Chapter 4

BCL11B IS REQUIRED FOR EARLY T CELL DEVELOPMENT AND MAINTAINANCE OF T CELL IDENTITY

4.1. Introduction

4.1.1. Notch signaling in T cell development

Notch signaling plays a key role in T cell development (Harman et al., 2003; Radtke et al., 2010; Rothenberg et al., 2008). Interactions between Notch1 and its ligands of the Delta and Jagged families trigger the proteolytic cleavage of Notch1 and release its Notch intracellular domain (NICD). Then the NICD translocates from the cytoplasm to the nucleus, where it associates with the DNA-binding protein CSL (Rbpj) to activate genes downstream of Notch signaling (Kopan and Ilagan, 2009). Notch signaling triggers the initiation of the T cell program in hematopoietic progenitor cells. OP9 stromal cells obtain the capacity to induce the differentiation of hematopoietic progenitors into T cells after the Notch ligand Delta-like-1 that activates Notch signaling are forcefully expressed in them (Schmitt and Zuniga-Pflucker, 2002). Overexpression of a constitutively active form of Notch1 in hematopoietic progenitors leads to the development of T cells in the BM and arrested B cell development (Pui et al., 1999). In contrast, deletion of Notch or CSL in the thymus causes the disruption of T cell development and the accumulation of B cells in

the thymus possibly by a cell-extrinsic pathway (Feyerabend et al., 2009; Han et al., 2002; Radtke et al., 1999). Similarly, enforced expression of Dtx1, an antagonist of Notch1, results in B cell development at the expense of T cell development (Izon et al., 2002). Collectively, these studies suggest that Notch signaling controls the T-versus-B cell fate decision in lymphoid progenitors.

Notch signaling is also required to sustain early T cell development (Maillard et al., 2005; Radtke et al., 1999; Rothenberg, 2007). Indeed, loss of Notch signaling in DN1 cells converts them into dendritic cells (Feyerabend et al., 2009). In committed T cells, Notch signaling favors αβ- versus γδ-T cell lineage (Washburn et al., 1997; Wolfer et al., 2002), influences CD4 versus CD8 lineage decisions (Fowlkes and Robey, 2002), and regulates T-helper-2 (Th2) cell development partly through Gata3 (Amsen et al., 2009; Ho et al., 2009).

A recent study in Drosophila has indeed identified CG6530, the Drosophila orthologue of *Bcl11* genes, as a direct downstream target gene of Notch signaling (Krejci et al., 2009). Gene expression analysis in thymocytes reveals that Bcl11b is the most upregulated transcription factor at the transition from ETP to DN2b cell stage, suggesting the potential function of Bcl11b in early T cell development and its possible connection with Notch signaling (Tydell et al., 2007) (David-Fung et al., 2009).

4.1.2. Key transcription factors in T cells

Besides Notch1, other transcription factors also participate in regulating T cell development. For example, the transcriptional repressor Gfi1 is required for the development of early T cell progenitors and the CD4/CD8 lineage decision in the thymus (Yucel et al., 2003). Similarly, the basic helix-loop-helix transcription factor HEBAlt is expressed in pro-T cells and enhances the generation of T cell precursors

(Wang et al., 2006). The zinc finger transcription factor Zbtb7b (Th-POK), which appears to be repressed by Runx complexes (Setoguchi et al., 2008), regulates the CD4-versus-CD8 T-cell lineage commitment (He et al., 2008). Tbx21 (T-bet), a T-box transcription factor, directs Th1 lineage commitment (Szabo et al., 2000). GATA-3 is necessary and sufficient for Th2 cytokine gene expression in $CD4^+$ T Cells (Zheng and Flavell, 1997). Eomes, another T-box transcription factor, controls effector $CD8^+$ T cell function (Pearce et al., 2003). Despite these advances, no single transcription had been identified for T cell lineage commitment and/or identity maintenance.

In the previous chapter, I showed different *Bcl11b* expression levels in T cell subsets. A recent study showed that Bcl11b binds to several regions within the *Zbtb7b* locus and possibly regulates differentiation from DP thymocytes to $CD4^+$ T cells through Zbtb7b (Kastner et al., 2010). Further studies on cross-talk between Bcl11b and other T cell transcription factors are required to fully understand the transcription factor network that regulate T cell development and homeostasis.

4.1.3. NK cell-associated genes

Although NK cell developmental pathways are not entirely clear, several transcription factors have been identified as necessary for NK cell development. For example, the helix-loop-helix transcription factor Id2, which antagonizes the bHLH E proteins Tcf3 (E2A) and Tcf12 (HEB), is essential for full NK cell development since *Id2*–deficient mice exhibit a severe peripheral NK cell deficiency (Ikawa et al., 2001; Yokota et al., 1999). Conversely, forced expression of Id2 or Id3 is able to redirect pro-T cells to NK cell differentiation (Fujimoto et al., 2007; Spits et al., 2000). The basic leucine zipper (bZIP) transcription factor Nfil3 (E4BP4), which acts in a cell-intrinsic manner ‘downstream’ of the IL-15 receptor and interacts with Id2, is

indispensable for the generation of the NK cell lineage. Overexpression of E4bp4 promotes NK cell generation from hematopoietic progenitor cells while a lack of E4bp4 impairs NK cell development and NK cell-medicated cytotoxicity (Gascoyne et al., 2009; Kamizono et al., 2009). Additionally, forced expression of a NK specific transcription factor, Zfp105, promotes differentiation from HSC to the NK cell lineage (Chambers et al., 2007). Additionally, deletion of *Sfpi1* (PU.1), *Cbfb* (CBFβ) or *Ets1* adversely affects NK cells but this is likely due to their important roles in the lymphoid lineages (Barton et al., 1998; Colucci et al., 2001; Guo et al., 2008).

