtypes, but their role in the development of NKT lymphomas is unexplored. Adoptive transfer experiments confirmed that the malignant cells are able to invade healthy tissues. cDNA Microarray analysis of NKT lymphoma and pre-malignant NKT cells revealed alterations in several cytokine signaling pathways during tumor progression. These findings indicate that regulation of E proteins by Id2 and Id3 may play important roles in the development of NKT lymphoma. To our knowledge, this study represents the first mouse model in which NKT lymphoma develops at such high frequency and fast kinetics. Our double knockout mice provide a unique model to study mechanisms of human NKT lymphoma progression.
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List of Abbreviations

AD activation domain
bHLH basic helix-loop-helix
BM bone marrow
BTB broad complex, tramtrack, and ric-à-brac
D diversity gene segment
DC dendritic cell
DL1 delta 1
DN double negative
DP double positive
ERK extracellular signal-regulated kinase
ETP early T cell progenitor
f flox, conditional allele
HMGB1 high-mobility group protein B1
HSC hematopoietic stem cell
HSTC Hepatosplenic T-cell lymphoma
Id inhibitor of DNA binding
iGb3 Glycolipid isoglobotrihexosylceramide
iNKT invariant natural killer T
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>IP</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>ISP</td>
<td>immature single positive</td>
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<tr>
<td>J</td>
<td>joining gene segment</td>
</tr>
<tr>
<td>LMPP</td>
<td>lymphoid-primed multipotent progenitor</td>
</tr>
<tr>
<td>MAIT</td>
<td>mucosal associated invariant T</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MHCI</td>
<td>MHC class I</td>
</tr>
<tr>
<td>MHCII</td>
<td>MHC class II</td>
</tr>
<tr>
<td>NFAT</td>
<td>nuclear factor for activation of T cells</td>
</tr>
<tr>
<td>NF-kB</td>
<td>nuclear factor kB</td>
</tr>
<tr>
<td>NHEJ</td>
<td>non-homologous end joining</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NKT</td>
<td>natural killer T</td>
</tr>
<tr>
<td>PLZF</td>
<td>promyelocytic leukemia zinc finger</td>
</tr>
<tr>
<td>PTC</td>
<td>peripheral T-cell lymphoma</td>
</tr>
<tr>
<td>Rag</td>
<td>recombination activating gene</td>
</tr>
<tr>
<td>RSS</td>
<td>recombination signal sequences</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SAP</td>
<td>SLAM-associated protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SLAM</td>
<td>signaling lymphocytic activation molecule</td>
</tr>
<tr>
<td>SP</td>
<td>single positive</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TdT</td>
<td>terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>TEA</td>
<td>T early α</td>
</tr>
<tr>
<td>V</td>
<td>variable gene segment</td>
</tr>
<tr>
<td>ZAP</td>
<td>zeta-associated protein</td>
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1. Introduction

The immune system provides the most important defense against disease; its functions include eliminating external pathogens and maintaining surveillance inside. It consists of two broad systems, the innate immune system and the adaptive immune system. The innate immune system responds to pathogen immediately, while the adaptive immune system responds more slowly, but develops immunological memory for much quicker and stronger responses against the same pathogen upon a second infection. Both of these systems incorporate multiple immune cell types derived from hematopoietic stem cells (HSCs). Among them, T lymphocytes (T cell) are one of the most important immune cells. The conventional T cells are essential for adaptive immunity, while the innate-like T cells may possess features similar to those of the innate immune system. These innate-like cells provide faster effector responses than conventional T cells, functioning alongside innate immune cells as a first line of defense.

There are two main classifications of T cells in thymus, αβ T cells and γδ T cells, according to the expression of an αβ or γδ T cell receptor (TCR), respectively on their surface. αβ T cells are subdivided into CD4 single positive (SP) cells, CD8 SP cells and nature killer T (NKT) cells according to different selection mechanisms and surface markers. NKT cells and one specific subset of γδ T cells, Vγ1.1 Vδ6.3+ T cells, which are also referred to as γδ NKT cells, belong to the innate-like T cell family (Alonzo and Sant’Angelo, 2011; Carpenter and Bosselut, 2010).
The following introduction will focus on the development of T cells, especially NKT cells in the thymus as well as how Id proteins and E proteins regulate this process.

1.1 The development of T cells in the thymus

1.1.1 T cell commitment and specification

All T cells originate from early T cell progenitors (ETP), which develop from HSCs in bone marrow (BM). ETPs derive from the lymphoid-primed multipotent progenitors (LMPPs) in the BM and then migrate into the thymus at the cortical-medullary junction, where they further differentiate into different T cell lineages (Petrie, 2003). Commitment to the T cell lineage is regulated by multiple transcription factors, which guide lineage differentiation programs in response to signals from thymic microenvironments.

The developmental process of T cells in the thymus is divided into three main sequential stages according to the surface expression of CD4 and CD8; these are double negative (DN), double positive (DP) and single positive (SP). The DN stage is further separated into four stages according to CD44 and CD25 expression, which are DN1 (CD44+CD25-), DN2 (CD44+CD25+), DN3 (CD44 CD25+) and DN4 (CD44 CD25-). This process is detailed in Figure 1. The DN1 and DN2 precursors retain the potential to develop into other lineages, such as NK cells, myeloid cells and dendritic cells (DCs). Commitment to the T cell lineage occurs in the DN2 to DN3 stage when TCRβ and TCRγδ rearrangement occur. The αβ T cell and γδ T cell lineages are specified after
expression of the pre-TCR composed of TCRβ and pre-TCRα or γδ TCR, respectively (Ciofani and Zuniga-Pflucker, 2010).

Transcriptional regulators such as PU.1, Ikaros, Notch, Gata3, E proteins, and Tcf1 coordinately regulate T cell commitment (David-Fung et al., 2009). Among them, Notch signaling is necessary for the transition from the ETP stage to the DN3 stage. Notch mainly supports T cell survival and metabolism through the phosphatidylinositol-3-OH kinase (PI(3)K) and Akt pathways. Notch signaling is activated by the interaction of Notch1 transmembrane receptor with its ligands, mainly Delta-like 1 (DL1) and DL4 for T cell precursors. Upon the ligation, the intracellular domain is cleaved to an active form, which binds to RBP-Jk and promotes transcriptional activation of target genes. In the absence of Notch1, the T cell precursors are able to adopt a B cell fate (Hayday and Pennington, 2007; Radtke et al., 1999). The OP9 stromal cells from bone marrow can support DN precursor differentiation into αβ or γδ T cells if Notch1 ligand DL1 or DL4 is ectopically expressed in the stromal cells (Schmitt and Zuniga-Pflucker, 2002). Known Notch signaling targets such as pre-Tα and c-Myc play important roles in DN T cell differentiation. Notch1 is regulated by E2A (Ikawa et al., 2006). E proteins are also required for early T cell commitment. They are critical for T cell development at multiple stages, which will be introduced in detail in chapter 1.2.
T cell lineage commitment occurs after the transition from ETP to DN2. There are two main developmental checkpoints for the αβ T cell lineage, the pre-TCR checkpoint and the TCR checkpoint.

![Diagram of T cell lineage specification and commitment](image)

**Figure 1: T cell lineage specification and commitment**

Figure 1. Thymic T cell developmental stages are defined by the expression of CD4 and CD8 as CD4<sup>-</sup>CD8<sup>-</sup> double negative, CD4<sup>+</sup>CD8<sup>-</sup> double positive and CD4<sup>+</sup>CD8<sup>-</sup> or CD4<sup>-</sup>CD8<sup>-</sup> single positive. The DN stage cells are further separated into DN1-4 stages according to the expression of CD25 and CD44. Immature single positive (ISP) is a transitional stage between DN and DP. DN2 and DN3 cells have the potential to differentiate into γδ T cells. The remaining cells are destined for the αβ lineage and are further selected in the DP stage, becoming CD4 SP, CD8 SP and iNKT cells. There are two essential checkpoints during this process: the pre-TCR checkpoint in the DN3 stage and TCR checkpoint in the DP stage.
1.1.1.1 pre-TCR checkpoint in the DN stage

After the DN2 stage, the pre-TCR selection checkpoint is the first step toward \( \alpha\beta \) lineage specification. During TCR\( \beta \) rearrangement in the DN2 to DN3 stage, cell cycle progression is halted until successful TCR\( \beta \) rearrangement is completed, at which point cells are able to pass this checkpoint and continue further differentiation. Therefore, this checkpoint is also known as the beta-checkpoint or beta selection (Carpenter and Bosselut, 2010).

The pre-TCR is composed of a functional TCR\( \beta \) paired with pre-TCR\( \alpha \) and the CD3 complex. The requirement of pre-TCR\( \alpha \) and TCR\( \beta \) for T cell development is illustrated by the study of pre-TCR\( \alpha \) or TCR\( \beta \) knockout mice. In the absence of pre-TCR signaling, thymocytes are arrested at the DN3 stage (Fehling et al., 1995; Mombaerts et al., 1992). Immediately downstream of the pre-TCR complex, Lck, Fyn and zeta-associated protein (ZAP)-70 are also required for pre-TCR signaling. pre-TCR signaling is able to activate transcriptional factors for the further differentiation and cell cycle regulation, including nuclear factor \( \kappa \)B (NF-\( \kappa \)B), nuclear factor of activated T cells (NFAT), cMyc and Id3 (Yamasaki and Saito, 2007).

Borowski’s research has demonstrated that the pre-TCR is not interchangeable with the \( \alpha\beta \) TCR, indicating that it has specific functions during the transition from the DN to the DP stage (Borowski et al., 2004). The pre-TCR checkpoint is enforced through the regulation of proliferation and cell survival by pre-TCR signaling. VDJ
rearrangement itself cooperates with the functions of E proteins to stop the cell cycle in the DN3 stage (Engel and Murre, 2004; Liu et al., 2009; Xi et al., 2006). Only those cells that finish TCRβ rearrangement and form a functional pre-TCR will be released into cell cycle for the next step. The pre-TCR activates the Ras/Raf pathway, which leads to the expression of the Egr1 transcription factor. Egr1 subsequently activates the E2A inhibitor Id3, which in turn suppresses the activity of E2A (Engel and Murre, 2004). Meanwhile, cMyc expression is upregulated by pre-TCR signaling to promote cell proliferation. The expression level of cell cycle related proteins during checkpoints are correlated to changes of cell proliferation (Dose et al., 2006). Another function of pre-TCR signaling is to regulate cell survival through regulation of NF-κB. NF-κB is required for the survival of those lymphocytes with a functional TCRβ. Inhibition of NF-κB results in the loss of beta-selected lymphocytes, while in Rag1−/− mice, enforced activation of NF-κB can allow DN cells to progress to the DP stage even without expression of the pre-TCR (Voll et al., 2000). Collectively, multiple regulatory pathways are coordinately activated by the pre-TCR to allow cells to pass the beta-selection checkpoint.

The DN3 stage can be further subdivided into DN3a and DN3b according to the expression of CD27, a surface marker that is upregulated in cells upon passing the pre-TCR checkpoint. Therefore, DN3 cells can be separated into the CD27lo DN3a (before checkpoint) and CD27hi DN3b (after checkpoint) subsets. The differential expression of
CD27 provides an easy way to purify and characterize cells during the pre-TCR checkpoint (Taghon et al., 2006).

During the DN2-3 stage, divergence of αβ T cells and γδ T cells also occurs, as the TCRγ and TCRδ genes are also rearranged at this stage and those obtaining a functional γδ TCR differentiate into γδ T cells. These cells will be discussed in detail in chapter 1.3.

1.1.1.2 TCR checkpoint and selection in the DP stage

Following pre-TCR selection, DN cells re-enter the cell cycle and rapidly proliferate for a brief period in the DN4 and ISP stages prior to entering the DP stage (Figure 1). The TCRα chain is rearranged during the DP stage to form a complete αβ TCR, which will be selected by different MHC or MHC like molecules during differentiation into specific lineages. This is the second checkpoint for αβ T cells. There are two selective events that occur in the DP stage. The first is positive selection, in which the αβ TCR must weakly recognize antigen presented by certain MHC molecules. The second is negative selection, which results in deletion of cells bearing an autoreactive TCR. Those cells without a functional TCR or with an autoreactive TCR cannot pass through positive or negative selection and die by neglect or apoptosis, respectively. After MHC-induced selection, DP cells differentiate into different T cell subsets. CD8 SP cells are MHCI-restricted, CD4 SP cells are MHCII-restricted, and NKT
cells are selected through CD1d, an MHCI-like molecule (Carpenter and Bosselut, 2010; Gascoigne and Palmer, 2011).

The signal strength of the TCR upon interacting with self-peptide presented by MHC is critical for selection. A particularly strong signal will initiate negative selection by way of activation-induced cell death, but partial activation induced by a weaker signal results in positive selection. It seems a threshold of TCR strength between positive selection and negative selection must be reached, which can be defined as the compartmentalization of RAS-MARK-Erk signaling or Ca\textsuperscript{2+} influx, two downstream pathways activated by TCR signaling. Several publications have indicated that the weakest signal capable of inducing negative selection is stronger than the signal required for positive selection (Daniels et al., 2006; Kane and Hedrick, 1996). However, it is still not thoroughly understood how various TCR signal strengths activate the different downstream responses.

The TCR signaling triggers several responses. First, it upregulates the antiapototic molecules, Bcl-2 family members, to prevent cell death (Campbell et al., 2012; Sohn et al., 2007). Second, it upregulates CCR7, which is critical for thymocyte migration from the cortex to the medulla during the transition from DP to SP. Third, Foxo1, Klf2 and IL-7Ra are expressed under the control of TCR signaling to initiate and maintain the status of SP cells. Fourth, Id3 is also a target of TCR signaling through the Erk pathways and inhibits E proteins, allowing the transition from DP to SP. Id3 also
plays roles in the CD4/CD8 lineage choice, which will be discussed in chapter 1.2 (Carpenter and Bosselut, 2010; Jones-Mason et al., 2012).

1.1.2 Innate-like T cells

Innate-like T cells are a set of T cell subtypes that are capable of providing more rapid effector functions than conventional αβ T cells. Unlike conventional T cells, innate-like T cells constitutively express activation markers on surface, such as CD44 and CD69, and do not need activation-induced differentiation to produce effector cytokines (Alonzo and Sant'Angelo, 2011).

Several types of innate-like T cells are studied frequently, including NKT cells, mucosal associated invariant T (MAIT) cells, Vγ1.1+Vδ6.3+ γδ T cells (also known as NK like γδ T cells), and innate-like CD8 T cells. Among multiple transcription factors that have been studied in the development and function of innate-like T cells, promyelocytic leukemia zinc finger (PLZF) has been identified as the most important signature transcription factor for innate-like T cells. PLZF is also named as ZBTB16, which belongs to the zinc finger and BTB (broad complex, tramtrack, and ric-ś-brac) family. It has N-terminal poxvirus and zinc finger (POZ) domain and a C-terminal functional domain (Kelly and Daniel, 2006). PLZF is expressed by the majority of these innate like T cells and is essential for the effector functions of these cells, including expression of activation markers and Th-like cytokine production. This is corroborated by the finding that NKT cells and Vγ1.1+Vδ6.3+ γδ T cells in the PLZF-deficiency mice fail to upregulate CD44
and fail to produce effector cytokines (Gordon et al., 2011; Kovalovsky et al., 2008; Kreslavsky et al., 2009; Savage et al., 2008).

In the following introductory chapters, we will focus on the development of NKT cells and innate-like Vγ1.1-Vδ6.3+γδ T cells.

### 1.1.3 NKT cells

#### 1.1.3.1 Overview of NKT cells

NKT cells are CD1d-restricted αβ T cells, which share the surface markers of both Nature killer (NK) cells and conventional αβ T cells, such as NK1.1, NKG2D and TCR. They produce multiple effector cytokines after antigen stimulation and go on to activate many other immune cells including NK cells, B cells, T cells, dendritic cells and macrophages. NKT cells have been shown to play important roles in many diseases, such as microbial infection, cancer, autoimmune disease and graft-versus-host disease (Brennan et al., 2013; Godfrey et al., 2010).

There are mainly two subsets of NKT cells, type I NKT cells and type II NKT cells (table 1). Type I NKT cells are also referred as invariant NKT (iNKT) cells or classical NKT cells, because they express an invariant TCRα chain with Vα14-Jα18 in mice and Vα 24-Jα18 in humans. The TCRβ chain for iNKT cells shows limited repertoire of several Vβ subtypes with a strong preference for Vβ2, Vβ7 and Vβ8.2 (Wei et al., 2006). These cells are usually in the form of CD4 SP or DN in mice, and typically reside in the thymus, spleen and liver. They are especially abundant in the liver, where
they take up 20-30% of the total lymphocytes in this organ. iNKT cells recognize glycolipid antigens presented by CD1d, for example, α-galactosylceramide (α-Galcer), which originates from the sea sponge Agelas mauritianus. Thus, CD1d tetramers loaded with α-Galcer are widely used as a reagent to identify iNKT cells (Das et al., 2010).

Type II NKT cells utilize a restricted TCRα repertoire such as Vα3-Jα9 and Vα8, along with Vβ6 and Vβ8. Sulfatide presented by CD1d tetramer can be recognized by the type II NKT cells, but this CD1dtet+ population does not include Vα3/Vα8+ NKT cells, indicating that other TCRα subtypes are used for type II NKT cells (Girardi et al., 2012; Jahng et al., 2004). Other antigens have also been reported to be recognized by type II NKT cells, such as lysosulfatide and glycosphingolipids (Rhost et al., 2012a; Roy et al., 2008). However, the function of type II NKT cells is largely unknown, although they have been reported to be involved in several infectious diseases, graft-versus-host disease, obesity and tumor immunity (Rhost et al., 2012b).

