Chapter 5

CHARACTERIZATION AND APPLICATION OF ITNKs

5.1. Introduction

5.1.1. Cancer immunotherapy

The immune system is alert to not only external biological invasion from virus infection but also transformed tumor cells within the host. Many groups in the area of cellular therapy have exploited the application of cancer immunosurveillance to treat cancer (Rosenberg et al., 2004; Zitvogel et al., 2006). There are three main approaches in cancer immunotherapy: non-specific immunomodulation, cancer vaccines, and adoptive cell transfer (ACT) (Rosenberg and Dudley, 2009). In non-specific immunomodulation, cancer patients are administrated with IL-2 that can activate endogenous tumour-reactive cells, mainly T cells and NK cells in vivo (Rosenberg et al., 1985; Rosenberg et al., 1998). Thanks to the identification of a large number of human cancer antigens, cancer vaccines that are based on immunizing cancer patients against their autologous cancers using either whole cells, proteins and peptides, have been developed since 1990s (Rosenberg and Dudley, 2009; van der Bruggen et al., 1991). However, these two approaches are not as effective or promising as strategies using ACT of anti-tumor lymphocytes that are grown ex vivo and then infused into the cancer patient. ACT has the following advantages (Rosenberg et al., 2008). Firstly, a small number of anti-tumour lymphocytes with the appropriate properties can be identified and then expanded to

large numbers ex vivo for treatment (Rosenberg and Dudley, 2009). Secondly, the lymphocytes can be activated in vitro to avoid endogenous inhibitory factors, such as T regulatory cells and TGFβ. In addition, ACT can often be combined with vaccines or growth factors that can augment the in vivo impact of the transferred lymphocytes. Finally, it is possible to manipulate the host before cell transfer to provide an optimal environment for the transferred cells (Rosenberg and Dudley, 2009). For example, prior to ACT, radiotherapy and chemotherapy are usually used to reduce tumor burden and deplete endogenous lymphocytes that compete with transferred lymphocytes for homeostatic cytokines (Hogan and Rothenberg, 2008; Rosenwasser and Rothenberg, 2010).

Cytotoxic T lymphocytes (CTLs) are the main source of cells in ACT (Rothenberg et al., 2008). However, in T cell-based therapy, direct immunological pressure from T cells on tumour cells can lead to the outgrowth of tumour clones which express low or no human leukocyte antigens (HLA) (Chang and Ferrone, 2007; Dunn et al., 2004; Smyth et al., 2006) or that have lost the targeted antigen (Yee et al., 2002). The development of ACT using tumour-infiltrating lymphocytes (TIL), which are mainly $CD8^+$ T cells and can be expanded in IL-2, improves the tumour-specific activity in transferred cells and the immunization of lymphocyte donors (Muul et al., 1987; Rosenberg et al., 1986). However, the persistence of the transferred TILs in vivo is short (Rosenberg et al., 2008).

Evading tumour cells which have way low or no HLA antigens are nevertheless ideal targets for NK cells, because NK cells detect and kill tumor variants which lack MHC class I expression (Karre, 2008). In some trials, it was more effective to use alloreactive NK cells in cancer immunotherapy due to the mismatch between inhibitory receptors on NK cells and HLA on tumour cell surface (Ruggeri et al.,

2002). However, there are at least two hurdles to overcome before successful application of NK cells in ACT. Adoptive NK cell therapy requires large numbers of cells with a well-defined phenotype and high purity to be produced. This is difficult to achieve practically because the isolation, culture, and expansion of clinical NK cell products has been hampered by their relatively low representation in the blood. The other reason for this difficulty is that the in vitro proliferation potential of NK cells in IL-2 and IL-15 is limited (Carlens et al., 2001), despite some recent advances (Alici et al., 2008). In addition, the transferred NK cells must persist long enough to affect tumor killing (Robbins et al., 2004). Even with IL-2, however, transferred NK cells gradually lose killing ability in about 2 weeks (Miller et al., 2005) (Yee et al., 2002). Therefore, neither T cells nor NK cells are ideal sources for ACT.