Besides transcription factors, several molecules including receptors, cytokines, and enzymes are also important for NK cell development and homeostasis. Il2rb (CD122), which is one of the receptor subunits for IL-2 and IL-15, is required for NK cell proliferation and differentiation (Waldmann and Tagaya, 1999). *Il2rb*$^{-/-}$ mice exhibited a reduction of peripheral NK cells and absence of NK cytotoxic activity in vitro (Suzuki et al., 1997). The cytokine lymphotoxin α (Lta), is secreted by lymphocytes including NK cells, and is important for NK cells as the number of NK cells is significantly reduced in *Lta*-deficient mice (Kuprash et al., 2002; Wang et al., 2008a; Ware et al., 1992). Plcg2, a member of Phosphatidylinositol (PI)-specific phospholipase C-γ enzymes is essential for NK cell cytotoxicity and innate immunity (Caraux et al., 2006; Wilde and Watson, 2001).

It is not known whether Bcl11b plays a role in NK cells though it is expressed in DN1 and DN2 thymocytes, which are considered as NK/T progenitors (Bell and Bhandoola, 2008; Wada et al., 2008). In the last chapter, I showed that *Bcl11b* was transiently expressed in some immature NK cells, but was absent in NKPs and mature NK cells. Based on Bcl11b expression profiles in T and NK cells, it is suggested that

Bcl11b may play a role in regulating the T-versus-NK cell lineage choice in DN1 and DN2 thymocytes.

4.1.4. Purposes of this chapter

In this chapter, I initially used microarray analysis to study the changes of global gene expression profile upon loss of Bcl11b in thymocytes using a Bcl11b conditional knockout mouse strain. Subsequently, I induced deletion of *Bcl11b* in early, committed and mature T cells in vitro to reveal function of Bcl11b in early T cell development and the maintenance of T cell identity. Then, I confirmed the function of Bcl11b in different T cell subsets using an in vivo tumor model. Finally, I showed that Notch signaling directly regulated Bcl11b.

4.2. Results

4.2.1. Bcl11b transcription regulation in T cells

To study Bcl11b functions in T cells, I used a *Bcl11b* conditional knockout mouse strain generated in the lab where exon 4 was floxed (Fig. 4.1). Exon 4 encodes three-quarters of the Bcl11b protein. *Bcl11b*$^{flox/flox}$ mice were then crossed to the *Rosa26-Cre-ERT2* mice (Hameyer et al., 2007; He et al., 2010). Rosa26, which was originally identified in a gene-trap screen in murine ES cells, is a mouse genomic locus commonly used to knock-in cDNA constructs in transgenic mice because it is ubiquitously expressed during embryonic development and in adult mouse tissues (Nagy et al., 1993). Controlled by the regulatory elements of the *Rosa26* locus, the Cre-ERT2 fusion protein combines Cre recombinase and estrogen receptor (ER) and is retained in the cytoplasm but translocates to the nucleus upon addition of Tamoxifen or OHT (Brocard et al., 1997). Consequently, *Bcl11b* was ablated in all

the cells from *Cre-ERT2; Bcl11b*$^{flox/flox}$ mice (referred to *flox/flox* in this study) after expression of Cre recombinase was induced by either Tamoxifen or OHT.

We cultured thymocytes from *flox/flox* mice and added OHT to the culture media. 48 hours after OHT treatment, Bcl11b protein was barely detectable on Western blot (Fig. 4.2). Deletion of *Bcl11b* was efficient in cells from the *flox/flox* mice. To probe gene expression changes immediately following *Bcl11b* deletion in T cells, we performed expression array analysis in whole thymocytes from *flox/flox* mice 24 and 48 hours following *in vitro* OHT treatment. Table 2 lists genes that exhibit at least a two-fold change in expression after *Bcl11b* deletion. 24 hours after OHT treatment, expression of T cell genes such as *Bcl11b*, *Tcrb* and *Cd3e*, had already downregulated. Within 48 hours after OHT treatment, expression of NK-cell-associated genes, such as *Id2*, *Nfil3*, *Klrd1*, *Lta*, *Plcg2*, *Ifng* (IFN-γ), and *Nkg7* that is expressed in activated T cell and NK cells (Cook et al., 2005; Turman et al., 1993), was significantly increased. The microarray results suggest that Bcl11b might suppress expression of NK cell-associated genes in T cells and might also induce or maintain the expression of T cell genes in T cells. Furthermore, Bcl11b expression became undetectable once DN2 thymocytes commit to NKPs while remained being at high level through T cell development. Taken together, I speculated that Bcl11b regulated the T-versus-NK cell lineage choice in NK/T progenitors.