Human NKT cells have several distinct features in comparison with mouse NKT cells. First, the invariant TCRα in human NKT cells is Vα24-Jα18, while the Vβ chain is mainly Vβ11. The cells may express CD4, CD8, or neither. Second, there are five CD1 molecules, CD1a, CD1b, CD1c, CD1d, CD1e in humans, but only CD1d in mice. Besides the CD1d-restricted type I and type II NKT cells, CD1a, CD1b and CD1c are classified as a separate group, which also present lipid antigen to select NKT-like cells with a diverse repertoire. However, the iNKT cells in humans show characteristics similar to mouse
iNKT cells, including the invariant TCR, CD1d-dependent development, α-GalCer reactivity and expression of IL-4 and IFNγ (Godfrey et al., 2010).

**Table 1: NKT cell subsets**

<table>
<thead>
<tr>
<th>subsets</th>
<th>TCRα</th>
<th>TCRβ</th>
<th>antigen</th>
<th>α-GalCer reactive</th>
<th>surface marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I NKT</td>
<td>Vα14Jα18(mouse)</td>
<td>Vβ2, Vβ7, Vβ8.2 (mouse)</td>
<td>iGb3, α-GalCer, β-GalCer, β-GluCer</td>
<td>Y</td>
<td>CD4, DN (mouse)</td>
</tr>
<tr>
<td></td>
<td>Vα24Jα18 (human)</td>
<td></td>
<td></td>
<td></td>
<td>CD4, DN, CD8 (human)</td>
</tr>
<tr>
<td>Type II NKT</td>
<td>Vα3Jα9, Vα8 (mouse)</td>
<td>Vβ8 (mouse) Vβ11 (human)</td>
<td>Sulfatide, Lyso-sulfatide, glycosingolipids</td>
<td>N</td>
<td>CD4, DN (mouse)</td>
</tr>
</tbody>
</table>

1.1.3.2 Development of iNKT cells

Two models have been proposed to explain the development of iNKT cells. One is named the “committed precursor” model, which indicates that iNKT cells are developed from unique precursors and pre-committed in the early embryogenesis. This model is supported by the detection of Vα14-Jα18 rearrangement in the prenatal mice (Hager et al., 2007; Makino et al., 1996). The other model is named the “TCR instructive” model, which suggests that iNKT cells share the same precursors as conventional αβ T cells until the DP stage. DP cells with functional Vα14-Jα18 rearrangement develop into the iNKT subset. Numerous publications demonstrated that DP cells are the immediate precursors of iNKT cells, providing strong supports to the instructive model (Godfrey and Berzins, 2007; Godfrey et al., 2010).

As mentioned in chapter 1.1.1, iNKT cells are selected at the DP stage through the interaction of specific TCR and CD1d. The essential event for the initiation of NKT
development is the proper rearrangement of Vα14-Jα18, which will be further introduced in chapter 1.1.3. The CD1d molecule is also necessary, as NKT cells are absent in CD1d deficient mice, but present in mice with CD1d expressed on cortical thymocytes (Coles and Raulet, 2000; Gapin et al., 2001; Xu et al., 2003). The ligand presented by CD1d during selection is still under investigation, but it is widely accepted that it is self-antigen. Glycolipid isoglobo trihexosylceramide (iGb3) has been suggested as one potential ligand (Cheng et al., 2007; Stanic et al., 2003). Selection is not only restricted to the invariant Vα14-Jα18; the Vβ repertoire also plays a role in selection as the affinity for the invariant TCRα with different Vβ is broad during positive selection (Mallevaey et al., 2009; Pellicci et al., 2009). It is still not well understood whether or even how iNKT cells undergo negative selection, but some studies indicate the existence of negative selection based on the depletion or decrease of iNKT cells that is observed when high affinity antigen is induced in the postnatal stage or when CD1d is overexpressed (Chun et al., 2003; Pellicci et al., 2003; Schumann et al., 2005).

Besides TCR-CD1d interaction, the activation of signaling lymphocytic activation molecule (SLAM) receptor and SLAM-associated protein (SAP) are also required for iNKT cell selection. SAP binds to the tyrosine-based motifs of SLAM receptor and recruits SAP interacting kinase Fyn (Chan et al., 2003; Latour et al., 2003). SLAM (encoded by Slamf1 gene) and Ly108 (encoded by Slamf6 gene) have been identified as the main SLAM receptors involved in NKT cell development. Disruption of Slamf1,
Slamf6, SAP and Fyn, all result in a block in iNKT cell development (Griewank et al., 2007; Nichols et al., 2005; Nunez-Cruz et al., 2008). Initiation of SLAM-SAP-Fyn signaling activates NFκB, which will be further discussed in the next chapter (Stanic et al., 2004b).

After selection, the NKT precursors undergo several sequential stages before maturation. The classical model suggests four stages according to the surface expression of CD24, CD44 and NK1.1 (Figure 2, upper panel). The first stage of NKT lineage development is stage 0 (CD24hiCD44loNK1.1); this subset is extremely small in numbers and does not proliferate. CD24 is subsequently downregulated when cells enter stage 1 (CD24loCD44loNK1.1), at which cells proliferate. CD44 is subsequently upregulated upon entry into stage 2 (CD24loCD44hiNK1.1). Stage1 and stage 2 are not fully distinctive, as CD44 expression gradually increases from low to high levels. A large fraction of stage 2 cells migrate to the periphery, while others remain in the thymus. The final maturation stage is stage 3 (CD24loCD44hiNK1.1*), when NK1.1 and other NK cell receptors are upregulated on the surface. Proliferative ability is reduced at this stage, accompanied with changes in cytokine expression. NKT cells express IL-4, but almost no IFNγ in stages 1 and 2, while they produce much more IFNγ and less IL-4 in stage 3. Expression of other cytolytic effectors and chemokine receptors, such as granzyme B, CCR5, CXCR3, CD122 and FasL are also increased during maturation to stage 3 (Bendelac et al., 2007; Godfrey and Berzins, 2007; Townsend et al., 2004).
This classical development model has been challenged recently by a new classification model (Figure 2, lower panel). The new model divides NKT cells into T-bet⁺NKT1, Gata3⁺NKT2 and RORγt⁺ NKT17 according to the expression of specific cytokines and transcriptional factors. NKT1 are the conventional stage 3 NKT cells, which express NK1.1⁺ and produce both IL-4 and INFγ. However, it is unclear regarding at which time point NKT2 and NKT17 branching out (Constantinides and Bendelac, 2013). Some publications indicate that the classical stage 2 NKT cells are terminally differentiated, as opposed to being immature NKT precursors. This hypothesis is supported by two lines of evidence. First, both NKT2 and NKT17 precursors express IL-17Rb, a component of the IL-25 receptor, while NKT1 precursors do not. Additionally, IL-17Rb deficient mice have almost no NKT2 and NKT17 cells, but still have NKT1 cells (Watarai et al., 2012). Second, IL-4 expressing NKT2 cells fail to upregulate T-bet after they are injected into the thymus. However, more data are needed to support this new lineage model (Lee et al., 2013b).

The mechanisms controlling iNKT cell homeostasis after differentiation and maturation are not thoroughly understood, but the homeostasis is thought to be dependent on cytokine IL-15, and to a less extent IL-7. However, homeostasis of hepatic iNKT cells is mainly dependent on the chemokine receptor CXCR6 and intergrin LFA-1 (Geissmann et al., 2005; Ohteki et al., 1999; Ranson et al., 2003; Sandberg et al., 2004).
Figure 2: The classical and modified models of iNKT cell development

Figure 2. NKT cells are derived from the common DP precursors with specific Vα14-Jα18 TCRα rearrangement and selected by CD1d molecules expressed on the other DP cells. These cells pass through several developmental stages. The classical model defines four stages according to the expression of CD24, CD44 and NK1.1. However, recent publications suggest a modified model after incorporating the NKT1, NKT2 and NKT17 cell subtypes, defined according to differential cytokine production and transcription factors expression. The divergence of these subtypes occurs in concert with altered expression of IL-17Rb.
1.1.3.3 Transcriptional regulation in iNKT cell development

Signaling events initiated by the TCR-CD1d interaction and SLAMF receptors finally activate multiple transcriptional factors that regulate iNKT lineage commitment and function acquisition. These transcriptional factors are involved in one or several stages including initiation, selection, expansion and maturation.

The first crucial event in iNKT cell development is the Vα14-Jα18 rearrangement in the DP precursors. Because Jα usage proceeds from the 5’ end to the distal 3’ end during the TCRα rearrangement and Jα18 is on the distal segment of the Jα locus, Vα14-Jα18 rearrangement can be influenced by the lifespan of DP cells. Several factors that affect DP cell survival have been demonstrated to control iNKT cell development. RORγt has been shown to prolong the lifespan of DP cells through upregulating Bcl-XL (Bezbradica et al., 2005). BATF and Fra-2 have the opposite functions as the negative and positive regulators for AP-1, which is essential for cell proliferation and apoptosis (Lawson et al., 2009; Williams et al., 2003; Zullo et al., 2007). Conditional deletion of HEB, a member of the E protein family of transcription factors, in DP cells results in reduced number of stage 0 iNKT cells mainly through regulating the RORγt-Bcl-XL signaling pathways (D’Cruz et al., 2010). cMyb-deficient DP cells show not only reduced Bcl-XL expression, but also altered the expression of SLAM, Ly108 and SAP (Hu et al., 2010). Overall, these transcription factors work together in DP cells to regulate the initiation and selection of iNKT cells (Figure 3).
Following selection by TCR with the V\textalpha{}14-J\textalpha{}18 chain, iNKT precursors undergo expansion and continue functional maturation. PLZF, NF-\kappa{}B, Egr2, T-bet, Gata3 and ROR\gamma{}t play essential roles during these late developmental stages. As mentioned in chapter 1.1.2, iNKT cells are capable of developing in PLZF\textsuperscript{−/−} mice, but fail to expand and acquire effector functions. This is confirmed by the PLZF transgenic expression in conventional \alpha{}\beta{} T cells, which results in the upregulation of CD44 and cytokine production without NK related markers upon primary activation (Kovalovsky et al., 2010; Kovalovsky et al., 2008; Raberger et al., 2008; Savage et al., 2008). The impacts of NF\kappa{}B have been shown in several ways. Expression of NF\kappa{}B dominant negative regulator IkBa or deficiency of NF\kappa{}B molecules causes a significantly reduced number of iNKT cells due to a block in the maturation of NKT cells from NK1.1\textsuperscript{−} to NK1.1\textsuperscript{+} (Stanic et al., 2004a; Vallabhapurapu et al., 2008). Egr2 is one of the downstream targets of TCR signaling. The deficiency of Egr2 or double deletion of Egr1 and Egr2 results in a reduction in numbers of iNKT cells. Egr2\textsuperscript{−/−} iNKT precursors fail to downregulate CD24 and their maturation is blocked. This developmental defect is attributed to failed upregulation of PLZF and IL-2 receptor beta chain, which is required for IL-15 responses (Lazarevic et al., 2009; Seiler et al., 2012). T-bet is involved in the terminal maturation of iNKT cells. T-bet expression increases from stage 1 to 3. iNKT cells lacking T-bet are blocked at stage 2 and fail to upregulate the NK cell specific markers and CD122, a component of the IL-15 receptors. They also fail to upregulate genes related to iNKT
effector functions, such as perforin, IFNγ and Granzyme B (Townsend et al., 2004). Gata3 is required for iNKT cell maturation in several ways. The deficiency of Gata3 in iNKT cells leads to the blockage of iNKT maturation and promotes apoptosis. Similar to its function in conventional αβ T cells, Gata3 is also essential for iNKT cells to produce Th2-like cytokines (Cen et al., 2009; Kim et al., 2006). RORγt is critical for the differentiation of NKT17 cells. These three transcriptional factors, T-bet, Gata3 and RORγt, are the key regulators of terminal differentiation for the three subtypes of iNKT cells.
Figure 3: Signaling pathways controlling iNKT cell selection and differentiation

Figure 3. After the interaction of Vα14-Jα18 bearing TCR and CD1d and subsequent ligation of SLAM family molecules, several signaling pathways are triggered, resulting in the iNKT cell selection, differentiation and maturation. Three major pathways are involved, the mitogen-activated protein kinase, NF-κB and calcium-flux pathways.
1.1.4 Innate-like γδ T cells

1.1.4.1 The divergence of αβ T cells and γδ T cells

γδ T cells are defined as lymphocytes with surface expression of the γδ TCR. For postnatal mice, γδ T cells differentiate from the DN2-3 stages. However, unlike αβ T cells, they do not have a pre-selection checkpoint. There are two models describing the divergence of αβ T and γδ T cells. One is the instructive model, which states that the T cell lineage is dependent on the direct instruction of the pre-TCR or γδ TCR. The other is the stochastic model, or pre-commitment model, which suggests that the linages are determined prior to TCR expression. Those pre-determined to be one specific type of cells will enter the lineage only when they express the matched TCR on their surface. Current studies support a modified version of the instructive model that the T cell fate is determined by TCR signal strength. Those cells receiving strong TCR signaling adopt the γδ T cell lineage, but cells receiving an attenuated signal proceed toward αβ T cell development (Ciofani and Zuniga-Pflucker, 2010; Haks et al., 2005; Narayan and Kang, 2007). However, γδ T cells mainly finish TCRδ rearrangement in the DN2 stage, and TCRγ rearrangement occurs in DN2 and DN3 stages, which allow functional γδ TCR formation before pre-TCR expression, but the number of γδ T cell does not increase even in the absence of functional TCRβ. This suggests the existence of additional regulatory mechanisms in γδ T cell development (Capone et al., 1998; Ueda-Hayakawa et al., 2009).
1.1.4.2 Overview of innate-like $\gamma\delta$ T cells

Different waves of $\gamma\delta$ T cells with specific $\gamma\delta$ rearrangement develop in different time frames and ultimately locate in certain tissues. The first wave of $\gamma\delta$ TCR rearrangement begins at embryonic day 13 (E13) for $V\gamma3$, and followed by E14 for $V\gamma4$, E16 for $V\gamma2$ and $V\gamma5$ (Prinz et al., 2013; Xiong and Raulet, 2007). One special $\gamma\delta$ T cell subset, $V\gamma1.1$-$V\delta6.3^+$ $\gamma\delta$ T cells, expands significantly during the first two weeks of the neonatal stage, although there is evidence that they are derived from fetal precursors (Grigoriadou et al., 2003). Similar to NKT cells, this population utilizes the $V\gamma1.1V\delta6.3$ TCR with limited diversity, expresses the activation marker CD44 and even the NK cell marker NK1.1 on surface, is able to produce IL-4 and IFN$\gamma$ cytokines upon stimulation, and shows high expression of PLZF. The innate phenotype of this population is also dependent on the expression of PLZF, as PLZF deficient $V\gamma1.1$-$V\delta6.3^+$ cells have defects in cytokine production. Because of these similar features between $V\gamma1.1$-$V\delta6.3^+$ $\gamma\delta$ T cells and iNKT cells, this $\gamma\delta$ T cell population is also named as $\gamma\delta$ NKT cells (Azuara et al., 1997; Grigoriadou et al., 2003; Kreslavsky et al., 2009; Verykokakis et al., 2010).

1.1.4.3 Transcriptional regulation of innate-like $\gamma\delta$ T cell development

Several transcriptional factors have been shown to regulate the development of the innate-like $V\delta1.1$-$V\delta6.3^+$ population. Deficiency of Id3 or the Tec kinase ITK results in a significant expansion of the $V\delta1.1$-$V\delta6.3^+$ population, and overexpression of Dok-1 also results in the same phenotype. Moreover, development of this population is also
regulated by TCR signal strength, as reduced strength of TCR signaling by forced-expression of a hypomorphic SLP-76 with mutation of key tyrosines in mice promotes the development of Vγ1.1-Vδ6.3+ cells. Id3, ITK and Dok-1 are all downstream of TCR signaling. ITK activates multiple pathways, including C-γ1, MAPK pathways as well as initiating calcium influx. Dok-1 is a negative regulator of TCR signaling. The phenotype of Id3-deficient, ITK-deficient or Dok-1 overexpressing mice confirmed that the development of Vγ1.1-Vδ6.3+ γδ T cells requires relatively low TCR signaling strength.

In addition to the regulation of TCR signaling, SAP is also important for innate like γδ T cell development in a manner similar to its roles in NKT cell development. Deletion of SAP in Id3−/−, ITK−/− or Dok-1 overexpressing mice suppresses the expansion of the Vγ1.1-Vδ6.3+ population (Alonzo et al., 2010; Besin et al., 2012; Verykokakis et al., 2010; Yin et al., 2013).

1.1.5 V(D)J recombination

1.1.5.1 Overview of V(D)J recombination

The development of a diverse TCR repertoire is dependent on V(D)J recombination, which is orchestrated by semi-random joining of variable (V), diversity (D) and joining (J) gene segments. In this way, a limited number of gene segments can generate a large number of different TCRs. The TCR is essential for the T cell to recognize antigen and for subsequent effector responses. The interaction of specific TCR
and self-peptide presented in the context of MHC or MHC like molecules is critical for the development of T cells, including innate like T cells (Dudley et al., 2005).

αβ T cells and γδ T cells utilize different types of TCR generated by separate antigen receptor genes. An αβ TCR is composed of TCRβ and TCRα, which are rearranged in the DN and DP stages, respectively. The mature αβ TCR is formed in DP cells. Both TCRγ and TCRδ are rearranged in the DN stage. Figure 4 shows the structure of the antigen receptor genes prior to recombination for αβ and γδ TCR. As the δ locus resides within the TCRα locus, it is also named TCRα/δ locus (Schatz and Ji, 2011).

