Chapter 6

GENERAL DISCUSSION

6.1. Summary

This chapter summarizes the thesis on *Bcl11b* expression and function in T cells, its regulatory networks, and its potential application in cancer immunotherapy. I will also evaluate the contribution of this thesis to the knowledge of T cell development and immunotherapy. Then, future experiments on the identification of upstream and downstream genes of Bcl11b, the gain-of-function studies, and approaches to reprogram human T cells to ITNKs will be discussed. Finally, I will make the conclusions of my study in this thesis.

6.1.1. Bcl11b expression in T cells

In Chapter 3, I characterized *Bcl11b* expression at the single cell level in a *Bcl11b-tdTomato* knock-in mouse, where the *tdTomato* cassette was inserted into the 3' UTR of the *Bcl11b* locus. In hematopoietic lineages, *Bc11b* expression was restricted to T cell compartments including almost all DN2-DN4 and DP thymocytes, $CD4^+$ and $CD8^+$ mature T cells, γδ−T cells, and NKT cells. Some immature NK cells also transiently expressed *Bcl11b* at low levels. However, neither ETPs nor $CD117^{++}$DN2 thymocytes expressed *Bcl11b*, suggesting that *Bcl11b* expression might be suppressed by c-Kit signaling, which often has important roles in progenitors (Kondo et al., 1997). A recent study from Kawamoto's lab showed that IL-7 signaling, which is essential for common lymphoid progenitors and early T cell

progenitors, suppresses induction of *Bcl11b* expression in early T cells (Akashi et al., 1998; Ikawa et al., 2010). Interestingly, Kit and IL-7 pathways directly interact with each other (Jahn et al., 2007). In T cells, activation of Kit induces strong tyrosine phosphorylation of γ and IL-7Rα in the absence of IL-7 (Jahn et al., 2007). Taken together, I speculate that *Bcl11b* expression marks that early T cell progenitors start to lose multipotency and differentiate towards T cell lineage.

I further described that *Bcl11b* was expressed in $CD4^+$ and $CD8^+$ mature T cells at different levels. Its expression in $CD8^+$ T cells was lower than that in $CD4^+$ T cells, as measured by RNA samples from T cell pools. Interestingly, at a single cell level, some activated T cells expressed *Bcl11b* at very low levels, suggesting that Bcl11b might suppress some T-cell-activation-associated genes thus inhibit T cell activation (Li et al., 2010).

6.1.2. Bcl11b functions in early T cells

Early T cell progenitors retain myeloid- and NK-cell potentials (Bell and Bhandoola, 2008) (Wada et al., 2008). In Chapter 4, I demonstrated that acute deletion of *Bcl11b* in T cell progenitors stopped T cell development at DN1-2 stages, as these *Bcl11b*-deficient T cell progenitors differentiated to NK-like cells instead of T cells in T cell culture conditions. Further studies from Rothenberg's lab shows that *Bcl11b*-deficient T cell progenitors initiates T cell specification normally, as indicated by upregulation of *Cd3e*, *Cd3g*, *Ptcra*, and *Rag1* (Li et al., 2010). Thus the blockage of T cell development is not due to a failure of initiation of T cell program, but rather a likely failure to repress alternative cell-lineage development. This is supported by the fact that *Bcl11b*-deficient thymocytes also express genes such as *Id2*, *Il2rb* and *Nifl3* that normally promote NK cell development (Li et al., 2010). However, it is possible that some *Bcl11b*-deficient DN2 thymocytes are able to differentiate further

to the DN3 stage before undergoing apoptosis. Indeed, DN3 thymocytes are detected in the *Bcl11b*-deficient fetal thymus (Wakabayashi et al., 2003b).

Recently, the block of T cell differentiation was observed in the DN2 fetal thymocytes from *Bcl11b* conventional knockout embryos (Ikawa et al., 2010). These *Bcl11b*-deficient DN2 thymocytes proliferated extensively and were able to differentiate to NK cells, dendritic cells and myeloid cells, but not T cells (Ikawa et al., 2010). However, once Bcl11b is reintroduced to the *Bcl11b*-deficient DN2 thymocytes, T cell development resumed (Ikawa et al., 2010). These results thus suggest that Bcl11b terminates non-T-cell-lineage potentials in T cell progenitors thus essential for early T cell development.