4.2.2. Bcl11b is required for early T cell development

To investigate the function of Bcl11b in NK/T progenitors, we treated whole thymocytes from *flox/flox* and control (CreERT2; *Bcl11b*$^{flox/+}$, referred to *flox/+*) mice with OHT for 48 hours. Subsequently, DN1 thymocytes that have both T cell and NK cell potentials were sorted and cultured on OP9-DL1 stromal cells in T cell media (5.0 ng/ml IL-7 and 5.0 ng/ml Flt-3) for 2 weeks (Fig. 4.3). OP9-DL1 stromal cells

express Delta-Like-1 Notch ligand and support robust T cell development (Schmitt and Zuniga-Pflucker, 2002) while normally do not support NK cell development in the absence of IL-2 or IL-15 (Carotta et al., 2006; Rolink et al., 2006). 10 days after OHT treatment, all OP9-DL1 stromal cells were killed in the OHT-treated culture of the *flox/flox* DN1 thymocytes without IL-2 or IL-15 in T cell media. Similar killing activities are also observed when OP9-DL1 stromal cells are co-cultured with NK cells (Rolink et al., 2006). Therefore we speculated that these killer cells were similar to NK cells. Strikingly, I detected expression of NK cell surface markers such as NK1.1 and DX5 on these killer cells. I also checked cell surface expression of NKp46, which is primarily expressed on NK cells (Walzer et al., 2007). Flow cytometry analysis showed that 24% of the OHT-treated cultured thymocytes from *flox/flox* mice expressed NKp46 (Fig. 4.4A). These $NKp46^+$ cells did not express T cell surface markers like CD3 or TCRβ (Fig. 4.4A and 4.4B), indicating that they did not acquire or had lost T cell features despite being co-cultured with OP9-DL1 stromal cells for 14 days. Based on NKp46 and CD3 expression, it was clear that these OHT-treated cultures of DN1 thymocytes were heterogeneous, including $NKp46^+CD3^-$, $NKp46^-CD3^-$ and $NKp46^-CD3^+$ populations. As *Bcl11b* deletion efficiency usually cannot reach 100%, I checked the *Bcl11b* deletion efficiency in these populations by genomic PCR to investigate whether *Bcl11b* deletion was related to expression of NKp46 and CD3. The genotyping results showed that both alleles of the *Bcl11b* exon 4 had been deleted in these $NKp46^+CD3^-$ cells, whereas at least one copy of the *flox* allele was intact in the $NKp46^-CD3^+$ population (Fig. 4.5). In contrast to the OHT-treated *flox/flox* DN1 cells, the untreated *flox/flox* DN1 cells (Fig. 4.4C) or the OHT-treated control *flox/+* DN1 cells (Fig. 4.4A) proliferated rapidly on OP9-DL1 stromal cells, and many acquired CD3 expression but none were $NKp46^+$. These

data thus demonstrated that the Bcl11b deficiency abolished T cell development at the early stage and produced NKp46^{+}CD3^{-} cells from DN1 thymocytes.

Similarly, the OHT-treated DN2 thymocytes from the *flox/flox* mice produced NKp46^{+}CD3^{-}TCRβ^{-} cells in the T cell culture (Fig. 4.6A and 4.6B). OP9-DL1 stromal cells were also destroyed. In contrast, the control *flox/flox* DN2 thymocytes without OHT treatment (Fig. 4.6C) and the OHT-treated *flox/+* DN2 thymocytes (Fig. 4.6A) proliferated extensively on OP9-DL1 cells and gave rise to CD3^{+} cells but not NKp46^{+}CD3^{-} cells. These results again demonstrated that Bcl11b was also required for DN2 thymocytes to differentiate towards T cells. During early T cell development, Bcl11b is thus an essential regulator to promote T cell development and/or to suppress NK cell development in NK/T progenitors.

4.2.3. Bcl11b is required for committed T cells

DN3 thymocytes lose NK cell and myeloid cell potentials, when they commit to the αβ-T cell lineage at this stage (Bell and Bhandoola, 2008; Huang et al., 2005; Wada et al., 2008). To determine whether committed DN3 thymocytes regain NK or other lineage potentials following the loss of Bcl11b, we repeated the *Bcl11b* deletion experiments using purified DN3 thymocytes and cultured them on OP9-DL1 stromal cells in T cell media. Within 14 days, 13% of the OHT-treated *flox/flox* DN3 cells became NKp46^{+}TCRβ^{-} (Fig. 4.7, right), while the OHT-treated *flox/+* DN3 cells (Fig. 4.7, left) and the OHT-non-treated *flox/flox* DN3 cells proliferated and differentiated towards TCRβ^{+} T cells (Fig. 4.8). The cells from the culture of OHT-treated *flox/flox* DN3 cells in the culture with IL-2 or IL-15 grew faster than the ones in T cell media only. Also, with IL-2 or IL-15, the percentages of NKp46^{+} cells increased and these cells started to kill stromal cells within 10 days after OHT treatment (Fig. 4.8A and 4.8B). Therefore supplementation of IL-2 or IL-15 in the culture media greatly

promoted proliferation and/or differentiation of the NKp46^{+}TCRβ^{-} cells. These results show that even committed DN3 T thymocytes exhibited NK cell properties upon loss of Bcl11b.