V(D)J recombination is carried out by two recombinase proteins, which are encoded by recombination activation gene 1 (RAG1) and RAG2. They recognize and bind to the recombination signal sequences (RSS). The RSS is composed of the conserved heptamer and nonamer sequences separated by a less conserved spacer of 12 or 23 base pairs. RSS recombination occurs between RSSs of 12 and 23 spacers, which is known as the 12-23 rules. The complex of RAG1, RAG2 and their cofactor high-mobility group protein B1 (HMGB1), binds to one RSS and recruits the second RSS to form a synaptic complex, or paired complex. Then double strand DNA is cleaved by RAG proteins between the VDJ gene segments and the RSSs, and the ends of the chromosome with gene segments are rejoined and connected by RAG proteins and non-homologous end joining (NHEJ) DNA repair factors. Meanwhile, the RSS ends form a circular signal joint. Extra non-template nucleotides may be added by terminal deoxynucleotidyl transferase
(TdT) or nucleotides may be lost during the formation of the coding joint, adding sequence diversity to the finished products (Dudley et al., 2005; Schatz and Swanson, 2011).

Because recombination errors may lead to lymphoma formation, recombination is strictly controlled by several ways. First, the expression of RAG proteins is restricted to developmental stages in which recombination occurs. RAG proteins are not expressed in all cell lineages except T or B cells and their expression is also controlled by the cell cycle. RAG2 is periodically degraded by cell cycle related proteins cyclinA/Cdk2, ensuring that it can only function in the G0/G1 phases of the cell cycle (Liu et al., 2009). Second, the accessibility of RSSs is strictly regulated so that only the transcribed region can be accessed by RAG proteins. The accessibility is correlated with changes in histone modification, DNA methylation, and germline transcription. In addition to transcriptional regulation, recombination is also regulated by the three dimensional architecture of the antigen receptor gene loci in the nuclei. Chromatin remodeling reorganizes DNA to promote recruitment of multiple control elements for recombination, such as enhancers, promoters, transcription factors and other cofactors (Schatz and Ji, 2011; Schatz and Swanson, 2011).
Figure 4: The structure of TCRβ and TCRα/δ receptor gene locus

Figure 4. The TCRβ and TCRα/δ loci are shown as schematic diagrams. Variable (V), diversity (D), and joint (J) gene segments are represented as rectangles, promoters are represented as triangles, and enhancers as ovals. The number of each V, D, J gene segments and approximate length are marked.
1.1.5.2 The TCRβ locus and Vβ-Dβ-Jβ rearrangement

TCRβ rearrangement is a critical step for the early development of αβ T cells. As Figure 4 shows, there are two Dβ-Jβ-Cβ clusters, controlled by Eβ and two promoters PDβ1 and 2 throughout the 25kb locus. TCRβ rearrangement requires two sequential steps, a Dβ-to-Jβ joint and then a Vβ-to-DβJβ joint. Both of these rearrangements are directed by the 23RSS and 12RSS flanking Dβ and Jβ, or Vβ and Dβ. Although what controls the order of Dβ-to-Jβ and Vβ-to-DβJβ is not well understood, deletion of the 23 RSS on the 3’ side of Dβ1 or c-Fos deficiency is able to trigger Vβ-to-Dβ1 recombination prior to Dβ1-to-Jβ1 recombination (Krangel, 2009; Wang et al., 2008).

Another feature of TCRβ rearrangement is allelic exclusion, meaning that each cell only has one functional TCR allele after the recombination. This is caused by asynchronous rearrangement during the initial recombination stages and inhibitory feedback following the generation of a functional TCRβ chain. Asynchronous rearrangement is due to the high frequency of one or two alleles associating with inhibitory subnuclear compartment, which has been proved to be the nuclear lamina. The peripheral positioning inhibits recombination and makes it unlikely to initiate Vβ-to-DβJβ recombination on both alleles simultaneously (Chan et al., 2013; Krangel, 2009). The feedback signal provided by the pre-TCR prevents rearrangement of the other allele through downregulating Rag1 and Rag2 and releasing cells into the cell cycle. Together they insure that only one functional TCRβ allele can be expressed in a given cell (Jackson
and Krangel, 2006; Khor and Sleckman, 2002; Schlimgen et al., 2008). Although Rag proteins are upregulated again to allow TCRα rearrangement in the DP stage, the chromatin structure and locus conformation of the TCRβ locus is altered to prevent RAG accessibility (Skok et al., 2007; Tripathi et al., 2002). This inhibition of accessibility can be overridden through enforced expression of one E protein, E47 (Agata et al., 2007). E proteins will be further discussed in chapter 1.2.

1.1.5.3 The TCRα/ TCRδ locus and Vα-Jα rearrangement

The TCRα/ TCRδ locus is much more complicated, as there are two recombination events for different TCRs that occur in two developmental stages. As shown in Figure 4, more than one hundred V gene segments, including both Vα and Vδ, as well as Dδ, Jδ and Cδ genes are located across 1.5 mb of the 5’ end in this locus, while 61 Jα and Cα take up 100kb on the 3’ end. Two specific enhancers, Eδ and Eα, control the recombination of Vδ-to-Dδ-to-Jδ in the DN stage and Vα-to- Jα in the DP stage, respectively (Hernandez-Munain et al., 1999).

Vα- Jα rearrangement is the essential event in DP cells, allowing a functional TCRα paired with the TCRβ chain to form a mature TCR. The TCRα locus can undergo several rounds of rearrangement. The primary rearrangements are biased toward the use of 3’Vα and 5’ Jα, while secondary rearrangements preferentially use 3’ Jα segments. Therefore, rearrangements using 3’ Jα require an extended time frame, allowing the lifespan of DP cells to affect the TCRα repertoire (Krangel, 2009). The primary Vα- Jα
rearrangement is controlled by two Eα-dependent promoters, the T-early α (TEA) promoter and the Jα 49 promoter. The TEA promoter is located 5’ of the Jα gene segments, while the Jα 49 promoter is located between Jα 50 and Jα 49. The accessibility of Jα gene segments is controlled by the transcriptional elongation initiated from TEA (Abarrategui and Krangel, 2006). TEA and possibly Jα 49 promoters are deleted by primary rearrangement, which introduce the Va promoter into the 5’ end of the Jα array, allowing transcription of the residual Jα segments during subsequent rearrangements. On average, approximately five rounds of Va-Jα rearrangement take place for each allele prior to positive selection. Therefore, the likelihood of successful positive selection increased, as initial recombination, if unsuccessful, paves the way for subsequent rounds of recombination (Guo et al., 2002; Hawwari et al., 2005; Hawwari and Krangel, 2007).

1.1.5.4 Va14-Jα18 rearrangement and iNKT cell selection

Proper Va14-Jα18 rearrangement is required for iNKT cell development. As one possible TCRα rearrangement outcome, any factors that affect the overall Va-Jα rearrangement also affect iNKT cell development. For example, iNKT cells are absent in the mice lacking Rag1, Rag2 or Jα18 genes (Cui et al., 1997; Godfrey et al., 2010). Additionally, because Jα18 is located at the 3’ end of the Jα array, its use necessitates multiple rounds of TCRα rearrangement, so any factors that affect the DP lifespan also affect the Va14-Jα18 rearrangement, which has been discussed in chapter 1.1.3.3. Finally, Jα18 accessibility is essential for the Va14-Jα18 rearrangement; therefore, any factors
that change the 5′-3′ bias on the Jα array also affect the Jα 18 usage. For example, in TEA or Jα 49 promoter-deficient mice, usage of Jα segments at the 5′ end is impaired while the usage of 3′ Jα segments is proportionally increased, including Jα 18. As a result, the number of NKT cells is increased in these mice (Hager et al., 2007).

1.2 Id proteins and E proteins

Inhibitor of DNA-binding (Id) proteins and E proteins are frequently studied regulators of many systems in numerous species. These systems include the development and differentiation of the neuronal and hematopoietic systems. Within the hematopoietic system, E and Id proteins play diverse roles in multiple immune cell types, including HSCs, NK cells, B cells, T cells, and DCs. This chapter will focus on the functions of Id and E proteins during T cell development, especially the proteins encoded by the Id2, Id3, E2A and HEB genes.

1.2.1 The structure of E proteins and Id proteins

E proteins, also known as class I HLH proteins, are named for their ability to bind a palindromic DNA sequence CANNTG, the E box. There are four E proteins found in mammals, E12, E47, HEB, E2-2. E12 and E47 are produced through alternative splicing of the E2A gene (also known as TCFE2A or TCF3). Both the HEB (also known as TCF12) and E2-2 (also known as TCF4) genes are able to produce two different proteins, canonical and alternative forms, through an alternative transcription start site. Id proteins are natural inhibitors for E proteins. There are also four Id proteins identified to
date, Id1, Id2, Id3, and Id4. All the E proteins and especially two Id proteins, Id2 and Id3, are widely expressed in the multiple immune cells, and play critical roles in their differentiation, proliferation and cell lineage choice (Kee, 2009; Wang et al., 2006).

All E proteins have one or two activation domains (AD), a basic-DNA-binding domain and a helix-loop-helix (HLH) domain (Figure 5A). The HLH domain enables protein dimerization. The majority of E proteins have two Ads. The alternative forms of HEB and E2-2 only have one AD, but they have a novel Alt domain, which suggests specific and unique functions for these alternative forms. E proteins can form homodimers to activate their downstream targets or heterodimers with class II basic HLH (bHLH) proteins to activate or inhibit transcription according to the cofactors they recruit. Dimerizing with tissue specific class II bHLH proteins may be one explanations for the specificity of E protein function in different tissues despite the wide expression of E proteins and high frequency of E boxes in genome. Id proteins have the same HLH domain, but no basic-DNA-binding domain; therefore, they can prevent E proteins from binding DNA by heterodimerizing with E proteins (Figure 5B). It is widely accepted that E proteins are the major binding partners for Id proteins, although other binding partners have been found (de Pooter and Kee, 2010; Kee, 2009; Lasorella and Iavarone, 2006; Loveys et al., 1996; Massari and Murre, 2000).
Figure 5: The structure and working model of E protein and Id protein

Figure 5. (A) Schematic diagrams for the each domain of E proteins and Id proteins. There are two activation domains (AD) and one basic-helix-loop-helix (bHLH) domain for E proteins and only helix-loop-helix (HLH) domain for Id proteins. (B) The working model for how E proteins work and how Id proteins inhibit E protein activity. The dimers of E proteins bind to the E box in promoters or enhancers to regulate its downstream targets, but once Id proteins heterodimerize with E proteins, DNA binding is inhibited.
1.2.2 The functions of Id proteins and E proteins in T cell development

1.2.2.1 The functions of Id proteins and E proteins in the DN stage

There are hundreds of publications about the multiple roles of Id proteins and E proteins in lymphopoiesis and immune responses. In T cell development, Id proteins and E proteins are involved in multiple stages and have various functions.

E proteins regulate lymphoid lineage development starting at the HSC stage. E2A is required for HSC maintenance and the development of lymphoid-primed multipotent progenitors (LMPPs). It also affects the fate choice of LMPPs to proceed toward the myeloid or lymphoid lineages, as E2A deficient LMPPs preferentially proceed to the myeloid lineage. T cell precursors, ETPs, which are derived from LMPPs, upregulate IL7R, Notch1 and CCR9 under the transcriptional control of E2A in concert with PU.1 and Ikaros (Dias et al., 2008; Ng et al., 2009; Semerad et al., 2009; Spooner et al., 2009). This process is defined as lymphoid priming and positions E2A as one of the important transcriptional factors at the very early stages of T cell development (de Pooter and Kee, 2010).

After the entry of ETP into the thymus for further differentiation, DN precursors still have the potential to develop into other immune cell types, such as NK cells and DCs, before they fully commit to the T cell fate and express the pre-TCR or γδ TCR. Id2 deficiency blocks the development of NK cells. Deletion of E2A in the Id2 deficient background can rescue this phenotype, while E2A−/− mice have significantly decreased
cellularity of ETPs and DN2 cells (Boos et al., 2007; Ikawa et al., 2001). Combined with the finding that numbers of LMPPs and CLPs are also decreased in E2A−/− mice, these data indicate that E2A promotes the T cell lineage fate and reduced activity of E2A by Id2-mediated inhibition promotes the NK cell fate (de Pooter and Kee, 2010; Dias et al., 2008).

Other E proteins also play roles during the T cell commitment stage. HEB single knockout mice have no severe defects before the immature ISP stage. However, mice expressing the dominant negative form of HEB (HEB^bm/bm mice), which can heterodimerize with E2A but cannot bind DNA due to the lack of the basic DNA binding domain, show a significant reduction in total thymocytes. These mice display a significant block at the DN3 stage along with impaired VDJ recombination. This phenotype indicates that the heterodimer of E2A and HEB is the major dimerization form of E proteins during the DN stages. Due to the incomplete block of DN cells observed in the E2A−/− mice, it is likely that E2A and HEB fulfill partially redundant functions in DN cells (Barndt et al., 1999; Barndt et al., 2000). These conclusions are confirmed by the conditional double knockout of E2A and HEB in the DN stage. Conditional elimination of E2A and HEB with LckCre in early T cell development results in a severe block at the DN stage and increased proliferation of DN cells. However, unlike HEB^bm/bm mice, these animals still contain DN4 cells and their TCRβ rearrangement is normal, which may be due to incomplete deletion by LckCre or
functional compensation by other E-proteins (Wojciechowski et al., 2007). E2-2 is also expressed in the DN stage, and E2-2⁻/⁻ mice show a partial block at the DN3 stage and reduced expression of pre-TCRa, a known target of E2A and HEB. Therefore, E2-2 may also fulfill redundant functions along with the other E proteins in the DN stage (Wikstrom et al., 2008). Overall, these publications demonstrate that E proteins work in concert to promote DN cell development and the cell cycle arrest before the TCRβ checkpoint.

Regarding the roles of Id proteins in the DN stage, the fact that single knockout of each Id gene does not show severe defect indicates their redundant functions. Enforced expression of Id1 or Id2 in T cells promotes proliferation and blocks T cell differentiation in the DN or ISP stage, indicating that a tight control of Id protein levels in early developmental stage is required (Kim et al., 1999; Morrow et al., 1999).

The mechanisms by which E proteins regulate T cell commitment and DN cell differentiation, although studied in many publications, are still incompletely understood. First, E proteins, mainly E2A, cooperate with Notch signaling during very early stages of development. Notch signaling is necessary and sufficient for T cell commitment and there is crosstalk between Notch1 and E2A. Before the beta-selection checkpoint, Notch1 is downstream of E2A, so E2A deficiency results in impaired expression of Notch1 and its targets, such as Hes1. Notch1 is downregulated in DN3 cells, mainly due to the fact that pre-TCR signaling upregulates Id3. Id3 inhibits E2A activity and thus inactivates
the transcription of Notch1 (Ikawa et al., 2006; Yashiro-Ohtani et al., 2009). Second, E2A and HEB are required for the proper V(D)J recombination of TCRβ and formation of the functional pre-TCR formation. In addition to regulating Rag1/2 and pre-TCRα, E2A also directly binds the TCRβ locus to regulate its accessibility. Enforced expression of E2A can break allelic exclusion, allowing TCRβ rearrangement (Agata et al., 2007; Schlissel et al., 1991; Tremblay et al., 2003). Third, E proteins have been found to maintain cell cycle arrest before the pre-TCR checkpoint in the DN3 stage by inhibiting cell proliferation and promoting cell survival. Only when functional pre-TCR signaling upregulates Id2 and Id3 through the extracellular signal-regulated kinase (ERK) MAPK pathway, is E protein activity inhibited and cells released into cell cycle (Bain et al., 2001; Engel et al., 2001; Engel and Murre, 2004; Prabhu et al., 1997). In conclusion, Id proteins and E proteins play key roles in the regulation of pre-TCR checkpoint in the DN stage.

1.2.2.2 The functions of Id proteins and E proteins in the DP stage

Several events during the DP stage have been found to be regulated by Id proteins and E proteins. First, E proteins are involved in regulating DP cell survival and TCRα repertoire. Because the lifespan of DP cells influences the TCRα repertoire as discussed in chapter 1.1.5 and RORγt is required for DP cell survival through activation of Bcl-xL expression, E proteins can affect DP cell survival through its direct target RORγt (Sun et al., 2000; Xi et al., 2006). Second, the dosage of E proteins influences the DP to SP transition. Early studies showed that deficiency of E2A enhances maturation
from DP to SP, while deficiency of Id3 yields the opposite phenotype (Bain et al., 1999a; Rivera et al., 2000). As E2A and HEB have critical roles before the DP stage, the functions of Id proteins and E proteins in the DP stage are more readily studied using conditional deletion to avoid the influence of defects arising in the DN stage. Combined deletion of E2A and HEB in the DP stage by CD4-Cre results in increased DP to SP transition, even bypassing the requirement of TCR (Jones and Zhuang, 2007). These studies demonstrate that a proper dosage of E2A and HEB in DP cells is necessary for acquisition of functional TCR and positive selection mediated by TCR-MHC interactions. Third, E proteins also affect the CD4/CD8 lineage choice of DP cells. Double deficiency of E2A and HEB in DP cells blocks CD4 lineage development, allowing only CD8 SP cells to be generated, while double deletion of Id2 and Id3 in the DP stage allows development of only CD4 SP cells. The bias toward the CD8 lineage in the absence of E proteins is not simply through regulating CD4 or CD8 expression, but through regulation of multiple migration and cell surface receptors like CCR7, IL7Ra and CXCR4, as well as the transcription factors regulating CD4/CD8 lineage selection, such as ThPOK and GATA3 (Jones and Zhuang, 2007; Jones-Mason et al., 2012). These results not only demonstrate the redundancy of Id proteins and E proteins in the DP stage, but also demonstrate that the proper dosage of E proteins is critical for the T cell lineage choice.
1.2.2.3 The functions of Id proteins and E proteins in NKT cell development

Because the initiation of NKT cell development is directly related to DP cell lifespan with respect to Vα14-Jα18 rearrangement, E proteins, especially HEB, have been found to regulate NKT cell development in the very early stages. HEB deficiency in the DP stage results in a significant decrease in the number of NKT cells. The impaired TCRα rearrangement with distal Jα, including Vα14-Jα18, in these mice is the major cause for this NKT reduction. This defect can be rescued by the expression of Vα14-Jα18 TCR transgene or Bcl-xL, which confirms that the defects of TCRα rearrangement due to shortened DP lifespan is the direct cause of impaired NKT development (D’Cruz et al., 2010). Id2 has also been shown to promote the homeostasis of NKT cells in the liver. The number of Id2−/− NKT cells in the liver is significantly decreased, which is mainly due to the increased apoptosis through downregulation of Bcl-2 and Bcl-xL (Monticelli et al., 2009). However, much remains unknown about the roles of Id2 and Id3 in NKT cells. This will be discussed in chapter 3 and 4.