6.1.3. Bcl11b functions in committed and mature T cells

In Chapter 4, I described that Bcl11b was not only required for early T cell development, but also essential for the maintenance of T cell identity in committed and mature T cells. Upon loss of Bcl11b, committed and mature T cells reprogrammed to ITNKs both in vitro and in vivo (Fig. 6.1) (Pentao Liu, 2010). *Bcl11b*-deficient DN3 thymocytes aborted T cell development and were reprogrammed to ITNK cells. These ITNK cells expressed other NK-cell-associated genes besides NKp46 and were similar to NK cells in morphology. In the single-cell-assay experiments, I demonstrated that every single *Bcl11b*-deficient DN3 thymocyte reprogrammed to ITNKs. And these ITNKs, similar to *Bcl11b*-deficient DN2 thymocytes, showed extensive in vitro expansion potentials. Besides DN3 thymocytes, ITNKs were also produced from DP thymocytes and $CD8^+$ mature T cells upon loss of Bcl11b in vitro. These ITNKs retained TCRβ and CD3 on the cell surface. However, I do not know the reprogramming efficiency of these ITNKs.

I also demonstrated that ITNKs from *Bcl11b*-deficient T cells was not an in vitro culture artifact, because the results from Chapter 4 showed that ITNK cells were detected in the spleen and thymus of Tamoxifen-treated *flox/flox* mice. Interestingly, these in vivo-derived ITNKs had higher proliferation potential than NK cells in NK culture condition. Moreover, similar to ITNKs reprogrammed from mature T cells in vitro, ITNKs that were reprogrammed from mature T cells in vivo also expressed TCRβ and CD3, although their TCR signaling appeared to be compromised.

I further demonstrated that reprogramming from T cells to ITNKs upon loss of Bcl11b was a cell-autonomous process, because ITNKs were present in *Rag2*$^{-/-}$*Il2rγ*$^{-/-}$ mice after being transferred with *Bcl11b*-deficent DP thymocytes. These ITNKs expressed TCRβ, CD3, CD8 and some members of Ly49 family receptors. The total number of ITNK cells did not vary substantially for at least 3 months, perhaps representing the homeostasis of ITNKs in the in vivo microenvironment.

In summary, acute loss of Bcl11b in T cells led to a failure of T-cell-lineage commitment and T-cell-identity maintenance. Possibly, Bcl11b maintains T cell identity by suppressing non-T cell potentials and sustains the T cell program in T cells. My results are different from the previous studies using constitutive expression of Cre recombinase in T cells. For example, in DP thymocytes, *Bcl11b* deletion using CD4-Cre causes defects in the initiation of positive selection (Albu et al., 2007). In another study, deletion of *Bcl11b* in early DP thymocytes using CD4-Cre causes expression of some genes that are found in mature SP T cells like *Zbtb7b* and *Runx3* (Kastner et al., 2010). Neither study revealed cells similar to ITNKs in these mutant mice.

6.1.4. Bcl11b transcription regulatory networks

Despite its importance in T cells, Bcl11b regulatory networks in T cells are not clear. Previous studies propose that Bcl11b is regulated by Notch signaling during T cell specification and commitment based on gene expression changes (Rothenberg, 2007). In Chapter 4, I showed NK-like cells instead of T cells grew out from *Bcl11b*-deficient DN1 and DN2 thymocytes on either OP9 or OP9-DL1 stromal cells. Therefore the lineage switch from T- to NK-cell upon loss of Bcl11b was independent of Notch signaling, suggesting that Bcl11b perhaps acted downstream of Notch signaling in T cells. Our ChIP analysis using antibodies to CSL confirmed that the canonical Notch signaling pathways directly regulated Bcl11b at the transcription level. Although *Bcl11b* expression is induced by Notch signaling, it is likely to be also regulated by other factors or signaling pathways in T cells. For example, the Bcl11b expression profile in early T cell progenitors from a *Bcl11b-tdTomato* knock-in mouse suggested that c-Kit signaling might suppress Bcl11b expression. IL-7 signaling is another candidate. In the stromal-free culture system higher concentration of IL-7 blocks DN2 thymocytes further differentiation into T cells. This block can be overcome by either lowering the concentration of IL-7 or by forcibly expressing Bcl11b, implicating that IL-7 signaling represses Bcl11b expression in early T cells (Ikawa et al., 2010).

Compared to upstream genes of Bcl11b, even less is known about its downstream genes. In Chapter 6, the microarray analysis showed that the global gene expression profile of ITNKs exhibited many NK cell features. Compared to their parental DN3 thymocytes, ITNKs had lower expression of many T-cell-lineage genes like *Gata3*, *Notch1*, *Dtx1* and *Hes1* and higher expression of NK cell-associated genes such as *E4bp4*, *Zfp105*, and *Id2*. Similar gene expression changes are also reported in

Bcl11b-dificient early T cells (Ikawa et al., 2010) (Li et al., 2010). In the future, ChIP assays will be required to determine whether these genes are the direct targets of Bcl11b.