To confirm that these NKp46^{+}CD3^{-} cells were directly reprogrammed from thymocytes rather than due to the presence of contaminating NK cells, we examined their TCRβ locus for the DNA rearrangements. These NKp46^{+}CD3^{-} cells retained the TCRβ V(D)J recombination even though the TCRβ was not expressed, thus genetically confirming the thymocyte origin of NKp46^{+}CD3^{-} cells (Fig. 4.9). We thus named these killer cells that were reprogrammed from T cells as Induced T-to-Natural-Killer (ITNK) cells.

Because loss of Bcl11b enables DN3 thymocytes to acquire NK cell potential, which is lost together with B cell and myeloid cell potentials during T cell commitment, I speculated that *Bcl11b*-deficient T cells might also possess potentials to differentiate to B cells or myeloid cells in proper conditions. To test these possibilities, I co-cultured the OHT-treated *flox/flox* DN3 thymocytes with OP9 stromal cells that support B cell proliferation and differentiation in B cell media (Nakano et al., 1994). After three weeks, NKp46^{+} ITNK cells, but not B cells (CD19^{+}), were detected in the cultures. In contrast, the OHT-treated *flox/+* control DN3 thymocytes expressed neither CD19 nor NKp46 and eventually died, as B cell potential was lost in DN3 thymocytes (Fig. 4.10). Similarly, the development of ITNK cells from *Bcl11b*-deficient thymocytes was an intrinsic property of the mutant thymocytes as ITNK cells were readily produced, but no myeloid cells were detected, from the OHT-treated *flox/flox* DN3 thymocytes culture in myeloid cell culture condition (Fig. 4.11). While the OHT-treated *flox/+* control DN3 thymocytes expressed neither CD11b nor NKp46 in myeloid culture. These results demonstrated

that Bcl11b-deficient DN3 cells reacquired NK-, but not B- or myeloid-cell properties, and suggested that Bcl11b maintains the T cell identity and specifically suppresses NK potentials in committed T cells.

4.2.4. Reprogramming efficiency from T cells to ITNKs upon Bcl11b ablation

To estimate the reprogramming efficiency of the *Bcl11b*-deficient DN3 thymocytes, I sorted single DN3 T cells from the OHT-treated whole thymocytes from *flox/flox* mice into individual wells of 96-well plates that were pre-seeded with OP9-DL1 stromal cells. These DN3 cells were cultured in T cell media with or without IL-2 supplementation (Fig. 4.12). Out of the 79 wells that had cells growing, 36 wells had many fast-proliferating T cells (Fig. 4.13). PCR genotyping confirmed that these T cells had deleted only one *flox* allele of *Bcl11b,* while the other conditional knockout allele remained intact (Fig. 4.14 Lanes T1 and T2). These cells, nevertheless, served as excellent controls for Cre toxicity because they had activated Cre recombinase but retained expression of Bcl11b for robust T cell development. In the other 43 wells, stromal cells were killed and most live cells were $NKp46^{+}CD3^{-}$, again demonstrating reprogramming of DN3 thymocytes to ITNKs (Fig. 4.13). The DN3-derived ITNKs had great proliferation potential, since up to 0.5 million ITNK cells were readily produced from a single DN3 thymocyte in 2-3 weeks post OHT treatment in the presence of IL-2. IL-2 or IL-15 was clearly able to promote proliferation of ITNKs substantially because, in absence of IL-2 or IL-15, only about 50,000 cells that also killed stromal cells were obtained from a single DN3 thymocyte under otherwise identical culture conditions. This result again demonstrated that the IL-2/15 signaling, which is essential for normal NK cell development (Waldmann, 2006), was dispensable for the generation and function of ITNKs. As expected, the

PCR genotyping results showed that the cells from all the 43 wells containing ITNKs had lost both *Bcl11b* alleles (Fig. 4.14 Lanes I1 and I2). Moreover, ITNKs of individual wells possessed unique rearranged TCRβ loci, thus confirming their independent thymocyte origins (Fig. 4.15). Therefore, once Bcl11b was deleted, the reprogramming efficiency of DN3 thymocytes to ITNKs could reach 100%.

4.2.5. Bcl11b is required in mature T cells

We next investigated whether Bcl11b was required for the maintenance of T cell identity in mature T cell subsets. The OHT-treated DP thymocytes, $CD4^+$, $CD8^+$ splenic T cells, and γδ T cells from *flox/flox* mice were sorted and co-cultured with OP9-DL1 in T cell media plus IL-2. ITNKs ($NKp46^+$) that effectively killed the stromal cells were found growing in the DP thymocytes cultures within 10 days after *Bcl11b* deletion (Fig. 4.16). These ITNKs, in contrast to those reprogrammed from early T cells, retained TCRβ expression. While the OHT-treated *flox/+* control DP thymocytes as control died eventually in the culture within 2 weeks. ITNKs that were $NKp46^+CD8^+$ could also be derived from the OHT-treated *flox/flox* $CD8^+$ T cells but not from the OHT-treated *flox/+* $CD8^+$ T cells in cultures (Fig. 4.17). Thus, Bcl11b is required to sustain T cell identity in mature T cell subsets. Moreover, the fact that ITNKs derived from mature T cell subsets retained T cell surface markers even after the acquisition of NK markers suggested that T cells reprogrammed to ITNKs directly, instead of dedifferentiating towards progenitors at first, upon loss of Bcl11b.