1.2.2.4 The functions of Id proteins and E proteins in γδ T cell development

Not only is αβ T cell development controlled by E proteins and Id proteins, the lineage choice of γδ T cells at the DN3 stage is also regulated by them, especially by E2A and Id3. In Id3−/− mice, numbers of γδ T cells are significantly increased, and the majority of them are Vγ1.1+Vδ6.3+ innate-like γδ T cells as discussed in chapter 1.1. This increased γδ T cell population is mainly dependent on E proteins, because when E2A is deleted in
the Id3 deficient background, this phenotype can be rescued. Additionally, deficiency in both E2A and HEB leads to the reduced numbers of γδ T cells (Ueda-Hayakawa et al., 2009; Verykokakis et al., 2010). Regarding the mechanisms of how E proteins regulate γδ T cell development, ectopic expression of E proteins in nonlymphoid cells may directly activate TCRγ and TCRδ rearrangement (Ghosh et al., 2001). In E2A− mice, the predominant usage of the Vγ2 and Vδ5 gene segments in adults is severely affected, but the usage of Vγ3 and Vδ1 gene segments in fetal mice remains normal. However, the mechanisms underpinning how E proteins regulate Vγ1.1+ Vδ6.3+ innate-like γδ T cell development is still not thoroughly understood (Bain et al., 1999b; Jones and Zhuang, 2009).
Figure 6: The functions of Id proteins and E proteins in T cell development

Figure 6. Id proteins and E proteins play multiple roles during T cell development. The expression level of two E proteins, E2A and HEB, and two Id proteins, Id2 and Id3, is shown in a gray scale. E2A and HEB have redundant functions in the DN3 and DP stages. E2A is critical for γδ T cell development, while HEB is required during the transition from ISP to DP and initiation stage of iNKT cell development.
1.3 NKT lymphoma

1.3.1 Overview of NKT lymphoma

Although NKT cells are well known for their anti-tumor functions, they may also be subject to malignant transformation in the absence of proper regulation. The current study about NKT leukemia-lymphoma is very limited. Early study of the peripheral T-cell lymphoma (PTCL) showed that some T lymphocyte type of the extranodal T/NK cell lymphoma express NK cell marker CD56 (Ohshima et al., 2002). Moreover, cell lines derived from patients with natural killer-like T cell leukemia/lymphoma expressed both T cell and NK cell markers (Matsuo and Drexler, 2003). Two publications showed that a defect in IL-15 signaling may cause over-proliferation of NKT cells, but the pathogenesis and mechanism of NKT lymphoma development remain much unknown (Matsuda et al., 2002; Yu et al., 2011).

1.3.2 The functions of Id proteins and E proteins in tumor progression

Id proteins and E proteins have been shown to regulate the progression of multiple tumors. Id proteins have been suggested as oncogenes due to their high expression in many tumor subtypes, such as prostate, breast, colon, neural tumors etc. Their expression in cancer is often related to the high level of known onco-proteins and growth factor activated signaling, and tumor suppressors such as FOXO3 and p53 repress Id protein expression. (Lasorella et al., 2014; Perk et al., 2005). Id proteins are required for the transition from G1 to S phase and have been demonstrated to release the
cell cycle arrest imposed by E proteins through the Rb and Ras pathways in neuronal cells and T cells (Bain et al., 2001; Hara et al., 1994; Lasorella et al., 2000; Ohtani et al., 2001). However, there is no direct evidence for Id proteins’ neoplastic effect on normal cells. Id3 mutations were found at a high frequency in human Burkitt’s lymphoma and Id3 knockout mice developed γδ Hepatosplenic T-cell lymphoma (HSTCL), suggesting that Id3 may also play a tumor suppressive role under certain circumstances (Li et al., 2010; Love et al., 2012; Richter et al., 2012). The majority of HSTCL cases display expression of a γδ T cell receptor, while some cases show lymphomas cells expressing an αβ TCR (Visnyei et al., 2013). Both of γδ and αβ HSTCL generally express NK cell-associated antigens and similar gene signatures, which indicate the similarity between αβ HSTCL and NKT lymphomas (Miyazaki et al., 2009; Morice et al., 2006; Travert et al., 2012). Moreover, the Id1 transgenic mice show a severe block in T cell development in the very early progenitor stages and enhanced T cell apoptosis, which makes the role of Id proteins in lymphocyte malignancy more complicated (Kim et al., 1999).

Regarding the roles of E proteins in tumorigenesis, E2A has been thought of as a putative tumor suppressor, capable of inhibiting cell proliferation. Loss of E2A expression has been observed in multiple human lymphoma subtypes, a finding consistent with Id proteins’ oncogenic effects (Loveys et al., 1996; Miyazaki et al., 2009; Steininger et al., 2011; Talora et al., 2003). E2A deficient mice develop T cell lymphoma at the age of 2 months. Overexpression of E2A in a human T cell lymphoblastic leukemia
cell line, Jurkat cells, promotes cell death through apoptosis (Bain et al., 1997; Park et al., 1999). However, E2A is also highly expressed in some cancers such as prostate and breast cancer, and in the cell lines derived from Burkitt’s lymphoma and natural killer-like T cell leukemia-lymphoma (Chen et al., 2011; Matsuo et al., 2004; Patel and Chaudhary, 2012; Steininger et al., 2011). These latter findings suggest that the roles of E proteins in oncogenesis may be cell type-specific.
2. Materials and methods

2.1 Mice

The LckCre transgenic allele and flox alleles for the Id3, Id2, E2A, and HEB genes have been previously described (Guo et al., 2011; Niola et al., 2012; Pan et al., 2002; Wojciechowski et al., 2007). Rag2\(^{-/-}\) mice were a gift from Dr. Qi-Jing Li’s lab, and MHCII\(^{-/-}\) mice were a gift from Dr. Weiguo Zhang’s lab. Mice used in this study have been maintained on 129/sv and C57Bl/6 mixed background in a SPF facility managed by the Duke University Division of Laboratory Animal Resources. All procedures have been conducted according to protocols approved by the Institutional Animal Care and Use Committee.

2.2 Id2 and Id3 deletion efficiency analysis

The genotyping and deletion efficiency of Id2 and Id3 have been described in Figure 7. The DN2, DN3 and DN4 populations were sorted from both Id2\(^{+/+}\)Id3\(^{+/+}\)LckCre\(^{+}\) and Id2\(^{+/+}\)Id3\(^{+/+}\)LckCre\(^{-}\) mice. DNA was extracted and analyzed by PCR for Id2 and Id3 deletion efficiency. Id2 deletion alleles and flox alleles were analyzed in separate PCR reactions. Id3 deletion and flox alleles were analyzed in a single PCR reaction with a common primer and allelic specific primers. Id3 deletion band (1.34kb) and floxed band (1.07kb) were detected by primers Id3-2, Id3-F and Id3-B. Id2 deletion band (0.9kb) was detected by primers Id2dF and Id2dR. Id2 floxed band (0.47kb) and wild-type band (0.43kb) were detected by primers Scr-1 and Scr-2 (table 2).
Table 2: Primer sequence for genotyping, RT-PCR and V(D)J rearrangement analysis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEB</td>
<td>JW1: CTGGGACAGAAGTTCAGCACTTATAGC&lt;br&gt; JW2: CATTCTTATACATCGGTTCTCC</td>
</tr>
<tr>
<td>E2A</td>
<td>YZ-104: ATGTGTGGGCTGGCACCACACCTTG&lt;br&gt; YZ-150: ACATGGCTGAATATCGACGGT&lt;br&gt; YZ-164: AA GAACGAGGCCCTTTCCGTC</td>
</tr>
<tr>
<td>Id2</td>
<td>Scr-1: TTTGTCAATAATTACGATCA&lt;br&gt; Scr-2: TTGGGAAGTCACATTGAGT&lt;br&gt; Id2-dF: GAGACGCGACAGTGTTCTTCAAA&lt;br&gt; Id2-dR: AAAGGCCATTCTGTGACCGAAGAC</td>
</tr>
<tr>
<td>Id3</td>
<td>Id3-2: GGATTTTCTCAAGATTATGTTGTCG&lt;br&gt; Id3-F: TTCTTCAATCTCCGACATCG&lt;br&gt; Id3-B: GGTCTTTCTTCAACCGACTG</td>
</tr>
<tr>
<td>LckCre</td>
<td>Lck-P: GCAGGAAGTGGTGAATGACTAATAC&lt;br&gt; Mx3: TCTCCCACGCAGTACGATGAGATATC</td>
</tr>
<tr>
<td>MHCII</td>
<td>OIMR6143: AGGGCTGTGTAGAACCTGCGTA&lt;br&gt; OIMR6144: AGGGCCAGCTCATCCTCCACT&lt;br&gt; OIMR6145: GCAGGAGCGGAAGTTGTAATT</td>
</tr>
<tr>
<td>Csf1r</td>
<td>Csf1r-5’: CTGGGACAGACAGGAAATAG&lt;br&gt; Csf1r-3’: CCTTCGAGAAAGTGAGGTAGG</td>
</tr>
<tr>
<td>Csf2rb2</td>
<td>Csf2rb2-5’: TGGAGCAATGAGTACATGG&lt;br&gt; Csf2rb2-3’: CTCCACTTCTGTATGTCCTG</td>
</tr>
<tr>
<td>Vβ5.5-Jβ2.7</td>
<td>Vβ5.5-5’: CCCAGCAGATTCTCA GTCCACAG&lt;br&gt; Jβ2.7-3’: TGAGAGCTGTCTCCTACTGATGG</td>
</tr>
<tr>
<td>Va14-Jα18</td>
<td>Va14-5’: GTGTCAGTCCCTGGTGTAG&lt;br&gt; Jα18-3’: CAAAAATGCAGCCTCCTAAG</td>
</tr>
<tr>
<td>Va3-Jα9</td>
<td>Va3-5’: CAGGGGCTGCAGCTGCTCTCAAG&lt;br&gt; Jα9-3’: CCGAGAGTACTGTACGGCC</td>
</tr>
</tbody>
</table>

2.3 RT-PCR

For gene expression analysis by RT-PCR, total RNA was extracted by using an RNAqueous Kit (Life Technology) according to manufacturer’s protocol, and reverse transcribed into cDNA by murine leukemia virus reverse transcriptase (Life Technology).
RT-PCR was performed by Fast-Start DNA master SYBR green kit and quantitative expression is normalized by β-actin. Primer sequence for each gene is in table 2.

2.4 Cell staining and flow cytometry

Intracellular staining was performed after 2% paraformaldehyde fixation and 0.5% saponin permeabilization. Staining for PLZF antibody (kindly provided by Dr. Derek Sant’Angelo) was carried out using a Foxp3 staining buffer set (eBioscience). CD1d tetramers with or without loaded PBS57 antigen were obtained from the tetramer facility of the National Institutes of Health. CD1d staining was done in the dark for 30 min at room temperature before antibody staining of other cell surface markers. Flow cytometry analysis was performed on a FACSCanto II (BD Biosciences). Doublets and dead cells were gated out before data analysis. Data were analyzed with the Flowjo software (Tree Star).

2.5 In vitro OP9-DL1 culture

FACS sorting of DN cells was performed after Dynabead biotin binder (Invitrogen) treatment to deplete CD4 SP, CD8 SP, and DP cells. The remaining cells were stained with CD44, CD25, TCRβ, TCRγδ, and CD27 antibodies. A cocktail of CD4, CD8, Gr-1, Mac-1, B220, and NK1.1 antibodies were included during staining as the dump channel to eliminate non-T cells and any residual CD4 SP, CD8 SP, and DP cells. Cells were cultured in MEM Alpha medium (10% FBS, Pen/Strep, and 5ng/µl IL-7 for all cultures and additional 5ng/µl Flt3L for the DN3 culture) on OP9-DL1 cell coated plates.
Lymphocytes were harvested and were then analyzed by FACS at the specified time points.

**2.6 In vivo BrDU incorporation assay**

Mice were sacrificed 4h after being injected with 1 mg BrdU. BrDU staining was performed according to manufacturer’s protocol (BD Biosciences). CD4 SP, DP and DN stage cells were pregated. For DN stage analysis, total thymocytes were stained with CD44 and CD25 after dumping lineage-positive cells and analyzed for BrdU incorporation.

**2.7 V(D)J rearrangement analysis**

**2.7.1 Jα repertoire analysis of DP cells**

cDNA from FACS sorted thymocyte fractions were prepared as for RT-PCR. For Vα14-Jα18 usage in CD1dtet sorted iNKT cells, cDNA were amplified with primers as described (D'Cruz et al., 2010; Hager et al., 2007). RT-PCR products were subcloned into TA TOPO vector (Invitrogen) and sequenced. Jα gene segment usage was determined using the V-Quest search program (Brochet et al., 2008) and verified by manual check. For Jα repertoire analysis of DP cells, a Vα8 or Vα14 specific primer and a Cα primer were used to generate libraries for Ion Torrent high-throughout sequencing. Data were converted to Fasta format (Blankenberg et al., 2010) on Galaxy platform (http://galaxyproject.org/) before being submitted to the High V-Quest search engine (Brochet et al., 2008).
2.7.2 V-D-J rearrangement analysis

TCRβ V-D-J rearrangement assay was performed with primers Vβ5-5' and Jβ2.7-3' according to the protocol described previously (Wojciechowski et al., 2007). Genomic DNA was extracted from FACS sorted cell fractions. The primer sequences for Vα14-Jα18 and Vα3-Jα9 have been described in previous publication and are in Table 2 (Hawwari et al., 2005).

2.8 Methylation analysis

Genomic DNA of DN TCRβ, DN TCRβlo and DP cells from Cre- mice, and DN TCRβlo Cells from L-DKO mice was extracted with the Quick-gDNA miniprep kit (Zymo Research). DNA was treated for bisulfite conversion by using the EZ DNA methylation-Gold kit (Zymo Research). The primers for CD8b promoter are as described (Pobezinsky et al., 2012).

2.9 Pathology analysis

Tumor-bearing and control mice were killed and thymus, spleen and liver tissues were immediately fixed with 10% phosphate-buffered saline (PBS)-formalin. After 24h, samples were embedded in paraffin and sectioned for immunohistochemical staining. Hematoxylin and eosin (H&E) staining was performed by the Department of Pathology, Duke University.
2.10 Adoptive transfer of lymphoma cells

Lymphoma cells were obtained from the enlarged thymus in tumor-bearing mice. Tissues were minced, filtered and resuspended in PBS. Red cells were lysed using lysing buffer (BD bioscience) according to the manufacturer’s protocol and washed with PBS twice. 5x10^6 cells per mouse were intraperitoneally injected into Rag2^-/- mice at 6-8 week of age. Pathology and FACS analysis were performed after 1-2 months to assess tumor growth.

2.11 Microarray analysis

Pre-malignant NKT cells were sorted from three Id2^{i/j}Id3^{i/j}LckCre^+ mice at 20 days of age with a TCRβ antibody and PBS57 presented in CD1d tetramer. NKT lymphoma tissues from Id2^{i/j}Id3^{i/j}LckCre^+ were a thymoma from LV40 and two enlarged peyer’s patch from LIV34 and LIV56. Total RNA from the sorted NKT cells and lymphoma cells were extracted using the RNAqueous Kit (Life Technology) according to the manufacturer’s protocol. Microarray analysis was performed by the Duke Microarray Core Facility. (http://www.genome.duke.edu/cores/microarray). Mouse Genome 430A 2.0 Arrays (Affymetrix) were used in this experiment. Partek Genomics Suite and DAVID software were used for the final data analysis.

2.12 statistical analysis

Two-tailed Student’s t-test was performed for statistical analyses.
3. The roles of Id2 and Id3 in iNKT cell development and expansion

The majority part of this chapter is adapted from “Combined Deletion of Id2 and Id3 Genes Reveals Multiple Roles for E Proteins in Invariant NKT Cell Development and Expansion”, Jia Li, Di Wu, Ning Jiang and Yuan Zhuang, Journal of Immunology. 2013, November; 191:5052-5064. doi: 10.4049/jimmunol.1301252

3.1 Introduction

NKT cells represent a distinct effector group that is capable of providing diverse and fast effector functions and thus is also classified as innate-like T cells (Brennan et al., 2013). A large fraction of NKT cells are type I NKT cells, also known as iNKT cells for the invariant Vα14Jα18-bearing TCR, which share the same developmental history with the rest of αβ T cells up to the DP stage. Expression and selection of an appropriate TCRα-chain at the DP stage have been shown to provide the driving force in iNKT cell development (chapter 1.1.3). Most of our understanding of iNKT lineage development is based on events during and after TCRα gene expression at the DP stage (Bezbradica et al., 2005; Gapin et al., 2001). It is not entirely clear whether the highly restricted Vα-Jα usage for NKT cells is simply a result of TCR-mediated selection or additional regulation prior to TCR selection (MacDonald, 2002; Makino et al., 1996).