5.1.2. Purposes of this chapter

In this chapter, I will characterize gene expression features of ITNKs and examine their capacity for killing tumour cells both in vitro and in vivo. The results from these experiments implicated ITNKs as a potentially new cellular product for ACT in cancer immunotherapy.

5.2. Results

5.2.1. Gene expression profile in ITNKs

In previous chapters, I showed that ITNKs ($NKp46^{+}CD3^{-}$) that were derived from *Bcl11b*-deficient DN3 thymocytes in vitro resembled NK cells, as they expressed NK cell surface markers, killed stromal cells, and grew in NK culture conditions. Another important criteria to judge the similarity between the two types of cells is to compare their gene expression profiles. Studies on gene expression profiles of ITNK cells could also help us dissect the molecular mechanisms involved in the

reprogramming from T cells to ITNKs. Therefore, I performed gene expression microarray using mRNA from DN3 thymocytes, normal splenic NK cells that were expanded in vitro after enrichment (lymphokine-activated killer, or LAK cells, composed of >90% NK cells), and DN3-derived ITNKs in microarray analysis. The heat map showed that the global expression profile of these ITNKs was much more similar to that of LAKs than to that of their parental DN3 thymocytes (Fig. 5.1). To quantify the similarity between ITNKs and LAKs, we conducted further analysis from the array data and identified 504 genes that were expressed at least two-fold higher in LAKs vs. DN3 thymocytes, and 366 genes in ITNKs vs. their parental DN3 thymocytes. 70% of these 366 genes in ITNKs were also found to be overexpressed in LAKs (Fig. 5.2).

Genes with differential expression levels between the parental DN3 thymocytes and ITNK cells were listed in Table 3. I selected some key T and NK genes from the list and validated their expression by performing qRT-PCR. The qRT-PCR results showed that the expression of many T lineage genes, such as *Notch1*, *Est1*, *Hes1*, *Gata3*, *Dtx1* and *Tcf1,* was decreased, while the expression of genes important in NK cells such as *Id2* (Boos et al., 2007), *Il2rb* (CD122), *Zfp105* (Chambers et al., 2007) and *Nfil3* (Gascoyne et al., 2009) was greatly upregulated in ITNKs, compared to their parental DN3 thymocytes (Fig. 5.3A). Among the upregulated genes in ITNKs, *Zbtb32* (Rog, Repressor of GATA), which prevents GATA3 from binding to DNA and regulates T cell activation, was highly expressed in ITNKs but absent in DN3 cells (Miaw et al., 2000; Omori et al., 2003). Expression of *Cdkn1c* (p57KIP2), a putative direct downstream target gene of Bcl11b (Topark-Ngarm et al., 2006), was barely detectable in DN3 thymocytes but drastically increased in ITNKs, confirming that Bcl11b suppresses its expression (Fig. 5.3B). These results thus collectively

demonstrated that Bcl11b was essential for maintaining the T cell expression profile and for suppressing NK cell-associated gene expression.

5.2.2. Characterization of ITNKs derived in vitro

Besides analysis of gene expression profiles, we found that ITNKs reprogrammed from single DN3 thymocytes were morphologically similar to LAK cells. ITNKs were larger in size and had larger cytoplasm than thymocytes (Fig. 5.4). Moreover, images from transmission electronic microscopy show that ITNKs had enlarged granules and showed evidence of high protein synthesis activity with abundant endoplasmic reticulum (Fig. 5.5).

To further examine NK features in ITNK cells, I examined the expression of more cell surface markers and genes that are expressed in NK cells. Flow cytometry analysis showed that ITNK cells derived from DN3 thymocytes in vitro expressed other NK genes such as NKG2A/C/E, Trail, perforin and IFN-γ, (Fig. 5.6, Table 4), but not some other key NK function genes, such as members of the Ly49 family (Fig. 5.7). Similarly, ITNK cells that were reprogrammed from DP thymocytes also expressed NKG2A/C/E, but did not express members of the Ly49 family (Fig. 5.8).