Interestingly, I was unable to obtain consistent reprogramming results from *Bcl11b*-deficient splenic $CD4^+$ T cells, or from thymic γδ-T cells, using the same culture condition. These T cells appeared prone to cell death once Bcl11b was deleted. A possible reason could be that our in vitro culturing conditions were not yet optimized for the reprogramming of these T cell subsets.

4.2.6. ITNKs detected in Tamoxifen-treated flox/flox mice

To assess whether different T cell subsets including splenic $CD4^+$ and thymic γδ-T cells can reprogram to ITNKs in vivo, we treated the *flox/flox* mice with Tamoxifen to induce *Bcl11b* deletion and culled the mice for analysis two weeks later (Fig. 4.18). This experiment could also help us rule out the possibility that the reprogramming from T cells to ITNKs was an in vitro artifact rather than the direct consequence of *Bcl11b* ablation. Compared to Tamoxifen-treated *flox/+* mice as controls, thymi of the *flox/flox* mice were smaller and contained less thymocytes, suggesting the importance of Bcl11b in thymocyte homeostasis. The size and cellularity of the spleen were not affected by loss of Bcl11b. It was possible that other hematopoietic cells compensated for the space left by T cells in the spleen. Similar phenotypes with respect to cellularity were also observed in other studies using different Cre systems to induce deletion of *Bcl11b* in vivo (Albu et al., 2007; Kastner et al., 2010). By FACS, we detected ITNKs in both the spleen ($NKp46^+CD3^+$) and thymus ($NKp46^+CD3^+$ and $NKp46^+CD3^-$) from *flox/flox* mice but not from *flox/+* controls (Fig. 4.19). Similar to ITNKs that were derived from DP thymocytes or mature T cells in vitro, the majority of ITNKs in *flox/flox* mice maintained expression of their T cell surface markers, such as CD3 and TCRβ. To confirm that the production of ITNK was caused by the loss of Bcl11b, we sorted different ITNK populations and examined whether the *Bcl11b* conditional knockout alleles had been deleted in ITNKs. PCR genotyping results showed that the in vivo reprogrammed ITNKs from the thymus had lost both copies of Bcl11b alleles, while the rest of the thymocytes from *flox/flox* mice retained at least one copy of *Bcl11b* allele (Fig. 4.20). Most of the $NKp46^+CD3^+$ cells in the spleen had Bcl11b deficiency, while analyzing the *Bcl11b*-deficiency in $NKp46^+CD3^-$ ITNKs was complicated due to the presence of

conventional NK cells (Fig. 4.20). Conventional NK cells constitute about 3% of splenocytes but cannot be distinguished from $NKp46^{+}CD3^{-}$ ITNKs that were reprogrammed from early T cells in the thymus and migrated out of the thymus to the periphery, thus these conventional NK cells might overshadow the presence of $NKp46^{+}CD3^{-}$ ITNKs in the spleen. Previously, we showed that $CD8^{+}$, but not $CD4^{+}$ splenocytes could reprogram to ITNKs after loss of Bcl11b in vitro. Here, ITNKs ($NKp46^{+}$) expressing CD4, CD8 (Fig. 4.21) and TCRγδ (Fig. 4.22) were found in the spleen of Tamoxifen-treated *flox/flox* mice, but not in *flox/+* controls, suggesting that $CD4^{+}$ mature T cells and γδ-T cells had the potential to be reprogrammed to ITNKs. Thus, the in vivo microenvironment facilitates the reprogramming from some T-cell subsets to ITNKs upon loss of Bcl11b better than the OP9-DL1 co-culture system.

Natural killer T (NKT) cells are a subgroup of T cells that share properties of both T and NK cells. However, ITNKs detected here were not NKT cells, because NKT cells do not express the cell surface marker NKp46 (Walzer et al., 2007). Additionally, ITNK cells did not recognize glycolipids presented by CD1d molecules. In fact, CD1d-restricted NKT cells decreased in *flox/flox* mice after being treated with Tamoxifen (Fig. 4.23), indicating that Bcl11b was required for both conventional T cells and CD1d-restricted NKT cells (Kastner et al., 2010).

The in vivo reprogrammed ITNKs could readily be expanded in NK culture conditions (100 ng/ml IL-2). The percentages of ITNKs ($NKp46^{+}CD3^{+}$) in splenocytes and thymocytes from *flox/flox* mice increased substantially after two weeks culture, whereas no ITNKs were detected in cultured splenocytes or thymocytes from *flox/+* mice (Fig. 4.24). In addition, we speculated that some $NKp46^{+}CD3^{-}$ cells in the culture of *flox/flox* splenocytes and thymocytes were ITNKs, which might be derived from early thymocytes. Furthermore, these ITNKs

stained negatively with CD1d dimer, further confirming that ITNKs were not NKT cells (Fig. 4.25).