As discussed in chapter 1.2.2, E proteins and their inhibitor Id proteins have been shown to play important roles at the pre-TCR, the γδ TCR, and the αβ TCR checkpoints. Especially two E protein genes, E2A and HEB, and two Id genes, Id3 and Id2, are
involved in producing E proteins and Id proteins, respectively, during T cell development (Jones and Zhuang, 2011). Although removal of a single E protein gene only resulted in partial defects in T cell development, deletion of both E2A and HEB genes in the early stages of T cell development resulted in nearly complete block in αβ lineage development and severe impairment in γδ lineage development (Wojciechowski et al., 2007). Conditional deletion of E2A and HEB at the DP stage with CD4-Cre also demonstrated an essential role for E proteins in CD4 lineage and iNKT lineage development (D’Cruz et al., 2010; Jones and Zhuang, 2007; Jones-Mason et al., 2012). In contrast to E protein gene knockout, deletion of Id3 early in T cell development resulted in a significant increase in γδ lineage T cells, although this increase is almost exclusively restricted to innate-like γδ T cells expressing the Vγ1.1Vδ6.3 TCRs (Ueda-Hayakawa et al., 2009; Verykokakis et al., 2010). These genetic studies clearly demonstrated that E protein dosage plays an important role in influencing the fate choice between the γδ and αβ lineages at the pre-TCR and γδ TCR checkpoints, reminiscent of E protein functions at the αβ TCR checkpoint (Bain et al., 2001; Lee et al., 2010; Rivera et al., 2000).

Given that Id2 has been shown to collaborate with Id3 in regulating the TCR checkpoint (Jones-Mason et al., 2012), it is speculated that Id2 could also collaborate with Id3 in regulating the pre-TCR and γδ TCR checkpoints. In this study, we used LckCre to delete both Id2 and Id3 at the pre-TCR and γδ TCR checkpoints. Deletion of both Id2 and Id3 resulted in a partial block at the pre-TCR checkpoint and increased production
of innate γδ T cells, suggesting opposing roles for Id genes in regulating the αβ lineage and the innate γδ lineage. More importantly, analysis of Id2 and Id3 double-deficient animals also revealed a novel role for Id2 and Id3 in regulating the development and expansion of iNKT cells. The mutant mice showed a dramatic increase in numbers of iNKT cells. A biased rearrangement involving Vα14-Jα18 was detected in preselected DP cells, indicating a role for Id proteins in regulating Vα14-Jα18 rearrangement prior to CD1d-mediated selection. Results presented in this study further suggest a dosage-dependent mechanism for Id genes in repressing the fate of innate-like γδ T cells versus iNKT cells during T cell development.

### 3.2 Results

#### 3.2.1 Conditional removal of Id2 and Id3 with LckCre is completed before DP stage

We hypothesized that Id2 may play a redundant role and functionally compensate for the loss of function of Id3 at the pre-TCR checkpoint. To test this hypothesis, we used LckCre to drive conditional deletion of Id3 and Id2 before the pre-TCR and γδ TCR checkpoints. PCR analysis of fractionated thymocytes demonstrated that LckCre–mediated deletion of the Id2 and Id3 floxed alleles started at the DN3 stage and achieved near completion by the DP stage (Figure 7). Mice carrying the LckCre transgene or the floxed alleles alone did not show any phenotype, which makes the LckCre− mice good controls in the following experiments (Figure 8).
Figure 7: Deletion efficiency of Id2 and Id3 genes in L-DKO thymocytes

Figure 7. The DN2, DN3 and DN4 population were sorted from both Id2<sup>f/f</sup>Id3<sup>f/f</sup>LckCre<sup>+</sup> and Id2<sup>f/f</sup>Id3<sup>f/f</sup>LckCre<sup>−</sup> mice and DNA was extracted. Id2 deletion allele and flox allele was analyzed in separate PCR reactions. Id2 floxed band (0.47kb) and wild-type band (0.43kb) were detected by primers Scr-1 and Scr-2. Id2 deletion band (0.9kb) was detected by primers Id2dF and Id2dR. Id3 deletion and flox alleles were analyzed in a single PCR reaction with a common primer and allelic specific primers. Id3 deletion band (1.34kb), and floxed band (1.07kb) were detected by primers Id3-2, Id3-F and Id3-B.
Figure 8: Effects of LckCre mediated Id2 and Id3 deletion on thymocyte development

Figure 8. Total thymocytes were analyzed with either CD4 and CD8a (top panel) or TCRβ and TCRγδ (bottom panel). The DN fractions (defined by CD4 CD8 NK1.1 B220 Gr-1 Mac-1) of thymocytes were displayed by CD44 and CD25 expression (middle panel). Samples are from 20-day-old Id2+/+Id3+/+LckCre+, Id2+/+Id3+/+LckCre-, Id2+/+Id3+/+LckCre, and Id2+/+Id3+/+LckCre+ mice as indicated on the top of each column. Percentages of cells in each quadrant are displayed.
3.2.2 T cell development is impaired by double deletion of Id2 and Id3

LckCre–induced Id2 deletion alone did not produce any developmental abnormalities, whereas deletion of both copies of Id3 plus one copy of Id2 resulted in a dramatic increase in γδ T cells and a reduction in αβ T cells (Figure 9, bottom panel), a result similar to a previous report of Id3 knockout mice (Ueda-Hayakawa et al., 2009). Deletion of both Id2 and Id3 with LckCre (referred to as L-DKO hereafter) resulted in a phenotype significantly different from these control groups. The total thymic cellularity of L-DKO mice was reduced to an average of 36% (among 1.5-mo-old young adults) or 43% (at the weaning age) in comparison with age-matched Cre− control mice (Figure 10A). Consistent with an earlier study involving CD4-Cre–mediated deletion of Id2 and Id3 at the DP stage, LckCre–mediated deletion of Id2 and Id3 at the DN stage also resulted in a complete block in CD8 lineage development (Figure 9, upper panel) (Jones-Mason et al., 2012). L-DKO mice also exhibited a significant increase in numbers and percentages of DN cells (Figure 9 middle panel). Further analysis showed that the increase in DN cells could be attributed mostly to a change in the CD4−CD8−CD25−CD44+ (DN4) fraction (from 6.7 ±1.1 x 10^5 in Cre− mice to 26.5 ± 5.2 x 10^5 in L-DKO mice; Figure 10B). The absolute numbers of DN3 and DP cells in L-DKO mice were decreased to approximately half of the Cre− controls (Figure 10B-C). Collectively, these results indicated that LckCre induced deletion of Id2 and Id3 perturbed T cell development at multiple stages, including the DN3 stage.
Figure 9: Double deletion of Id2 and Id3 impairs thymocyte development

Figure 9. Total thymocytes were analyzed in 2-D plots of either CD4 and CD8a (upper panel) or TCRβ and TCRγδ (bottom panel). The DN fractions (defined by CD4<sup>-</sup>CD8<sup>-</sup>NK1.1<sup>-</sup>B220<sup>-</sup>Gr-1<sup>-</sup>Mac-1<sup>-</sup>) of thymocytes were displayed by CD44 and CD25 expression (middle panel). Samples are from 2-month-old Id2<sup>+/+</sup>Id3<sup>+/+</sup>LckCre<sup>-</sup>, Id2<sup>f/f</sup>Id3<sup>+/+</sup>LckCre<sup>-</sup>, Id2<sup>+/+</sup>Id3<sup>f/f</sup>LckCre<sup>+</sup>, and Id2<sup>f/f</sup>Id3<sup>f/f</sup>LckCre<sup>+</sup> mice as indicated on the top of each column. Percentages of cells in each quadrant are displayed.
Figure 10: Cell number of Total lymphocyte and subpopulations is affected by Id2 and Id3 double deletion
Figure 10. (A) Cell counts of total thymocytes in 1.5-mo-old and 20-d-old mice. The mean value for 1.5-mo-old Cre control mice is $2.4 \pm 0.2 \times 10^8$ (n = 7), and for Cre L-DKO mice is $0.85 \pm 0.13 \times 10^8$ (n = 8). The p value of the t test between the two groups is <0.0001. The mean value for 20-d-old Cre control mice is $13 \pm 1.1 \times 10^7$ (n = 5), and for Cre L-DKO is $5.6 \pm 1.2 \times 10^7$ (n = 5). The p value of the t test between the two groups is 0.0017. (B) Cell counts for CD4 SP, γδ T, DN3 and DN4 fractions in both Cre control and Cre L-DKO mice at the age of day 20. (C) Total numbers of DP cells from the same animals used in B were counted. N is 5 for each genotype. Two tailed and non-paired student t test was performed to generate the p values shown in the plots.
3.2.3 Accumulation of CD4⁺CD8⁻TCRβlo cells occurs during neonatal life

Further examination of L-DKO thymocytes showed that their CD4⁺CD8⁻ cells expressed TCRβ at a level lower than that of CD4 SP in Cre⁺ controls (Figure 11A). TCRβlo cells were also observed among most CD4 CD8⁻ cells and peripheral CD4 T cells in L-DKO mice. Both L-DKO and Id2⁻/⁻/Id3/⁻/LckCre⁺ mice also showed a small fraction of TCRβ⁻ cells among the CD4 T cells in the thymus and periphery. A separate FACS analysis showed that these TCRβ⁻CD4⁺CD8⁻ T cells were innate-like γδ T cells as reported in Id3 knockout mice (Verykokakis et al., 2010). To uncover the primary developmental defects and to limit the potential cross-regulation from effector T cells generated in the mutant mice, we switched to examination of neonatal animals (Figure 11B). Among day 3 neonates, CD4 cells were not detected in L-DKO mice, indicating a developmental block in the transition from DP to CD4 SP. CD4⁺CD8⁻TCRβlo T cells were absent at this early stage. In contrast, a dramatic increase in CD4⁺CD8⁻TCRβlo T cells and γδ T cells became apparent 10 d after birth concomitant with the appearance of CD4 cells. By day 20 (weaning age), both CD4⁺CD8⁻TCRβlo T cells and CD4 cells exhibited a further increase in percentages relative to other populations in the thymus of L-DKO mice. CD4 cells in the mutant mice expressed a lower level of TCRβ in comparison with the CD4 cells in the Cre⁺ control mice (Figure 11C). Based on these findings, we chose 20-d-old animals to further dissect T cell development in L-DKO mice.
Figure 11: TCRβlo population is accumulated in the DN and CD4 population from neonatal stage
Figure 11. (A) Expression level of TCRβ among indicated cell fractions isolated from thymus, spleen, and lymph nodes of the mice in Figure 9. (B) FACS analysis of day 3, 10, and 20 neonatal Cre- and Cre Id2<sup>lo</sup>Id3<sup>lo</sup> thymocytes. Plots shown are CD4/CD8a staining of total thymocytes (top panel) and TCRβ/TCRδ staining of DN fractions (bottom panel). Percentages of cells in each quadrant are shown. Results are representative of three Cre- and Cre+ pairs at each time point. (C) Cre- (red) and Cre+ (blue) samples from day 20 shown in (B) were further analyzed in an overlay histogram to display TCRβ expression in DP and CD4 SP cells.
3.2.4 A partial block at the pre-TCR checkpoint

Given that LckCre initiates Id2 and Id3 deletion at the DN3 stage, we first evaluated the effect of Id deletion on pre-TCR selection. We used CD27 to separate DN3 cells into DN3a (CD27lo) and DN3b (CD27hi) fractions (Figure 12A). CD27 upregulation is tightly correlated with pre-TCR selection among DN3 cells (Taghon et al., 2006). Analysis of L-DKO mice showed that the percentage of DN3b cells within the DN3 fraction was reduced to approximately one-half of that in Cre- control littermates (Figure 12B).

To determine whether this block in pre-TCR selection was due to any major perturbations of TCRβ gene rearrangement, we examined TCRβ gene usage based on V-D-J rearrangements (Wojciechowski et al., 2007). A random pattern of Jβ usage was detected among DN3 and DN4 cells in Cre- mice and DN3 cells from L-DKO mice (Figure 12C). This result indicated a relatively normal TCRβ usage among DN3 cells in L-DKO mice. However, both CD4+CD8- TCRβ+ and CD4-CD8+ TCRβ- cells in L-DKO mice showed a perturbed pattern of D-J usage.
Figure 12: pre-TCR checkpoint is partial blocked
Figure 12. (A) Separation DN3a and DN3b fractions with CD27 marker. DN cells were defined as CD4-CD8- NK1.1-B220-Gr-1-Mac-1- in FACS sorting. DN3 fraction was first gated as a c-Kit-CD25+ fraction of DN thymocytes before analysis with CD44 and CD27. (B) Summary of DN3b percentages in total DN3 from four independent sortings. Means±SE are 13±1.3% for Cre- and 7.2±0.86% for Cre+ samples with p = 0.01. (C) TCRβ D-J rearrangement assay with Vβ5 and Jβ2.7 primers. Lane orders are: M, size marker; L-DKO DN3; L-DKO DN4 TCRβ+; L-DKO DN4 TCRβ-; Cre- DN3; Cre- DN4; Rag2-/- The predicted size for each rearrangement is: Jβ2.7, 282 bp; Jβ2.6, 495 bp; Jβ2.5, 638 bp; Jβ2.4, 730 bp; Jβ2.3, 869 bp; Jβ2.2, 1135 bp; Jβ2.1, 1338 bp.
3.2.5 Developmental delay at the pre-TCR checkpoint in ex vivo culture

To further evaluate the efficiency of pre-TCR selection, we tested the differentiation capability of DN3 cells in an OP9-DL1 culture system (Schmitt and Zuniga-Pflucker, 2002). DN3 cells from both Cre⁻ control and L-DKO samples expanded dramatically and differentiated into the DP stage within 6 d in culture (Figure 13A-B). However, DN3a cells from L-DKO mice progressed from DN to DP in a slower kinetics in comparison with the Cre⁻ controls (Figure 13A). The kinetic difference between these two genotype groups was also evident among the sorted DN3b cells (Figure 13B), which represents cells having undergone β-selection (Taghon et al., 2006). Collectively, these data indicate that LckCre-mediated deletion of Id2 and Id3 impaired the pre-TCR checkpoint.

Figure 13: DN3a and DN3b cells in vitro culture on OP9-DL1
Figure 13. (A) OP9-DL1 culture of DN3a thymocytes sorted from Cre− controls (left panel) and L-DKO mice (right panel). Twenty thousand cells were seeded in each well for the time course experiments. CD4 and CD8 staining of total cultured cells are shown for days 2, 4, and 6 in culture. Results are representative of three repeats of independently sorted cells. (B) OP9-DL1 culture of DN3b thymocytes sorted from Cre− controls (left panel) and L-DKO mice (right panel). Four thousand cells were seeded in each well for the time course experiments. Analysis was carried out as described in (A). Results are representative of three repeats of independently sorted cells.
3.2.6 CD4⁺CD8⁻TCRβ⁻ and CD4⁻CD8⁻TCRβ⁻ cells in L-DKO mice are mostly iNKT cells

iNKT cells are known to express lower levels of TCR than those of conventional αβ T cells and are phenotypically classified into either the CD4⁺CD8⁻ or CD4⁻CD8⁻ compartment. The development of iNKT cells also begins in the neonatal stage with a time frame similar to the appearance of CD4⁺CD8⁻ TCRβ⁻ cells and CD4⁻CD8⁻ TCRβ⁻ cells in L-DKO mice (Koseki et al., 1990). We therefore examined the possibility that the TCRβ⁻ cells in L-DKO mice were iNKT cells. FACS analysis of thymocytes revealed that most CD4⁺ cells and a third of CD4⁻CD8⁻ cells in L-DKO mice were recognized by an Ag-loaded CD1dtet that specifically binds to the canonical TCR on iNKT cells (Figure 14A). PLZF has been shown to be a signature transcription factor for innate T lymphocytes, including Vγ1.1⁺Vδ6.3⁺ γδ T cells and iNKT cells (Alonzo and Sant’Angelo, 2011). Intracellular staining with anti-PLZF Ab demonstrated an overall increased in expression of PLZF in CD4⁺CD8⁻ and CD4⁻CD8⁻ cells but not DP cells in L-DKO mice (Figure 14B). PLZF expression was found in both αβ⁺ and γδ⁺ fractions of CD4⁻CD8⁻ cells in the mutant mice. Furthermore, a small fraction of TCR⁻CD4⁻CD8⁻ cells also expressed PLZF, indicating their possible lineage relationship with TCR⁺ PLZF-expressing cells. On average, the absolute numbers of CD1dtet⁺ iNKT cells in CD4 SP and DN fractions in L-DKO mice were ~9- and 28-fold higher, respectively, than those of the age-matched Cre⁻ controls (Fig. 14C). Because most CD4 SP and DN cells in L-DKO mice expresses PLZF, we conclude that Id2 and Id3 double deletion leads to a significant increase in numbers
of PLZF-expressing thymocytes. Sequence analysis of cDNA products amplified with Va14- and Jα 18-specific primers confirmed the exclusive usage of the canonical invariant Va14-Jα18 TCR in CD4 CD1dtet+ cells isolated from L-DKO mice (Figure 15B).