6.1.5. Potentials of ITNKs in immunotherapy

Both T cells and NK cells have been used to treat cancer in adoptive cell transfer (ACT) therapy (Rosenberg et al., 2004). However, due to the limitations of T cells and NK cells, new cell sources are required to further explore the potential of cancer immunotherapy. The unique properties of ITNKs shown in Chapter 5 made them an attractive cell source for this purpose. In contrast to limited availability of NK cells for ACT, a large quantity of T cells could be readily obtained from either peripheral blood or thymus to produce ITNKs. Every *Bcl11b*-deficient DN3 T cell could be reprogrammed to ITNK cells, according to our single cell assay results. Furthermore, ITNKs could be expanded extensively *in vitro* and exhibited potent killing ability on various tumour cell lines, regardless of MHC-I molecules expression status. Finally, ITNKs appeared to be able to distinguish normal cells from the tumour cells, and were not malignantly transformed, because host mice with ITNKs did not show any abnormalities.

6.2. Significance

6.2.1. Novel roles of Bcl11b in the maintenance of T cell identity

This study demonstrates for the first time that ablation of a single transcription factor, Bcl11b, abolished maintenance of T cell identity, resulting in reprogramming of T cells at different developmental stages to ITNKs. IL-2 and IL-15 were

dispensable for, but could stimulate the growth of ITNKs. ITNKs share similar gene expression profiles with NK cells, and were potent killers for three tested tumour cell lines. However, ITNKs were not NK cells. They could be produced from DN1 and DN2 thymocytes in the presence of Notch signaling and are independent of cytokines such as IL-2 or IL-15 for survival and proliferation. Moreover, ITNKs from DP thymocytes or mature T cells retained some signatures of their T cell origin, such as expression of some T cell genes and compromised TCR function.

6.2.2. Clinical potential of ITNKs

NK-cell-based therapies hold promise in cancer and virus infection, such as hepatitis C virus infection, treatment (Barrett and Le Blanc, 2010; Rosenberg et al., 2008; Salem et al., 2010). We are now able to reprogram T cells to ITNKs, which could be extensively expanded but were not malignantly transformed. Moreover, they effectively killed tumor cells in vitro and eliminate metastatic cells in mice but did not appear to attack normal cells from hosts. Because *Bcl11b* sequence is highly conserved in both the mouse and human, we expect that ITNKs can also be produced from human T cells, where BCL11B is inactivated by genetic and non-genetic approaches such as RNAi, zinc finger nuclease (Urnov et al., 2005), and small molecule inhibitors. These ITNK cells may serve as a new cell source for cancer immunotherapy and other cell-based therapies (Fig. 6.2).

6.3. Future experiments

The results presented in this thesis demonstrate that Bcl11b was essential for early T cell development and the maintenance of the T cell identity in committed and mature T cells. Future work is required to probe the regulators of Bcl11b and its downstream genes and pathways. ITNKs that were produced from *Bcl11b*-deficient T

cells could efficiently kill tumor cells both in vitro and in vivo, regardless of MHC-I expression. To further explore the clinical application of ITNKs in cancer immunotherapy, future experiments are required to demonstrate whether human T cells can be reprogrammed to ITNK cells through genetic or non-genetic modification. Furthermore, it is necessary to test whether these human ITNKs are capable of eliminating human cancer cells efficiently in vitro and in tumor bearing animal models.

6.3.1. Upstream genes of Bcl11b

In Chapter 4, I showed that Notch1 induces *Bcl11b* expression through CSL in T cells, as CSL directly bind to the *Bcl11b* locus. Luciferase assays can be performed to examine whether enforced expression of CSL or Notch1 can induce *Bcl11b* expression at the cellular levels, or assess whether overexpression of a dominant negative form of NICD inhibits *Bcl11b* expression.