4.2.7. Reprogramming from T cells to ITNKs in vivo

The above experiments in which *flox/flox* mice were treated with Tamoxifen showed the in vivo ITNK production from various T cell compartments. However, analyzing ITNKs in these mice was complicated by the presence of host T and NK cells. To address this problem, we transferred 2-4 million DP thymocytes from OHT-treated whole thymocytes of *flox/flox* mice ($CD45.2^+$) into *Rag2*$^{-/-}$*Il2rγ*$^{-/-}$ mice ($CD45.1^+$) that have no B, T or NK cells (Fig. 4.26) (Colucci et al., 1999). These recipient mice thus enabled us to easily identify and analyze the reprogrammed ITNKs. I chose DP thymocytes because they usually account for more than 75% of total thymocytes and could be efficiently reprogrammed to ITNKs in vitro. Additionally, as committed T cells, DP thymocytes have lost NK cell potential. Two weeks post transplantation, we found that around 5% of splenocytes were from the donor cells ($CD45.2^+$). Approximately 47% of the donor-derived splenocytes expressed NKp46 and CD3 (Fig. 4.27). Moreover, these $NKp46^+CD3^+$ cells had lost both copies of *Bcl11b* while the donor-derived $NKp46^-$ cells still retained at least one copy of the *Bcl11b* allele (Fig. 4.28). Therefore the reprogramming from *Bcl11b*-deficient DP thymocytes to ITNKs in immune deficient mice was a cell-autonomous process.

In *Rag2*$^{-/-}$*Il2rγ*$^{-/-}$ mice, the majority of $NKp46^+$ ITNKs expressed CD8 and TCRβ, and no $CD4^+$ ITNKs were detected (Fig. 4.29), suggesting the reprogramming from *Bcl11b*-deficient DP thymocytes to $CD4^+$ ITNKs was not favored in this in vivo microenvironment. However, we did not know whether these $CD8^+NKp46^+$ ITNKs were derived directly from Bcl11b deficient DP thymocytes. Alternatively, these DP

thymocytes might have differentiated to $CD8^+$ T cells before reprogramming to ITNKs. It was also possible that the differentiation and reprogramming processes could take place simultaneously.

Besides the spleen $NKp46^+$ ITNK cells were also detected in the bone marrow and peripheral blood (Fig. 4.30). As there were about 5 million splenocytes in each receipt mouse, we estimated that there were 200,000 ITNK cells in the spleen alone. No $NKp46^+$ cells were found in control mice transplanted with OHT-untreated DP thymocytes (Fig. 4.27). ITNK cells were maintained in the recipient mice for at least 3 months without changes to the cell number, perhaps reflecting a homeostasis of ITNKs. Importantly, the recipient mice did not show any noticeable abnormalities, indicating that ITNK cells did not indiscriminately kill normal host cells or exhibit any malignant transformation in the recipient mice.

4.2.8. Bcl11b is positively regulated by Notch signaling

During T cell specification and commitment, it is proposed that Bcl11b is regulated by Notch signaling (Rothenberg, 2007). The fact that ITNKs were readily produced from *Bcl11b*-deficient DN1, DN2 and DN3 thymocytes in co-culture with either OP9 or OP9-DL1 stromal cells (Fig. 4.31 and 4.10) demonstrated that T cells would reprogram to ITNKs upon loss of Bcl11b in vitro regardless of the presence of external Notch ligands. Thus Bcl11b might be one of the downstream genes regulated by Notch signaling in T cells.

To confirm this, we searched within the *Bcl11b* gene locus for the putative CSL (Rbpj)-binding sites (CGTGGGAA) (Tun et al., 1994). Several CSL sites were identified which were conserved between the mouse and human *Bcl11b* genes (Fig. 4.32). ChIP assays were subsequently performed using a CSL polyclonal antibody to pull down the genomic DNA fragments from T cells cultured on OP9-DL1 stromal

cells. The primers flanking the putative CSL binding regions were designed to amplify the ChIP pull-down genomic DNA. Three regions were greatly enriched in the T cell samples using the CSL antibody compared to the control (Fig. 4.33). The ChIP results thus strongly suggested that the canonical Notch signaling directly regulated Bcl11b at the transcription level. Moreover, it is reported that deletion of CSL (*Rbpj*) in Mx-Cre transgenic mice after injection of poly(I)-poly(C) results in a block of T cell development at the earliest stage and an increase of B cell development in the thymus (Han et al., 2002). This phenotype is very similar to that in a Notch1 knockout mouse using the Mx1-Cre system (Radtke et al., 1999). However, we could not rule out the possibility that Bcl11b was regulated by other signaling pathways. Therefore, we proposed the following model to summarize the regulation and function of Bcl11b in T cells (Fig. 4.34).