The lipid Ag used in the CD1dtet specifically recognizes iNKT cells. Type II NKT cells have been shown to use several highly restricted VaJα rearrangements, including Va3 to Jα9 rearrangement (Arrenberg et al., 2010; Park et al., 2001). Therefore, we examined Va3-Jα9 usage in the TCRβloCD1dtet-CD4-CD8- and CD4+CD8- cells in the mutant mice. Va3-Jα9 products but not Va14-Jα18 products were readily detected by PCR in TCRβloCD1dtet- cells (Figure 15A). Sequence analysis of Va3-Jα9 products from TCRβloCD1dtet- cells indicated that a third of them were in-frame and the remaining two-thirds were out-of-frame Va3-Jα9 rearrangements (Figure 15C). Therefore, other Va rearrangements must also be involved in generating functional TCRα-chains in the TCRβloCD1dtet- fraction.

To further test lineage identity of the expanded TCRβlo cells in L-DKO mice, we bred L-DKO mice to the MHC class II-deficient background. Both TCRβloCD1dtet+ and TCRβloCD1dtet- cells in L-DKO mice were generated in the absence of MHC class II selection (Figure 16), demonstrating that these cells were unrelated to the conventional helper T cell lineages. Thus, we conclude that LckCre-mediated deletion of Id2 and Id3 resulted in increased generation of iNKT cells. Further investigation is still needed to determine whether the TCRβloCD1dtet+ fraction is type II NKT cells or not.
Figure 14: CD4⁺CD8⁻TCRlo and CD4⁺CD8⁻TCRlo cells in L-DKO mice are NKT cells
Figure 14. (A) Representative staining of total lymphocytes with CD4, CD8, TCRβ, and CD1dtet. CD4 and CD8 staining of total thymocytes were used to define DN and CD4 SP gates. DN and CD4 fractions of each genotype were analyzed with either unloaded CD1dtet as a control or PBS57-loaded CD1dtet. The percentages of CD1dtet+ or TCRβ+CD1dtet- cells in CD4 and DN fractions are displayed in the plots. (B) Intracellular staining of PLZF in CD4 SP (CD4+CD8-), DP (CD4+CD8+), and DN (CD4-CD8-) fraction of total lymphocytes. DN fraction is further separated by TCRβ and TCRδ. DN TCRβ-TCRδ-, DN TCRβlo, and DN TCRδ+ populations were analyzed. (C) Cell counts for CD1dtet+ iNKT cells in CD4 SP and DN fractions in 20-d-old pups. The mean values for CD4 SP iNKT cells are 1.43±0.31×10^5 and 12.76±2.34×10^5 in Cre- controls and L-DKO, respectively, with p = 0.0013. The mean values for DN iNKT cells are 0.64±0.15×10^5 and 17.81±5.08×10^5 in Cre- controls and L-DKO, respectively, with **p = 0.0097. Five mice of each genotype group were used in the analysis.
Figure 15: Sequence for CD4+CD8+ TCRβlo and CD4−CD8+ TCRβlo cells in L-DKO mice

Figure 15. (A) PCR analysis of Vα14-Jα18 and Vα3-Jα9 rearrangement in CD1dtet+ and CD1dtet− fraction of DN and CD4 SP cells. Results are representative of two independent experiments involving separate L-DKO mice. (B) Sequence results of Vα14-Jα18 junctions from cDNA of DN CD1dtet+ and CD4 CD1dtet+ populations in L-DKO mice. N additions are shown in bold. (C) Sequence results of Vα3-Jα9 junctions from cDNA of TCRβlo CD1dtet− population in L-DKO mice. N additions are shown in bold.
Figure 16: Development and expansion of iNKT cells in L-DKO mice is independent of MHC class II
Figure 16. Total thymocytes were analyzed with either CD4 and CD8a (first row across) or CD1dtet and TCRγδ (second row across) staining. The CD4+CD8− (third row across) and CD4−CD8− (fourth row across) fractions of thymocytes were displayed by CD1dtet and TCRβ expression. Samples are from 20-d-old Id2+/Id3+/LckCre−, Id2+/Id3−/LckCre+ MHC class II−/−, and Id2+/Id3+/LckCre+MHC class II+/+ mice as indicated on the top of each column. Percentages of cells in each quadrant are displayed. n = 2 for each genotype.
3.2.7 Assessing DN4 proliferation and differentiation in OP9-DL1

Because part of the DN TCRβ·TCRγδ· population also express PLZF (Figure 14B), to further assess the developmental transition from DN3 to DP, we isolated TCRβ·TCRγδ· DN4 cells from L-DKO mice and assessed their proliferation and differentiation capacity in OP9-DL1 culture. The sorted cells were labeled with CFSE before they were seeded on the OP9-DL1 stromal layer. The entire population of Cre− control cells showed efficient proliferation as evidenced by a uniformed dilution of CFSE signals and differentiation into the DP stage at day 2 and 3 in culture (Figure 17A). In contrast, only a fraction of Cre+ DN4 cells diluted CFSE signals became DP cells under the same culture conditions (Figure 17B). Another fraction of DN4 cells remained undivided as CFSE high cells and failed to develop into the DP stage. These CFSE high non-proliferating cells gradually gained CD4 expression within the window of culture (Figure 17B, lower panel). Both DN and CD4 fractions of the CFSE high cells expressed surface TCRβ. TCRβ expression was detected exclusively among the non-dividing CFSE high cells. These results suggested that the DN4 fraction of thymocytes in L-DKO mice contains two distinct populations: the true proliferating DN4 cells, which behave in a similar manner as the DN4 cells from Cre− control mice, and a novel non-proliferating fraction that readily upregulates TCRβ expression in OP9-DL1 culture.
Figure 17: A non-proliferative fraction of DN4 cells revealed in OP9-DL1 culture.
Figure 17. (A) DN4 TCRβ- TCRδ- cells from Cre- control mice were sorted by FACS, labeled with CFSE and cultured on OP9-DL1 for 1 to 3 days. CFSE signal intensity and CD4/CD8 expression were analyzed each day. Samples from day-3 culture were further separated into CD4 positive and DN fractions for analysis of TCRβ expression (lower right panel). (B) DN4 TCRβ-TCRδ- cells from L-DKO mice were analyzed as in (A) except dividing cultured cells into CFSElo (middle panel) and CFSEhi (lower panel) fractions.
3.2.8 The non-cycling DN4 cells are CD24\textsuperscript{lo} and NKT lineage committed cells

We further characterized the non-dividing DN4 fraction cells with additional markers that are differentially expressed according to the maturation status of developing T cells. CD24 is highly expressed in developing T cells and is typically downregulated upon lineage differentiation.

Analysis of TCR\textsuperscript{−}DN4 cells clearly separated them into CD24 high and CD24 low fractions (Figure 18A). A majority of TCR\textsuperscript{−}DN4 cells from Cre\textsuperscript{−} control mice were phenotypically CD24\textsuperscript{hi}, and they readily expanded and differentiated into DP cells in OP9-DL1 culture (Figure 18B). Only 4\% of CD4 expressing cells became CD1dtet reactive NKT cells under these culture conditions. In contrast, the majority of TCR\textsuperscript{−}DN4 cells from the Cre\textsuperscript{+} mutants were phenotypically CD24\textsuperscript{lo} and readily differentiated into CD1dtet reactive cells without proliferating in OP9-DL1 culture (Figure 18A-B, bottom panel). This study further indicates that the non-cycling CD24\textsuperscript{lo} cells in L-DKO mice are fully committed iNKT lineage cells.

3.2.9 DN TCR\beta\textsuperscript{lo} population has gone through DP stage

As shown in Figure 18, the DN CD24\textsuperscript{lo} population represents NKT committed cells. To determine whether the DP stage is a necessary step in the development of iNKT cells in L-DKO mice, we examined the epigenetic markers associated with DP stage
development. Development from DN to DP is coupled with irreversible demethylation of the CD8 locus. If iNKT cells are derived from the DP subset, their CD8 locus should remain demethylated even though they have stopped expressing the CD8 gene (Pobezinsky et al., 2012). Sequencing analysis of bisulfite-treated DN TCRβlo cells from Cre+ mice showed a pattern of demethylation similar to that of DP cells (Figure 18C). This result supports the idea that DN NKT cells found in L-DKO mice have gone through the DP fate prior to losing CD4 and CD8 expression.
Figure 18: CD24lo non-proliferating cells are NKT committed and post-DP cells
Figure 18. (A) Sorting strategy for CD24<sup>hi</sup> or CD24<sup>lo</sup> cells among DN4 TCRβ<sup>-</sup>-<br>TCRγδ<sup>-</sup>CD1d<sup>-</sup> fractions. (B) Direct conversion from DN4 TCRβ<sup>-</sup>-TCRγδ<sup>-</sup>CD1d<sup>-</sup> cells into<br>iNKT cells in OP9-DL1 culture. Results of 3-day OP9-DL1 culture from CD24<sup>hi</sup> Cre<sup>-</sup><br>(upper panel), CD24<sup>hi</sup> L-DKO (middle panel), and CD24<sup>lo</sup> L-DKO (lower panel) samples<br>are displayed in CD4 and CD8 plots (left column). CD4 positive (middle column) and<br>DN (right column) fractions were further analyzed for CD1dtet and TCRβ staining. (C)<br>Analysis of methylation status at the CD8 locus. Percentage of demethylated CpG in the<br>CD8b promoter are shown for sorted DN, DP, and DN TCR<sup>+</sup> cells from Cre<sup>-</sup> control and<br>DN TCR<sup>+</sup> cells from L-DKO mice. Each dot represents the results of 5 CpG sites within a<br>single sequencing product.
3.2.10 Expansion of stage 1 iNKT cells in L-DKO mice

Further analysis showed that most iNKT cells developed in L-DKO mice do not express NK1.1 and DX5 even though they have downregulated CD24 (Figure 19). FACS analysis also showed that iNKT cells in L-DKO mice expressed low levels of CD44 (Figure 9). These features together with PLZF expression indicated that iNKT cell development in L-DKO mice has progressed to stage 1 (Das et al., 2010; Godfrey and Berzins, 2007), where cells undergo proliferative expansion (Benlagha et al., 2002).

Indeed, in vivo BrdU pulse labeling revealed a significantly higher fraction of cycling cells among the CD4+ population in L-DKO mice (most of which are iNKT cells) in comparison with the conventional CD4 SP cells in Cre- controls (Figure 20A-B). The percentage of cycling cells was also higher in the DN TCRlo fraction of L-DKO mice (most of which are presumed type II NKT cells) in comparison with the conventional CD4 SP cells in Cre- controls. The same analysis indicated a relative normal pattern of cell cycle for L-DKO mice at the DN2, DN3, and DP stages of T cell development (Figure 20-21). This proliferative behavior of NKT cells was observed at both weaning and young adult age (Figure 20). Repertoire analysis with a panel of TCR Vβ isotype-specific Abs revealed a broad pattern of Vβ usage among CD4+ cells of L-DKO mice, indicating that the expanded iNKT cells in L-DKO mice remain polyclonal (Table 3). However, the overall patterns of Vβ usage in L-DKO mice were different from the conventional Vβ8>Vβ7>Vβ2 rule (Mallevaey et al., 2009), indicating the possibility of an altered
selection during development or expansion phase of these iNKT cells. Furthermore, five of six L-DKO mice showed increased Vβ usage involving Vβ8.3, Vβ11, or Vβ13. Taken together, these results indicated that Id2 and Id3 deletion resulted in an expansion of immature iNKT cells.

Figure 19: The increased iNKT population in L-CKO mice is immature

Figure 19. Total thymocytes from Cre\(^{−}\) control L-DKO mice were displayed for CD1d tet and CD24 expression (left column), NK1.1 and TCRβ expression (middle column), and DX5 and TCRβ expression (right column).
Figure 20: DN and CD4 iNKT cells in L-DKO mice are proliferative
Figure 20. (A) BrdU incorporation among DP and CD4+ SP thymocytes analyzed at 4 h after BrdU injection of 20-d-old (left panel) or 2-mo-old mice (right panel). The percentage of BrdU+ cells for each subpopulation is shown in histograms. (B) Summary of BrdU+ percentages in DN2, DN3, DP, CD4+CD8-, and DN TCRβ+ fractions from three independent experiments of 20-d-old pups. Significant difference was observed between L-DKO and control mice among the CD4+CD8- fraction (mean value, 3.85±0.61% and 8.56±0.72% for Cre- controls and L-DKO, respectively, with **p = 0.0076). Numbers of DN TCR+ cells in the Cre- control group were too small to be included in this analysis.
Figure 21: DN2 and DN3 population in L-DKO showed similar proliferation ability as controls.

Figure 21. BrdU incorporation among DN thymocytes analyzed at 4-hours post BrdU injection of 20-day mice. The DN fractions (defined by CD4<sup>−</sup>CD8<sup>−</sup>NK1.1<sup>−</sup>B220<sup>−</sup>Gr-1<sup>−</sup>Mac-1<sup>−</sup>) of thymocytes were displayed by CD44 and CD25 expression. The percentage of BrDU-positive cells for DN2 and DN3 subpopulation is shown in histograms. N=3 for each genotype.
Table 3: TCR Vβ repertoire analysis of L-DKO CD4+ thymocytes

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<td>0.97</td>
<td>3.69</td>
<td>4.73</td>
<td>3.63</td>
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<tr>
<td>Average ±SD</td>
<td>±1.22</td>
<td>±0.48</td>
<td>±1.06</td>
<td>±1.06</td>
<td>±0.24</td>
<td>±0.61</td>
<td>±1.32</td>
<td>±0.64</td>
<td>±0.26</td>
<td>±0.84</td>
<td>±0.74</td>
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<tr>
<td>LDKO (LIII24) 2m</td>
<td>2.01</td>
<td>2.67</td>
<td>2.54</td>
<td>2.94</td>
<td>3.23</td>
<td>4.48</td>
<td>4.36</td>
<td>18.2</td>
<td>2.29</td>
<td>2.48</td>
<td>8.13</td>
<td>2.96</td>
<td>3.04</td>
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<td>3.74</td>
<td>1.78</td>
<td>4</td>
<td>7.31</td>
<td>2.04</td>
<td>0.25</td>
<td>0.56</td>
<td>12.7</td>
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<tr>
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<td>2.06</td>
<td>1.86</td>
<td>2.47</td>
<td>3.05</td>
<td>4.19</td>
<td>6.57</td>
<td>7.87</td>
<td>1.45</td>
<td>1.76</td>
<td>27</td>
<td>2.61</td>
<td>10.8</td>
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<td>0.79</td>
<td>1.83</td>
<td>2.06</td>
<td>1.98</td>
<td>3.32</td>
<td>10.7</td>
<td>2.45</td>
<td>1.38</td>
<td>0.93</td>
<td>9.91</td>
<td>0.99</td>
<td>8.44</td>
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<td>0.99</td>
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<td>4.22</td>
<td>4.13</td>
<td>11.7</td>
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<td>0.7</td>
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<td>2.9</td>
<td>3.33</td>
<td>14.5</td>
<td>3.29</td>
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<td>1.16</td>
<td>2.84</td>
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<td>10.9</td>
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<tr>
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<td>±0.58</td>
<td>±0.89</td>
<td>±0.48</td>
<td>±3.76</td>
<td>±6.33</td>
<td>±0.71</td>
<td>±0.73</td>
<td>±8.81</td>
<td>±1.94</td>
<td>±3.71</td>
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</tbody>
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Frequencies of Vβ usage are shown as percentages among CD4+CD8- fraction of thymocytes isolated from five control mice and six L-DKO mice. The average and standard deviation (SD) are calculated for each group. “m” indicates mouse age in month. Numbers in parentheses are identifiers of the mice used in the experiment.

Antibodies used in the analysis are TCR Vβ screening panel from BD Pharmingen™.

3.2.11 Id2 and Id3 deletion results in a biased rearrangement toward Va14-Jα18

Given that iNKT cell development is dependent on Va14 to Jα 18 rearrangement, which typically occurs during the DP window of thymocyte development, we asked whether Id2 and Id3 deletion affects TCRα rearrangement. We assessed the Jα usage by performing high-throughput sequencing of rearrangement products involving either
Vα8 or Va14 genes from the preselecting CD4+ CD8+CD69+ DP cells. CD1dtet+ and CD69+ postselecting cells were gated out to avoid the contamination of NKT cells or mature αβ T cells (Figure 22). Jα usage was captured by PCR amplification with a Vα-specific primer and a Cα primer. A broad pattern of Jα usage involving Vα8 rearrangements was detected in both Cre- control and L-DKO cells (Figure 23). Because Vα8 is one of the commonly used Vα genes (Guo et al., 2002), the random distribution of Jα usage indicates that a significant fraction of DP cells undergoes relatively normal TCRα rearrangements. In contrast to the Vα8 result, Vα14 rearrangements exhibited a highly skewed pattern toward Jα 18 usage in L-DKO mice (Figure 24). This bias toward Jα 18 was associated with Vα14 but not Vα8 (Figure 23). It remains a possibility that some of these Vα14-Jα 18 rearrangements may come from NKT cells that have downregulated their surface TCR. However, this argument cannot fully explain the fact that a significant number of unproductive Vα14-Jα 18 rearrangements were also observed in three independent L-DKO DP samples (Jones and Zhuang, 2011; Wojciechowski et al., 2007), (61%; Figure 24, lower panel). In particular, one of the three samples (Figure 24, center pie chart) showed more unproductive rearrangements (61%) than productive rearrangements (37%), which presents a typical preselecting repertoire. In contrast, unproductive Vα14-Jα18 rearrangements were detected at a much lower frequency in three wild-type control samples (0, 0.01, and 5%; Figure 24, upper panel). This result
suggests that Id2 and Id3 deletion promotes \( V\alpha 14 \) to \( J\alpha 18 \) rearrangements among developing T cells when \( V\alpha 14 \) is involved in rearrangements.