Besides Notch signaling, other signal pathways may regulate *Bcl11b* expression positively and negatively in different T cell compartments. Bcl11b was absent or expressed at undetectable levels in ETP and $CD117^{+}$DN2 thymocytes. There are several candidates that may suppress Bcl11b expression in these populations. I propose that IL-2Rβ and its downstream JAK/STAT signaling pathways (Benczik and Gaffen, 2004), are candidates to repress *Bcl11b* expression in early T cell progenitors based on the following evidence. Wild type DN1 and DN2 thymocytes differentiate to T cells upon co-culture with OP9-DL1 stromal cells that provide Delta-like 1, the Notch ligand, to activate Notch signaling in these thymocytes. However, supplementation of IL-15 (30 ng/ml) or IL-2 (100 ng/ml) in this culture forces the early T cell progenitors to differentiate to NK cells instead of T cells, suggesting that the activation of IL-2Rβ signaling by its ligands can abolish T cell development in the

presence of Notch signaling. Mutual antagonism between the Notch and JAK/STAT signaling pathways is reported in drosophila. Notch signaling inhibits JAK/STAT signaling by preventing STAT nuclear translocation, while signaling by JAK/STAT reduces Notch signaling (Assa-Kunik et al., 2007; Gutierrez-Avino et al., 2009; Sotillos et al., 2008). Moreover, Bcl11b was induced by Notch signaling and was essential for T cell development. Therefore we speculate that IL-2Rβ signaling and JAK/STAT signaling may suppress *Bcl11b* expression through Notch signaling. In this case, extremely high concentrations of IL-2, IL-7, and IL-15 in culture, which all activate JAK/STAT pathways (Waldmann, 2006), may suppress Bcl11b expression in T cells and produce ITNKs. Nevertheless it is also possible that IL-2Rβ signaling repressed downstream genes of Bcl11b rather than Bcl11b expression directly. To investigate these possibilities, we can use flow cytometry or RT-PCR to measure *Bcl11b* expression changes in early T cell progenitors from *Bcl11b-tdTomato* knock-in mice when IL-2Rβ signaling is activated or inhibited. Similarly, we can examine the effect of IL-7Rα and c-Kit signaling on Bcl11b expression in gain- and loss-of-function studies. For example, we can measure Bcl11b expression in early T cells expressing both CD127 and CD117 with high concentration of IL-7 or SCF. We can also block receptors of IL-7 and c-Kit with antibodies and then assess Bcl11b expression in the T cells.

Furthermore, it was shown that Bcl11b expression in activated T cells is lower than that in naïve T cells in the Chapter 3. Consistent with this, induction of T cell activation using anti-CD3 antibodies promoted expansion of the $Bcl11b^{low}$ activated T cells (Shannon Burke, observation). Taken together, these results suggested that TCR signaling might suppress Bcl11b expression during T cell activation. Thus further

experiments are required to validate whether TCR signaling antagonizes Bcl11b in mature T cells.

6.3.2. Downstream genes of Bcl11b

Because Bcl11b is a transcription factor, ChIP-seq experiments using Bcl11b antibodies are the most straightforward method to study its downstream targets. The ChIP-seq results from Gross's laboratory showed that Bcl11b bound to several regions within the *Zbtb7b* locus, including the distal regulatory element (Kastner et al., 2010). However, NK-cell-associated genes are not found in the list of Bcl11b putative binding sites. To refine the list of Bcl11b downstream genes, we can combine the microarray analysis of gene expression profiles that were obtained from loss- and gain-of-function studies on Bcl11b in T cells and the ChIP-seq results together to probe overlapping candidates. Moreover, we should focus on the known NK-cell- and myeloid-cell-associated genes and T-cell genes from the Bcl11b downstream candidates listed in the Chapter 5 and two other recent publications (Ikawa et al., 2010; Li et al., 2010). For example, IL-2Rβ signaling, the prospect upstream of Bcl11b, may also be regulated by Bcl11b. *Bcl11b*-deficient DN1 or DN2 thymocytes differentiated to ITNKs that were IL-2 or IL-15 independent, suggesting that IL-2Rβ signaling is constitutively activated without the stimulation of its ligands after Bcl11b ablation. Therefore Bcl11b may suppress IL-2Rβ signaling in early T cell progenitors. And this repression cannot be bypassed in committed T cells by stimulation through IL-2Rβ, as DN3 thymocytes fail to switch to NK-cell lineage despite of high concentration of IL-2. This suggests that IL-2Rβ signaling cannot suppress Bcl11b expression once T cell program, possibly including Bcl11b self-sustain feedback loop, has been established in committed T cells.