4.3. Discussion

In this Chapter, I demonstrated that Bcl11b had essential roles in early T cell development and in the maintenance of T cell identity. Positively regulated by Notch signaling, Bcl11b suppressed key NK cell-associated genes expression and might positively regulate key T cell genes in T cells. Acute loss of Bcl11b made DN1 and DN2 thymocytes lose their T cell potential and differentiate to NK-like cells. Strikingly after Bcl11b ablation, DN3, DP thymocytes, and mature $CD8^+$ T cells also lost T cell identity and reprogrammed to ITNKs in OP9-DL1 co-culture system. Similarly, ITNKs reprogrammed from various T cell subsets were also detected in vivo models. Additionally, transplantation experiments showed the reprogramming was a cell autonomous process. In summary, T cells at different developmental stages reprogrammed to ITNK upon loss of Bcl11b. Therefore Bcl11b was crucial to sustain T cell development in different T cell subsets (Fig. 4.35).

4.3.1. Deletion of Bcl11b using different Cre systems

T cell development is impaired beyond the DN3 stage, while DN1, DN2 and γδ-T cells seems to be intact, in the fetal thymus of a Bcl11b germline knockout mouse (Wakabayashi et al., 2003b). However, a recent study using the same knockout strain shows that *Bcl11b*-deficient thymocytes are arrested at DN2 stages and acquired self-renewal properties in vitro (Ikawa et al., 2010). Our results, together with a study from Rothenberg's lab, show that *Bcl11b*-deficient DN1 and DN2 thymocytes stopped T cell development and acquired other cell potentials in the adult thymus (Li et al., 2010). Taken together, both fetal and adult early T cells needed Bcl11b to sustain T cell development and suppressed non-T cell potentials. However, unlike *Bcl11b*-deficient fetal γδ-T cells, adult γδ-T cells also reprogrammed to ITNKs upon loss of Bcl11b, suggesting that fetal and adult γδ-T cells may require different transcription networks during development.

Using different Cre system to delete Bcl11b generates different phenotypes. For example, in *CD4-Cre; Bcl11b*$^{flox/flox}$ mice, a reduction of $CD4^+$ and $CD8^+$ SP thymocytes is observed and *Bcl11b*-deficient DP thymocytes commit to apoptosis (Albu et al., 2007; Kastner et al., 2010). Additionally, $CD8^+$ T cells have reduced clonal expansion and cytolytic activity when Bcl11b ablation is induced by dLck-iCre (Zhang et al., 2010). Expression of both CD4 and Lck is detected in early T cells (Lee et al., 2001; Wildin et al., 1995), thus DP thymocytes and mature T cells may have lost Bcl11b since they were early T cells in these two studies. In contrast, after acute loss of Bcl11b, we found that DP thymocytes and mature T cells reprogrammed to ITNKs both in vitro and in vivo. Thus deletion of Bcl11b in T cells at different stages may thus cause different phenotypes.

4.3.2. Possible factors affecting reprogramming efficiency

The efficiency of reprogramming from T cell to ITNK varies in different T cell subsets. For example, DN3, DP thymocytes, and $CD8^+$ splenic T cells could be efficiently reprogrammed to ITNKs upon loss of Bcl11b in vitro, whereas *Bcl11b*-deficient $CD4^+$ splenic and γδ-T cells underwent apoptosis instead of reprogramming to ITNK in the same culture condition. Here are three possible reasons to explain why different T cell subsets had different abilities of reprogramming.

First, a suitable in vitro reprogramming condition has not been identified yet for *Bcl11b*-deficient $CD4^+$ T cells and γδ-T cells. It is possible that different T cells require different reprogram conditions. For example, OP9-DL1 stromal cells do not normally support $CD4^+$ T cells for proliferation and differentiation because they do not express MHC-II molecules that are required for differentiation of $CD4^+$ T cells (Schmitt and Zuniga-Pflucker, 2002). It is possible that MHC-II molecules are also required for *Bcl11b*-deficient $CD4^+$ T cells to reprogram to ITNK cells. This may explain why $CD4^+$ T cells failed to reprogram to ITNK in the OP9-DL1 co-culture system. In fact, *Bcl11b*-deficient $CD4^+$ T cells were able to reprogram to ITNKs in vivo, where MHC-II molecules are available. However, this hypothesis cannot explain the blockage of reprogramming from γδ-T cell to ITNK in vitro, because OP9-DL1 stromal cells support γδ-T cells development.

The second explanation could be that *Bcl11b*-deficient mature $CD4^+$ T cells and γδ-T cells are more prone to apoptosis than other *Bcl11b*$^{-/-}$ T cell subsets in vitro. In contrast, the apoptosis in these cells could be prevented in vivo, possibly because the in vivo microenvironment can provide suitable cytokines and cell-cell interactions at physiological conditions. This may explain why $CD4^+$ and $TCR\gamma\delta^+$ ITNKs were

detected in OHT-treated *flox/flox* mice but not in OP9-DL1 co-culture system. If this hypothesis is true, the addition of apoptosis inhibitors in culture should facilitate reprogramming from $CD4^+$ and γδ-T cell to ITNKs. The apoptosis caused by loss of Bcl11b could also depend on the cellular context. For example, Bcl11b might induce *Gata3* expression as loss of Bcl11b caused downregulation of Gata3 (shown in Chapter 5). Also, ChIP-seq experiments suggest that Bcl11b directly regulates Zbtb7b, which is essential for $CD4^+$ T cells and γδ-T cells but not for other T cell subsets (He et al., 2008; Park et al., 2010). Consequently, a decrease of Gata3 or Zbtb7b might specifically cause apoptosis of *Bcl11b*-deficient $CD4^+$ T cells and γδ-T cells.