**Figure 22:** Sorting strategy for CD4\(^+\)CD8\(^+\)CD69\(^-\) DP cells used in high-throughput sequencing

Figure 22. The sorting strategy for CD4\(^+\)CD8\(^+\)CD69\(^-\) DP cells was shown in flow chart. Doublet and 7AAD\(^+\) cells were gated out and CD1dtetCD69\(^-\) cells were further gated by CD4 and CD8. The post-sort cells were run for the purity.
Figure 23: Jα repertoire analysis for Vα8 of DP cells
Figure 23. Jα repertoire analysis of Va8-Cα PCR products amplified from a Cre−
control (top) and L-DKO (bottom) CD69-DP cells. Bar graphs depict relative percentages
of each Jα gene usage separated by productive and unproductive rearrangements. Jα
genes are shown according to their relative positions in the TCRα locus starting with the
Va-proximal Ja61 gene. Analysis was based on 24,124 and 17,482 sequence reads for Cre−
control and L-DKO cells, respectively.
Figure 24: Jα repertoire analysis for Vα14 of DP cells

Figure 24. Jα repertoire analysis of Vα14-Cα PCR products amplified from a Cre- control (top) and L-DKO (bottom) CD69 DP cells. Each pie chart represents result of a single animal. The relative percentages of J gene usage were depicted in shaded slices with Jα18 highlighted in yellow. Sequence reads were 37,811, 10,994, and 7,468 for wild-type samples and 37,758, 46,141, and 7,960 for mutant samples.
3.2.12 Increased iNKT development in Id2- and Id3-deficient mice is driven by high levels of E proteins

The major targets of Id proteins are E protein transcription factors, although E proteins are not the only Id-interacting proteins reported thus far (Lasorella and Iavarone, 2006). To test whether enhanced iNKT development is indeed regulated by E proteins, we lowered the E protein dosage by removing one copy each of the E2A and HEB genes on the L-DKO background (referred to as L-DKO50%E). This genetic change effectively reduced iNKT cell numbers almost back to the level seen in Cre- controls and concurrently enhanced numbers of γδ T cells dramatically (Figure 25A-B). Further analysis confirmed that the expanded γδ T cells in L-DKO50%E mice belong to the innate γδ T lineage that uses exclusively the Vδ6.3 TCR (Figure 25A, right panel). This result indicates that innate γδ T and iNKT lineages are regulated by different levels of E proteins. To further evaluate the necessity of E proteins in the generation of innate γδ T cells, we generated Id2\textsuperscript{f/f}Id3\textsuperscript{f/f}E2A\textsuperscript{f/f}HEB\textsuperscript{f/f}LckCre\textsuperscript{+} quadruple-deficient mice (referred to as L-QKO mice). Deletion of E2A and HEB effectively prevented αβ T and iNKT lineage development (Figure 26A). Numbers of γδ T cells in L-QKO mice were also reduced to 10% of the Cre- controls (Figure 26B), a phenotype similar to the previously defined LckCre-mediated E2A and HEB knockout mice (Wojciechowski et al., 2007). This result demonstrated that the Id2 and Id3 genes control iNKT lineage development through inhibition of E protein activities exclusively.
Figure 25: E protein dosages at the DN3 stage control lineage outcomes
Figure 25. (A) Effects of E protein dosage on T cell development revealed by analysis of Cre− control, L-DKO, and L-DKO50%E (Id2flofloId3flofloE2A+floHEB+floLckCre+) mice. Total thymocytes were analyzed with either CD4 and CD8 staining (far left column), CD1dtet and TCRβ staining (middle left column), or CD1dtet and TCRδ staining (middle right column). TCRδ+ cells were further analyzed for Vδ6.3 expression (far right column). (B) Cell counts of each T cell fractions as defined in (A). Three mice for each genotype group were included in the analysis.
Figure 26: E protein is required for γδ T cell development

Figure 26. (A) FACS analysis of L-QKO mice. Results of CD4 and CD8 analysis of total thymocytes (left column) are shown along with the CD1dtet and TCRβ analysis of gated DN fractions (right column). Results are representative of three pairs of animals. (B) Cell counts for total thymocytes (top) and γδ T cells (bottom) in the thymus of 20-d-old L-QKO mice. n = 3 pairs, p< 0.0005 for both plots.
3.3 Conclusion and Discussion

Following a recent publication demonstrating an essential role for Id3 and Id2 at the TCR selection checkpoint (Jones-Mason et al., 2012), the present study provided new genetic evidence that Id2 and Id3 are also collectively involved in regulating the pre-TCR checkpoint. Furthermore, our study also revealed a previously unanticipated role for Id3 and Id2 in regulating iNKT cell development.

Two models have been proposed to explain the development of iNKT cells. The “pre-commitment” model postulates that iNKT cell fate is predetermined prior to CD1d-mediated selection. This idea has been supported by the finding that $\text{V}_\alpha 14$-$\text{J}_\alpha 18$ rearrangements can be detected prior to the appearance of conventional $\alpha\beta$ T cells in the mouse fetus (Makino et al., 1996). However, this finding is inconsistent with the fact that most $\text{V}_\alpha 14$-$\text{J}_\alpha 18$ rearrangements occur as secondary rearrangements in DP cells, from which iNKT cells are generated continuously in postnatal life (Bezbradica et al., 2005; Egawa et al., 2005; Gapin et al., 2001). The “mainstream” (or TCR-instructive) model argues that iNKT cells acquire their lineage identity upon CD1d-mediated TCR selection of DP cells that have successfully produced the canonical $\text{V}_\alpha 14$-$\text{J}_\alpha 18$ TCR resulting from the sequential rearrangement of the TCR$\alpha$ gene segments. Recent studies further demonstrated that a strong TCR signal is associated with activation of NKT lineage-specific transcription factors such as PLZF (Seiler et al., 2012). Mounting evidence supports the idea that CD1d-mediated selection, together with signaling events
involving the SLAM receptors, drive iNKT lineage differentiation (Das et al., 2010). In light of these previous findings, our observation of a biased $V\alpha 14-J\alpha 18$ rearrangement in L-DKO mice provides an alternative view to the existing models. We propose that $V\alpha 14-J\alpha 18$ rearrangement is not a random event and is subject to repression by concerted activity of Id3 and Id2.

The expansion of iNKT cells in L-DKO mice could also be attributed to other T lineage cells developing along with iNKT cells that may inadvertently affect the development and expansion of iNKT cells (Lee et al., 2011). These include the innate $\gamma\delta$ T cells that developed in the neonatal stage and the small number of conventional CD4 SP cells made through positive selection (Jones-Mason et al., 2012). Indeed, CD4 SP cells that developed in Id3-deficient background have been shown to exhibit various effector phenotypes (Miyazaki et al., 2011), which could potentially influence the development and expansion of iNKT cells. To resolve this issue, we have tested our L-DKO mice on an MHC class II-deficient background and observed a similar expansion of iNKT cells as in L-DKO mice. The effect of innate $\gamma\delta$ T cell on the development of iNKT cells in our L-DKO model could be further investigated in the future by crossing the L-DKO mice to the TCR$\delta$-deficient background.

Under our experimental conditions, the innate $\gamma\delta$ lineage and iNKT lineage are selectively expanded in response to increasing levels of E proteins. The tight correlation between E protein dosage and unique TCR types such as $V\gamma 1.1V\delta 6.3$ of innate $\gamma\delta$ T cells
and Vα14Jα18 of iNKT cells provides a genetic frame-work for further understanding how TCR rearrangement, expression, and signaling are coupled with E protein-mediated lineage differentiation programs.

Investigation of PLZF expression shed new light on the lineage relationship between Vγ1.1 Vδ6.3 γδ T cells and iNKT cells (Alonzo et al., 2010; Kreslavsky et al., 2009). Although these two innate lineages seem to be developed independently during thymopoiesis, they clearly share a similar developmental blueprint by employing PLZF-mediated transcriptional regulation and possess similar innate-like features such as restricted TCR usage, acquisition of effector memory like phenotypes upon maturation, and coexpression of IL-4 and IFNγ (Alonzo and Sant'Angelo, 2011). Furthermore, a recent RNA expression profiling analysis classified iNKT cells as a lineage closely related to γδ T cells (Bezman et al., 2012). This result supports the idea that NKT cells could be evolutionarily closer to innate γδ T cells than the conventional T cells that perform adaptive immune functions. Our study raised the possibility that E protein-mediated regulation may function upstream of PLZF and other innate lineage-relevant transcription factors. The genetic models established in this study identified E proteins as an important transcriptional switch controlling lineage choice between iNKT and other alternative T cell lineages.
4. The roles of Id2 and Id3 in NKT lymphoma formation

4.1 Introduction

NKT lymphoma is a rare lymphoma, the etiology and pathogenesis of which are poorly understood. The limited characterizations of NKT lymphoma undertaken to date have been done using cell lines derived from patients with natural killer-like T cell leukemia/lymphoma, which express both T cell and NK cell maker profiles. Additionally, early study of peripheral T-cell lymphoma (PTCL) indicated that extranodal T/NK cell lymphomas express the NK cell marker CD56 (Matsuo and Drexler, 2003; Ohshima et al., 2002). IL-15 signaling has been shown as a possible inducer for over-proliferation in NKT cells. However, the pathogenesis and the mechanism of the NKT lymphoma development remain unknown (Matsuda et al., 2002; Yu et al., 2011).

E proteins and Id proteins have been demonstrated to regulate the development of multiple lymphocyte lineages, including NKT cells (de Pooter and Kee, 2010; Kee, 2009). One of the E proteins, HEB, has been proven to regulate iNKT cell development during the initiation stage (D’Cruz et al., 2010). Id2 and Id3 are two main Id proteins expressed in lymphocytes, and they coordinately regulate the DN T cell precursors at the pre-TCR checkpoint as well as the life span of DP cells (Jones-Mason et al., 2012). In chapter 3, we have shown that combined deletion of Id2 and Id3 in the DN stage promotes the expansion of immature NKT cells (Li et al., 2013).
Previous studies have demonstrated that Id proteins and E proteins not only regulate lymphocyte development, but are also involved in the development of multiple tumors. Genes encoding Id proteins have been suggested as oncogenes, because their high expression in many subtypes of tumor can release the cell cycle arrest imposed by E proteins (Bain et al., 2001; Lasorella et al., 2000; Ohtani et al., 2001; Perk et al., 2005). However, Id proteins may have regulatory effects on tumorigenesis, as Id3 mutations were found in numerous cases of Burkitt’s lymphoma, and Id3 deficient mice developed \( \gamma \delta \) hepatosplenic T-cell lymphoma (HSTCL)(Li et al., 2010; Love et al., 2012; Richter et al., 2012). E2A has been considered as a tumor suppressor, but it is also highly expressed in some cancers, such as prostate and breast cancers and the cell lines derived from Burkitt’s lymphoma and natural killer-like T cell leukemia-lymphoma (Chen et al., 2011; Matsuo et al., 2004; Patel and Chaudhary, 2012). Thus far, there has not been any report demonstrating that Id proteins and E proteins play causative roles in NKT lymphoma formation.

Here we demonstrate that Id2 and Id3 not only regulate NKT cell development, but also play an important role in NKT lymphoma progression. Infiltrating NKT cells were found in multiple lymphoid and non-lymphoid organs of Id2 and Id3 double knockout mice and these malignant cells were able to invade healthy tissues in the RAG deficient host upon adoptive transfer. Therefore, this mouse model may provide a useful
tool to study the developmental mechanisms of NKT lymphoma development and the functions of Id proteins during lymphoma progression.

**4.2 Results**

**4.2.1 Double deletion of Id2 and Id3 by LckCre results in NKT lymphoma in mice**

Because Id2 and Id3 double deletion by LckCre (L-DKO) has been shown to promote NKT cell development and expansion in young mice, we further explored how Id2 and Id3 affect the NKT cells in aged mice. Unexpectedly, mice began to die after 3 months. The majority of them died between five to seven months and all of them died within 11 months (Figure 27A). Splenomegaly and hepatomegaly were apparent in all mice, and several other organs were infiltrated and enlarged, including the thymus, peyer’s patches, lungs and kidneys (Figure 27B and Table 4). Statistical analysis showed that the weight of livers and spleens from L-DKO mice with tumor was significantly increased compared to the livers and spleens from control mice (Figure 27B). Histopathology analysis with H&E staining showed that the thymus, spleen and liver from L-DKO mice with tumors were infiltrated by lymphoma cells and their normal structure was destroyed (Figure 28C). These data indicate rapid lymphoma formation in L-DKO mice.
Figure 27: Id2 and Id3 double deficiency in the early stage of T cell development results in lymphoma in mice.
Figure 27. (A) Survival curve for Id2/Id3 LCKCre+ mice (control mice N=32, L-DKO mice N=48). (B) Comparison of the size and weight of spleens and livers from L-DKO mice and wild type controls (the size pictures for spleen and liver are from one representative. For weight measurement, N=4 for WT, N=5 for L-DKO). (C) Representative pictures of H&E staining of spleens and livers from L-DKO mice and WT controls.
Table 4: Summary of lymphoma phenotype in L-DKO mice

<table>
<thead>
<tr>
<th>Orginal tumor</th>
<th>#</th>
<th>Sex</th>
<th>Age(w)</th>
<th>T cell markers</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TCRβ</td>
<td>CD4</td>
</tr>
<tr>
<td>LII10^*</td>
<td>F</td>
<td>19</td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
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<td>M</td>
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<td></td>
<td>+</td>
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</tr>
<tr>
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<td>F</td>
<td>27</td>
<td>T S L LN BM</td>
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</tr>
<tr>
<td>LII65</td>
<td>M</td>
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<td>10%</td>
</tr>
<tr>
<td>LII66</td>
<td>M</td>
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<td>T S L</td>
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<td>-</td>
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<tr>
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<td>T S L LN BM</td>
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</tr>
<tr>
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<td>F</td>
<td>27</td>
<td>T S L LN BM</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
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<td>M</td>
<td>24</td>
<td>T S L G LN BM</td>
<td>+</td>
<td>-</td>
</tr>
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</tr>
<tr>
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<td>F</td>
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<td>T S L G LN BM</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>LV27</td>
<td>M</td>
<td>25</td>
<td>T S L LN BM</td>
<td>+</td>
<td>-</td>
</tr>
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</tr>
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</tr>
<tr>
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<td>78%</td>
</tr>
<tr>
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<tr>
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<td>T S L G LN BM</td>
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</tr>
<tr>
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</tr>
<tr>
<td>LV21^*</td>
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<td>37</td>
<td>T S L G LN BM</td>
<td>+</td>
<td>70%</td>
</tr>
</tbody>
</table>

+: positive -: negative ND: not done
T: thymus S: spleen L: liver G: gut LN: lymph node BM: bone marrow
*: The mice have enlarged lymphocyte-infiltrated kidney
^: The mice have lymphocyte-infiltrated lung
^: The mice have lymphadenopathy
4.2.2 Characterization of the NKT lymphoma

FACS analysis of lymphoma samples showed that the lymphoma cells in the majority of L-DKO mice were TCRβ-positive (Table 4). Because NKT cells are gradually accumulated in the L-DKO mice during the neonatal life, we then assessed the possibility that this lymphoma is derived from NKT cells. The FACS analysis showed that in 36.8% of L-DKO mice, TCR-positive cells from the tumor were almost all CD1d tetramer positive (Figure 28A, Table 4). PLZF is the characteristic transcription factor for innate-like T cells, including NKT cells (Gleimer et al., 2012). Intracellular staining showed that all of the TCRβ+ lymphoma cells had high levels of PLZF expression (Figure 28B). The majority of the lymphoma cells expressed intermediate levels of CD44 and did not express CD25 (Table 4), even though these surface markers are expressed in NKT cells at 20 days of age. To further confirm the lineage identity of CD1d tetramer positive lymphomas, we analyzed their VαJα usage with primers specific to the Vα14 and Jα18 loci. The results showed that all CD1d tetramer positive lymphoma cells were positive for Vα14-Jα18 rearrangement, but CD1d tetramer negative lymphoma cells were not (Figure 28C). In conclusion, these data indicate that the CD1d tetramer positive lymphoma observed in L-DKO mice are derived from NKT cells.
Figure 28: iNKT lymphomas are detected in Id2 and Id3 double deficient mice
Figure 28. (A) Representative staining of thymocytes from a wild type and two L-DKO mice with CD4 and CD8 markers (left panel). CD4 and DN fractions are further analyzed in double staining with TCRβ and CD1d tetramer without or with antigen. (B) Histogram of intracellular PLZF staining of TCRβ⁺ and TCRβ⁻ populations in the same mice with lymphoma as in figure A. (C) DNA analysis for Vα14-Jα18 rearrangement by PCR. Primers for Vα14 and Jα18 exons were used and lymphoma samples are divided into two groups according to CD1d tet⁺ and CD1d tet⁻. CD14 is used as a loading control.
4.2.3 Lymphoma cells from Id2 and Id3 double knockout mice are able to invade healthy tissues