Recently, thymic NK cells that originate in the thymus were identified by expression of Gata3 and CD127 (Vosshenrich et al., 2006). DN3 thymocyte-derived ITNKs, however, did not express CD127 and were therefore unlikely to be related to thymic NK cells (Fig. 5.9). Interestingly, unlike conventional mature NK cells that are $CD27^{-}CD11b^{+}$, most DN3-derived ITNKs expressed CD27 but not CD11b, both of which are used to define maturity of NK cells (Colucci et al., 2003) (Fig. 5.10). These results showed the distinctions between ITNKs and normal NK cells.

5.2.3. Characterization of ITNKs derived in vivo

As I showed in Chapter 4, different T cell subsets reprogrammed to ITNKs in vivo in a cell-autonomous manner upon loss of Bcl11b. Flow cytometry analysis showed that these ITNKs expressed NK surface receptors such as members of Ly49 family including Ly49C/I and Ly49G2 (Fig. 5.11) (Table 4), which were absent on the in vitro reprogrammed ITNKs (Fig. 5.7 and Fig. 5.8). These results suggested that in vivo microenvironment could facilitate reprogrammed ITNK cells to acquire inhibitory NK-cell receptors.

The majority of ITNKs from the spleen and thymus of Tamoxifen-treated *flox/flox* mice retained expression of CD3 and TCRβ, suggesting that T cell identity was not completely lost after Bcl11b ablation. To further define the T cell identity of the in vivo DP thymocyte-derived ITNK cells, I analyzed the expression of several key T cell and NK cell-associated genes. Compared to wild type $CD8^+$ mature T cells, ITNK cells had lower expression of *Il7r*, *Tbx21* and *Cd8a*, which are all important for $CD8^+$ T cells (Fig. 5.12). For example, IL-7 receptor signaling is indispensable for survival of $CD8^+$ memory T cells (Carrio et al., 2007; Kaech et al., 2003). Tbx21 is essential for effector and memory $CD8^+$ T cells (Intlekofer et al., 2005; Szabo et al., 2002). In contrast, Zfp105 (Fig. 5.12), the NK-associated gene (Chambers et al., 2007) was upregulated in ITNK cells. These results showed that ITNK cells lost or decreased some key T cell genes expression and acquired expression of some NK specific gene, indicating a loss of T cell identity.

TCR signaling is a unique hallmark of T cells, which leads to increased intracellular calcium (Lewis, 2001). Therefore we measured TCR-mediated calcium signaling to assess whether TCR signaling was functional or not in ITNK cells derived in vivo. The results of intracellular calcium flux assays showed that the

calcium response in ITNKs was not as robust as T cells from the controls (cells from Tamoxifen-treated *flox/flox* or *flox/+* mice) (Fig. 5.13). Moreover, we noticed that even calcium response of T cells that did not express NKp46 but had lost Bcl11b from Tamoxifen-treated *flox/flox* was lower than that in Tamoxifen-treated *flox/+* mice (Fig. 5.13), further suggesting that Bcl11b was required for full TCR signaling. Taken together, these results indicate that TCR signaling was impaired, at least partially, in ITNK cells.

Besides the calcium flux assays, we also performed a proliferation assay to further analyze the TCR signaling in ITNKs as wide-type $CD8^+$ T cells blast and proliferate after being activated by CD3 and CD28 antibodies. Unlike wild-type $CD8^+$ T cells, in vivo derived ITNKs died rather than proliferated upon activation by CD3 and CD28 antibodies in vitro. Although more investigation is needed to fully address to the observed loss of T cell identity in ITNKs, we can conclude that ITNKs from mature T cells did not simply acquire expression of NK associated genes, rather reprogramming was also accompanied by loss of T cell genes and functions.

Primary NK cells can be expanded for up to 7-10 days as LAKs with supplement of IL-2 or IL-15. Beyond this time, LAKs gradually lose proliferation and killing abilities. When 10 million whole splenocytes from Tamoxifen-treated *flox/flox* mice were cultured in NK culture conditions, most splenocytes died in the first 3 days, reflecting the low percentages of ITNKs in splenocytes initially (Fig. 5.14). However, within 7 days, about 12 million $NKp46^+TCR\beta^+$ ITNKs were obtained, most of which also expressed NK1.1 and NKG2D (Fig. 5.15). The ex vivo expanded ITNKs continued proliferating for at least 3 weeks while still retaining their killing ability (Fig. 5.14). ITNKs thus have a longer lifespan and greater proliferation ability than normal NK cells. Because T cells normally have longer lifespan than NK

cells in culture, it is possible that some retained T cell features made the ITNKs have long lifespan in culture.