Thirdly, I believe that the more similar two cell types are, the less changes in cellular components, transcription networks, protein synthesis, and epigenetic modifications are required for the reprogramming from one to the other. Therefore the similarities between two types of cells affect the reprogramming feasibility upon genetic manipulation. For example, induced pluripotent stem (iPS) cells have been generated from human somatic cells by overexpression of Oct3/4, Sox2, Klf4, Nanog and other defined factors (Takahashi et al., 2007; Yu et al., 2007). However, compared to other stem cells, such as HSC, neural stem cells (NSC) exhibit more similar transcriptional profiles to ES cells. Overexpression of Oct4 alone thus is sufficient to facilitate their reprogramming to iPS cells (Kim et al., 2009; Ramalho-Santos et al., 2002). Compared to mature $CD4^+$ T cells, cytotoxic $CD8^+$ T cells are more similar to NK cells. Both CTLs and NK cells express Eomes and T-bet at higher levels than $CD4^+$ cells (Intlekofer et al., 2005; Szabo et al., 2000), while $CD4^+$ expressed higher level of Zbtb7b than $CD8^+$ and NK cells (Setoguchi et al., 2008). In addition, CTLs and NK cells use polarized secretion of the cytotoxic granules

containing granzymes and perforin to destroy virus-infected and tumorigenic target cells. These molecules are usually absent in $CD4^+$ T cells. They also use a novel secretory mechanism, with the centrosome polarizing to the precise site of secretion within the immunological synapse (Blott and Griffiths, 2002; Griffiths et al., 2010; Stinchcombe et al., 2006). Taken together, it is not difficult to conceive that it may be easier for $CD8^+$ T cells, especially activated ones assembling with more NK features, to reprogram to ITNKs than $CD4^+$ T cells.

4.3.3. "Unconventional NKT cells" in wild type mice

Previous studies show some mature $CD8^+$ T cells can exhibit some NK features following activation, indicating the similarity between NK cells and activated $CD8^+$ T cells. For example, it is reported that some $NK1.1^+TCR\alpha\beta^+CD8^+$ cells can be derived from activated $CD8^+$ T cells upon stimulation with IL-2, IL-4, or IL-15 in vitro. These 'unconventional NKT' cells express CD122 and most Ly49 receptors (Assarsson et al., 2000). Similar to $CD8^+NK1.1^+$ cells, these non-CD1d-restricted cells can also be derived from tumor-bearing mice (Stremmel et al., 2001). These "unconventional NKT" cells can produce large amount of IFN-γ following activation and have a potent NK-like cytotoxic activity against multiple tumor targets regardless to MHC-I or non-classical MHC-I molecules (Stremmel et al., 2001). However, these "unconventional NKT" cells need further characterization for their expression of Bcl11b, more NK cell surface markers, such as NKp46, and for their global gene expression profiles before claiming that they are regular NK cells or ITNKs.

4.3.4. Dynamic balance of ITNKs in vivo

It is interesting to note that the total number of ITNKs in *Rag2*$^{-/-}$*Il2rγ*$^{-/-}$ mice did not either correlate with the initial number of injected *Bcl11b*-deficient DP T cells or

change markedly overtime in the hosts. It was likely that the homeostasis of ITNK cells was well maintained in *Rag2*$^{-/-}$*Il2rγ*$^{-/-}$ mice. There are many factors likely to contribute the dynamic balance of ITNK cells *in vivo*. Due to the similarity between NK cells and ITNKs, the cytokines and cell-cell interactions that are important for NK cells may play a role for the homeostasis of ITNKs in vivo. For example, signals from IL-2 receptors provide essential positive homeostatic functions for NK cells and promoted proliferation of ITNKs as shown in the chapter 4 (Lodolce et al., 1998). Thus the availability of IL-15 and IL-2 can be one of the limiting factors. Other cytokines like IL-12, IL-18, IL-21 and TGFβ might also regulate ITNK cell homeostasis, as they are important for NK cell homeostasis (Laouar et al., 2005; Zwirner and Domaica, 2010). The expansion of ITNKs might also be affected by limited chemokines such as CCL19 and CCL21, of which co-stimulation can promote the proliferation of CD56^{lo}CD16^{+} NK cells in human (Robertson, 2002). The total number of ITNKs in *Rag2*$^{-/-}$*Il2rγ*$^{-/-}$ mice might also be determined by the availability of adhesion molecules on the cell surface of stromal cells in the BM and spleen, as these adhesion molecules are known to affect NK cell homeostasis (Huntington et al., 2007). Additionally, the cross-talks between NK cells and other lymphocytes may also regulate the homeostasis of NK cells (Ferlazzo and Munz, 2009). Although adaptive immunity is absent in the *Rag2*$^{-/-}$*Il2rγ*$^{-/-}$ host mice, it is possible that some dendritic cells participated to control the total number of ITNKs in host (Degli-Esposti and Smyth, 2005; Di Santo, 2008).