To determine the malignancy of the NKT lymphoma in Id2 and Id3 double knockout mice, we adoptively transferred NKT lymphoma cells into Rag2<sup>−/−</sup> mice. The survival curve showed that most Rag2<sup>−/−</sup> mice receiving lymphoma cells died between 7 and 12 weeks. The remaining 30% of mice surviving after 12 weeks were found not to contain lymphoma cells after dissection (Figure 29A). The H&E staining of the tissues from mice with lymphoma showed characteristic lymphocytic infiltration (Figure 29B) as in Figure 27. FACS analysis was performed to characterize the secondary lymphoma arising after transfer. Because lymphoma cells were CD45.2<sup>+</sup> and Rag2<sup>−/−</sup> mice were on the CD45.1<sup>+</sup> background, the CD45.2<sup>+</sup> cells present in Rag2<sup>−/−</sup> mice must be derived from the donor cells. The CD5<sup>+</sup> cells were found in the thymus, spleen, liver and bone marrow in the Rag2<sup>−/−</sup> mice, which developed lymphoma after receiving donor cells. The percentage of infiltrated lymphoma cells in these organs varied between different hosts, but spleen and liver were all infiltrated by large numbers of lymphoma cells (Figure 30A). Both lymphoma cells from CD1dtet<sup>+</sup> (donor 1) or CD1dtet<sup>−/−</sup> (donor 2) donors are able to form secondary lymphoma in the Rag2<sup>−/−</sup> host. Moreover, CD45.2<sup>+</sup> cells from donor 1 in Rag2<sup>−/−</sup> mice recipients consisted of CD1dtet<sup>−/−</sup>TCR<sup>+</sup> cells and this population also had high PLZF expression, indicating the formation of the secondary iNKT lymphoma in the host mice (Figure 30B). In conclusion, these data demonstrate that the lymphoma cells from L-DKO mice have the ability to invade normal tissues and develop into NKT lymphoma.
Figure 29: NKT lymphoma is adoptively transferred into Rag2<sup>−/−</sup> recipients

Figure 29. (A) The survival curve for Rag2<sup>−/−</sup> host after receiving 5X10<sup>6</sup> lymphoma cells from L-DKO mice (N=10), three of them did not develop lymphoma. Rag2<sup>−/−</sup> mice without injection are used as control (N=3). (B) Representative pictures of H&E staining for spleen and liver from, WT control, L-DKO mice with lymphoma, Rag2<sup>−/−</sup> control and Rag2<sup>−/−</sup> transferred with lymphoma cells.
Figure 30: Characterization of the secondary lymphoma arising in Rag2<sup>−/−</sup> recipients
Figure 30. (A) Representative flow cytometric analysis with CD45.1, CD45.2, CD5 and Thy1.2 markers of cells recovered from RAG2<sup>−/−</sup> recipients two month after receiving lymphoma donor cells. Cre<sup>−</sup> control (first row) and unmanipulated RAG2 deficient mice (second row) are included as controls. Two representative recipients injected with 5X10<sup>6</sup> lymphoma from different donors are shown in third and forth rows. Donor 1 is CD1dtet<sup>+</sup> (third row) and donor 2 is CD1dtet<sup>−</sup> (forth row). Both of the donor lymphoma cells are CD45.2<sup>+</sup>, while the Rag2<sup>−/−</sup> recipients are CD45.1<sup>+</sup>. (B) CD45.2<sup>+</sup> cells in recipient injected lymphoma from the donor 1 are gated for the CD1dtet and TCRβ analysis. Comparison of the intracellular PLZF expression in Cre<sup>−</sup> control CD45.2<sup>+</sup>CD1dtet<sup>−</sup>TCRβ<sup>+</sup>, Cre<sup>−</sup> control CD45.2<sup>+</sup>CD1dtet<sup>−</sup>TCRβ<sup>+</sup> and CD45.2<sup>+</sup> cells in Rag2<sup>−/−</sup> recipients are shown. Data are representative on 3 recipients analyzed.
4.2.4 Altered signaling profiles associated with NKT lymphoma progression

In order to further explore the signaling pathways involved in the transition from non-malignant NKT cells to NKT lymphoma, we performed microarray analysis on the total RNA extracted from NKT cells in neonatal 20 d L-DKO mice and NKT lymphoma cells from L-DKO mice. According to the results, there were 587 genes that changed more than 2 fold with a P value <0.01. We further divided these genes into sub-groups according to the signaling pathways they involved. There are more genes upregulated than downregulated in the NKT lymphoma compared to the pre-malignant NKT cells. The most frequently upregulated genes in NKT lymphoma cells are associated with cytokine-cytokine receptor interaction (Figure 31A-B). Among them, the expression of Csf1r and Csf2rb2 were confirmed by RT-PCR (Figure 32). Csf1r gene encodes the receptor for macrophage colony-stimulating factor-1 (M-CSF, CSF-1), which is also known as CD115. The CSF signaling have been shown to regulate the differentiation, growth and survival of monocytes and macrophages, and it has been considered as a proto-oncogene because of the aberrantly expression in Hodgkin’s lymphoma (Hamilton, 2008; Lamprecht et al., 2010). CSF2Rb is a common subunit for type I cytokine receptors, including GM-CSF receptor, IL-3 receptor and IL-5 receptor. All three signaling pathways have been shown to promote tumor growth in different tumor types(Broughton et al., 2012; Lee et al., 2013a; Wu et al., 2000). Therefore,
deficiency of Id2 and Id3 may promote NKT lymphoma through these cytokine signaling pathways.
Figure 31: Microarray analysis reveals upregulation of cytokine signaling genes upon malignant transformation from NKT cells to NKT lymphoma

Figure 31. RNAs were extracted from pre-malignancy NKT cells at 20 days and NKT lymphoma from L-DKO mice. (A) The hierarchical clustering of genes expressed differently between the two groups (P<0.01, fold change >2). (B) Signaling pathways involved according to the gene list in (A) indicated by DAVID.

Figure 32: RT-PCR analysis for the expression of cytokine receptors in NKT cells and NKT lymphoma

Figure 32. Quantitative RT-PCR analysis of cytokine receptor csf1r and csf2rb2 expression in sorted NKT cells from 20d L-DKO mice and NKT lymphoma cells from 5m L-DKO developed with lymphoma. Samples were normalized to the expression of β-actin. Data are from two independent experiments (n=4). P<0.001.
4.3 Conclusion and Discussion

We showed that mice with combined deletion of Id2 and Id3 in early stages of T cell development developed into aggressive NKT lymphoma. NKT lymphoma is rare but aggressive in humans, and little is known about the mechanisms of its development and etiology. Our microarray analysis showed that Id proteins might regulate tumor progression through cytokine signaling. Although the underlying mechanisms of Id2 and Id3 in promoting NKT lymphoma still need further exploration, the cytokine signaling pathways are promising targets for subsequent study and therapy.

Id proteins have been demonstrated to regulate the development of two subtypes of the innate-like T cells, NKT cells and Vγ1.1-Vδ6.3+ γδ T cells, mainly through inhibition of E protein activity. E protein dosage not only regulates the proliferation of αβ and γδ T cells, but also controls the lineage choice of NKT cells and Vγ1.1-Vδ6.3+ γδ T cells. Our data, along with that of other groups, has demonstrated that the loss of Id proteins can result in γδ T cell or NKT cell malignancy, which may provide insight into their roles in the malignant transformation of innate-like cells.

The Vγ1.1-Vδ6.3+ γδ T cell population expands in Id3−/− mice develops into γδ HSTCL, a phenomenon similar to the expansion and malignant transformation of NKT cells in Id2 and Id3 double knockout mice. These findings indicate that the malignancy of innate-like T cells may lead to HSTCL. Previous publications have highlighted the similarity between the γδ and αβ HSTCL by pathological characterization and gene
expression profiling (Miyazaki et al., 2009; Travert et al., 2012). The pathological analysis of our L-DKO mice indicated that the NKT lymphoma is quite similar to one subtype of HSTCL, the αβ HSTCL. Indeed, the observed splenomegaly, hepatomegaly and infiltration of lymphoma cells into multiple organs, especially the spleen and liver, are consistent with the main clinical features of human HSTCL. Although our L-DKO mice may mimic human HSTCL, more pathological analysis is needed to classify the NKT lymphoma developed in the L-DKO mice.

Because of the low incidence of NKT lymphoma, standardized treatment for this disease is not well established. Therefore, an animal model may become a useful tool to facilitate investigation of new therapies. In our work, the rapid cell expansion and aggressive lymphoma development in the adoptive transfer experiment provides a good model for further study of NKT lymphoma pathogenesis and therapeutic interventions.
5. Future direction

In chapter 3 and 4, we have revealed multiple roles of Id2 and Id3 in iNKT cell development and NKT cell lymphoma formation in mice. These two Id genes control the dosage of E proteins in the developing T cells and thereby regulate the development and expansion of iNKT cells. These new discoveries also raised more questions to be further addressed.

5.1 How do Id2 and Id3 specifically regulate the Va14-Ja18 rearrangement?

Multiple factors may contribute to the overall increase in numbers of iNKT cells in L-DKO mice. The Jα repertoire analysis of L-DKO DP cells clearly revealed a biased usage of Va14-Jα18 when Va14 is used in rearrangement. Because Jα18 usage was not dramatically altered when Va8 was used in rearrangements, the biased Va14-Jα18 rearrangement in D-LKO mice cannot be simply due to targeted regulation at the Jα18 site. Given that each Va gene is regulated by an independent promoter, we propose that Va14 may be subject to targeted regulation in L-DKO mice. However, Va14 must be working in concert with Jα18 in L-DKO mice to promote Va14-Jα18 usage. This biased Va14-Jα18 usage seems only to affect a small fraction of DP cells because the pattern of Va8 rearrangements, a relatively common Va, seems unperturbed in L-DKO mice. If E-proteins are directly involved in regulating Va14-Jα18 rearrangement, E-protein recruitment to the Va14 and/or Jα18 sites alone is not sufficient to explain why only a small fraction of DP cells are promoted to enter the iNKT lineage in L-DKO mice.
Therefore, there should be other factors involved in the Id and E regulation network to define the specificity of V\(\alpha\)14-J\(\alpha\)18 rearrangement in a fraction of DP cells. These other factors may be regulated by E-proteins and differentially expressed in DP cells in L-DKO mice. Identifying E protein targets in DP cells may help to solve this problem. Moreover, we found that deletion of Id2 and Id3 by CD4-Cre does not cause a significant increase of iNKT cells (data not shown) as seen in L-DKO mice. This finding indicates that E-protein targets involved in promoting iNKT lineage development must be turned on or off prior to the DP stage. Therefore, analysis of gene expression changes between the CD4-Cre and LckCre mediated Id2 and Id3 knockout mice may provide more meaningful candidates relevant to iNKT lineage specification and development.

The phenotype difference between Id gene deletion by CD4-Cre and LckCre suggests a possible role for E protein dosage in promoting iNKT cell development at the pre-DP stage. The DN3 stage of thymocyte development has long been recognized as the major branching point between the \(\alpha\beta\) and \(\gamma\delta\) lineages. Our study provides new evidence that the lineage potential of iNKT cells may also be influenced at this early stage. These various T cell lineages seem to respond to different dosages of E-proteins. Previous studies have demonstrated that Id mediated down regulation of E-proteins in response to pre-TCR or \(\gamma\delta\) TCR signals is required for \(\alpha\beta\) and \(\gamma\delta\) lineage differentiation, respectively. Our study of L-DKO mice now suggests that Id gene upregulation before DP stage, possibly in the DN3 stage, is also important to suppress innate T lineage
development. Under our experimental conditions, the innate $\gamma\delta$ lineage and iNKT lineage are selectively expanded in response to increasing levels of E-proteins. We propose that a graded increase in E-protein activities leads to activation of different lineage programs and expression of different antigen receptor genes. The tight correlation between E-protein dosage and unique TCR types such as $V\gamma1.1-V\delta6.3$ of innate $\gamma\delta$ T cells and $V\alpha14-J\alpha18$ of iNKT cells provides an important clue for further understanding of how TCR rearrangement, expression, and signaling are coupled with E-protein mediated lineage differentiation programs.

5.2 How do Id2 and Id3 regulate the expansion of iNKT cells at the immature stage?

Altered $V\alpha14-J\alpha18$ usage alone is not sufficient to explain the overall increase in iNKT cell numbers. Our study has further revealed that most iNKT cells detected in the thymus of L-DKO mice are proliferating immature iNKT cells. The expansion of immature iNKT cells after TCR$\alpha$ rearrangement could be the main reason for the dramatic increase of iNKT cells in L-DKO mice. According to the new classification of iNKT subsets, the iNKT cells in the immature stage of the classical model contain the final-stage iNKT cells classified as the $\text{Gata3}^+\text{NKT2}$ and $\text{ROR}\gamma^+\text{NKT17}$ cells. Indeed two recent papers published back to back with our story have documented a significant alteration of these iNKT subsets in response to increased E-protein activities in developing T cells. A proportional increase of NKT2 and NKT17 subsets was shown in the study of ET2 knockin mice, which express a fusion protein composed of the
transactivation domains of E47 and the bHLH domain of SCL/Tal1 (Hu et al., 2013).
Likewise, Verykokakis’ s paper showed that Id3 is required for the T-bet+ NKT1 cell
development (Verykokakis et al., 2013). Comparing our data with the data in these
papers, it is highly possible that the increased CD44+NK1.1+ cells in our model includes
the NKT2 and NKT17 subsets.

Two interesting points are worth mentioning when considering the role of Id
proteins in regulating cell expansion after iNKT lineage commitment. First, the timing of
the gene manipulation is critical. As mentioned previously only double deletion of Id2
and Id3 by LckCre, but not by CD4-Cre, shows this phenotype. Second, an increase in
the CD44+NK1.1+ stage 1 cells is observed in both the ET2 knockin model and Id
knockout model. This observation indicates that NKT progenitors may also be regulated
by the E-protein dosage, presumably under a mechanism different from that controlling
the expansion of immature iNKT cells. Therefore, multiple regulatory events may occur
at different stages of iNKT lineage development, leading to the observed expansion of
iNKT cells in L-DKO mice.

Two specific hypotheses may be proposed to explain the increase of immature
iNKT cells and the differentiation bias. First, c-myc is involved in the first round of
expansion of iNKT cells in the early stage (Dose et al., 2009; Mycko et al., 2009).
Although c-myc is not the direct target of E proteins, it is downstream of the E protein
regulatory pathways (Weng et al., 2006; Yashiro-Ohtani et al., 2009). Second, Id3
upregulation in response to the TCR signaling may be the direct switch leading to
different NKT subset fate choice. The compartmentalization of RAS-MARK-Erk
signaling or Ca2+ influx needs to be evaluated to determine the TCR signal strength in
the differentiated iNKT cells. Although these two hypotheses may not be inclusive, they
provide a starting point for experimental approach in future studies.

5.3 What is the lineage identity of the TCRβloCD1dtet- fraction in Id2 and Id3 double deficiency mice?

The current data indicates that TCRβloCD1dtet- cells are not selected by MHCII.
These cells are enriched in the typical Vα3-Jα9 rearrangement for type II NKT cells,
although only one third of them are in-frame. Furthermore, we found that this
population is independent of CD1d selection (data not shown). Because type II NKT
cells are also selected by CD1d, the lineage identity of TCRβloCD1dtet- cells found in our
L-DKO mice remains a mystery. One hypothesis for this population is that these cells are
mucosal-associated invariant T (MAIT) cells. MAIT cells are also derived from thymus
and have very similar development pathway as iNKT cells except that they are selected
by MHC class Ib related molecules (MR-1) (Seach et al., 2013; Treiner et al., 2003). They
express CD44 and have the activated effector phenotype as NKT cells. Moreover, MAIT
cells also express the invariant TCR with Vα19-Jα33 (mice) or Vα7.2-Jα33 (human) and
can be CD4 SP, DN or CD8αα in humans (Martin et al., 2009). Therefore, repertoire
analysis of the TCRβloCD1dtet- cells can provide useful clues to understand this
population. To test whether this population is selected by MR-1, a more informative
experiment would be to test Id2 and Id3 deletion on the MR-1 deficiency background. If MAIT cells indeed account for the observed TCRβloCD1dtet- cells in L-DKO mice, we need to reconsider the developmental relationship between MAIT cells and iNKT cells. Perhaps, these innate-like T cell subsets are much closer than the current understanding and are all controlled by the E protein dosage.

Prior to the accumulation of iNKT cells and TCRβloCD1dtet- cells, an expansion of innate like Vγ1.1′Vδ6.3′ cells was also observed in L-DKO mice at the neonatal stage. NKT cells and innate like Vγ1.1′Vδ6.3′ cells are closely related lineages according to RNA expression profiling (Bezman et al., 2012). They clearly share a similar developmental blueprint by employing PLZF mediated transcriptional regulation. They possess similar innate features such as restricted TCR usage and acquire effector memory like phenotypes upon maturation. It would be interesting to determine the underlying mechanism leading to lineage switch from the Vγ1.1Vδ6.3 γδ lineage to the TCRβloCD1dtet- and iNKT lineages during the neonatal window of life. Further study of L-DKO neonates may gain evidence that put several different innate-like T cell lineages in the same window of T cell development.

5.4 How do Id proteins regulate the initiation and progression of NKT lymphoma?

Detailed pathological analysis is still needed to classify the lymphoma developed in Id2 and Id3 double deficiency mice. The outcome may help determine whether malignant transformation arises from the immature or mature stage of NKT cell
development. Several cytokine signaling pathways have been revealed from the microarray analysis of the pre-malignant NKT cells and NKT lymphoma. Additional work is still needed to determine which pathway is functionally relevant to the tumor phenotype. In addition to cytokine signaling, genes involved in other pathways such as lysosome and hematopoietic cell lineage are also significantly changed and these pathways may be related to tumor formation in multiple different ways. Id proteins and E proteins have been shown to have the complicated functions in tumorigenesis of different types of tumors. It would be interesting to compare pathways revealed from our study of the NKT lymphoma in Id3 and Id2 double deficient mice with the γδ T cell lymphoma in Id3 deficient mice. Such analysis could help reveal common pathways shared by lymphomas originating from innate lymphocytes. Findings from these studies may reveal important signature genes that can facilitate early diagnosis or treatment of NKT lymphomas in humans. Although NKT lymphoma in humans is very rare, the disease develops rapidly, which makes the treatment difficult. If our model can mimic NKT lymphoma in humans, it will be a powerful tool to test new therapies.
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