6.3.3. Overexpression of Bcl11b in ITNKs and NK cells

In this thesis, I have used a loss of function approach (acute loss of Bcl11b) to identify Bcl11b function in T cells. However, it remains unknown whether overexpression of *Bcl11b* in NK cells would reprogram them to T cells. Therefore future experiments can include construction of a Bcl11b overexpression vector with a GFP marker in the *piggyBac* (PB) transposon, which can carry a large DNA cassette and integrate into mammalian genome efficiently with the help of transposase (Ding et al., 2005; Wang et al., 2008b). Additionally, the PB transposon can jump between genomic sites by a "cut and paste" mechanism, and can excise itself precisely, leaving no footprint behind (Thibault et al., 2004). Thus we can use the amaxa nuclear transfection technology to deliver this vector into the genome of NK cells efficiently. These transfected NK cells that overexpress Bcl11b can be sorted and cultured in T cell condition, or injected into *Rag2*$^{-/-}$*Il2r*$\gamma^{-/-}$ mice. T cells may result from these NK cells if Bcl11b expression disrupts the NK-cell transcription program and establishes the T-cell transcription program.

6.3.4. Reprogramming human T cells to ITNKs

To use ITNKs as a source of cell-based therapy for human cancer, we need to derive ITNKs from human T cells by inactivating BCL11B. Because the conditional knockout technology is not available for human cells, we have to explore alternative methods to ablate *BCL11B* in human T cells. With zinc finger nuclease (ZFN) technology (Klug, 2010), it is possible to design ZFNs that specifically bind to and introduce mutations at the *BCL11B* locus, thus abolishing BCL11B transcription completely in human T cells. Additionally, single cell assays can be conducted to assess the efficiency of reprogramming from human $CD8^+$ T cells to ITNKs. However, due to the low mutation efficiency and high undesired-mutation rate in ZFN

technology, it is more practical to use non-genetic approaches to inactivate BCL11B in human T cells. Though small molecules have been widely used to inhibit protein function, there are only few successful examples for transcription factors (Lee et al., 2010). Compared to the two approaches discussed above, it is more feasible to choose another approach, such as RNAi technology, at least in the short term. BCL11B can be knockdown efficiently in both normal human T cells and human T cell leukemia by expressing *BCL11B*-specific shRNAs (Grabarczyk et al., 2007). We plan to use the similar approach to knockdown BCL11B in human T cells and examine whether these T cells can be reprogrammed to ITNK cells. Since the knockdown efficiency normally does not achieve 100%, and if a complete loss of BCL11B is a prerequisite for T cells to be reprogrammed to ITNKs, we may not obtain ITNKs from human T cells using RNAi, or at least not very efficiently. Moreover, in some T cells, knockdown efficiency could be around 50%. It is known that mice heterozygous for *Bcl11b* deletion develop T cell leukemia. Thus it is very important to test whether these BCL11B knockdown cells have cancer potential as a population or as single cells in humanized mouse models.

6.3.5. Testing the tumor-killing ability of human ITNKs

Once we successfully obtain human ITNK cells, we will next need to investigate the persistence of human ITNKs in animal models and determine how long human ITNKs are able to survive and remain functional in vivo. For safety reasons, it is also required to examine whether human ITNKs have cancer or oncogenic potential, as described above.

Another important factor for consideration is from which human T cell compartments are best production of ITNKs for cancer therapy. In the mouse, ITNKs can be obtained from various T-cell subsets, and these ITNKs have distinct gene

expression profiles. For example, DN3 thymocytes-derived ITNKs did not express TCR, while those derived from $CD8^+$ mature T cells retained a functional but compromised TCR complex. TCR signaling in $CD8^+$ ITNKs may still be used for targeting ITNKs to tumour cells expressing specific antigens. In this case, using a tumor-specific chimeric receptor or transgenic TCR may enhance the killing potency of ITNKs. Alternatively, this could be achieved by deriving ITNKs from T cells enriched for tumor-specific $CD8^+$ T cells or tumor infiltrating T cells (Fig. 6.2).

In addition, it is important to know whether ITNKs are suitable to a variety of tumor types. Thus we need to perform killing assays to measure how efficiently human ITNKs kill various human tumor cells. We also need to inject these ITNKs into humanized mice that are engrafted with human tumors and to examine whether ITNKs eliminate these tumors in vivo.

6.4. Conclusions

Bcl11b has now been identified as a critical transcription factor for T cell lineage commitment. Loss of Bcl11b in T cell progenitors allowed expression of genes of alternative lineages. Moreover, deletion of *Bcl11b* in committed or mature T cells caused loss, or decreased expression, of T-cell genes with concomitant expression of genes usually associated with NK cells. These ITNKs reprogrammed from T cells had enormous proliferation potential in vitro and potent killing ability that is MHC-independent, and yet killed normal cells. Therefore this thesis uncovered a critical transcription factor, Bcl11b, in T cells. ITNKs described in this study may provide a new cell source for cell-based therapies. Since ITNKs were a new type of killer cells, many exciting questions remain unanswered.