5.2.4. Killing ability of ITNK derived in vitro

As important cellular mediators of innate defense, NK cells efficiently kill some viral-infected and transformed tumor cells in a serial manner by releasing perforin, granzymes and IFN-γ to targets, and by expressing Fas ligand (CD178) and Trail on their cell surface (Colucci et al., 2003). Perforin and granzymes are major weapons of NK cells, and their killing ability by these means can be quantified using standard ^{51}Cr-release assays in which ^{51}Cr released by lysed targets is measured and correlated to killing activities of effectors. To measure the killing ability of reprogrammed ITNKs from DN3 thymocytes in vitro, Dr. Burke helped me to perform standard ^{51}Cr-release assays with three cell lines as targets: B16F10 melanoma (MHC-I low or negative) (Taniguchi et al., 1986), RMA lymphoma, which express MHC class I molecules, and RMA-S lymphoma (TAP-1-deficient variant), which have much reduced MHC class I presentation (Karre et al., 1986; Ljunggren and Karre, 1985). LAKs discriminate between MHC-class I positive and negative cells, sparing the former and killing the latter (Fig. 5.16). Similar to LAK cells, ITNKs selectively killed MHC-I negative B16F10 and RMA-S cells, but did not kill MHC-I positive RMA lymphoma cells (Fig. 5.16). Compared to LAKs, ITNKs derived in vitro appeared to have slightly lower killing potency.

5.2.5. Killing ability of ITNK derived in vivo

To assess the killing ability of in vivo reprogrammed ITNK cells, we expanded ex vivo ITNK cells that were reprogrammed from *Bcl11b*-deficient DP thymocytes in *Rag2*$^{-/-}$*Il2rγ*$^{-/-}$ mice in LAK culture conditions as described in Chapter 4 and

performed standard ^{51}Cr-release assays with three cell lines as targets: B16F10 melanoma, RMA lymphoma, and RMA-S lymphoma. Strikingly, these ITNK cells exhibited greatly elevated cytotoxic potential compared to in vitro reprogrammed ITNKs and LAKs against each of the target cells (Fig. 5.17). Moreover, unlike LAKs, these ITNKs killed RMA cells with almost the same efficiency as killing RMA-S cells (Fig. 5.17), despite expression of some inhibitory Ly49 receptors that recognize MHC-I. ITNK cells maintained their capacity to kill tumours even after extensive ex vivo expansion. Taken together, these results show that in vivo reprogrammed ITNK cells were potent killers of tumour cells regardless of MHC-I molecules expression on targets.

5.2.6. ITNKs prevent tumour expansion in vivo

Transplantable murine melanoma B16 cell lines are well-established models for studying experimental cancer therapies and NK cell tumour surveillance function (Gorelik et al., 1982). Intravenous injection of B16 cells into *Rag2*$^{-/-}$*Il2rγ*$^{-/-}$ mice leads to rapid formation of 'metastatic' foci on the lungs (Lakshmikanth et al., 2009). To investigate the tumour-killing ability of ITNK cells in vivo, we injected two million OHT-treated or -untreated DP thymocytes from *flox/flox* mice into *Rag2*$^{-/-}$*Il2rγ*$^{-/-}$ recipients to allow reprogramming of thymocytes to ITNKs in vivo. Two weeks later, each recipient was injected with 50,000 B16F10 melanoma cells (Fig. 5.18). Four weeks after the initial thymocyte transplantation, recipients were sacrificed and analyzed. Mice injected with PBS or with untreated DP thymocytes had about 200 metastatic foci in the lungs. In sharp contrast, mice injected with OHT-treated DP thymocytes had only about 20 tumour colonies on the lung (Fig. 5.19 and Fig. 5.20). Therefore ITNKs were potent killers of tumour cells in vivo and prevented cancer outgrowth.

5.3. Discussion

In this chapter, I demonstrated that the gene expression profiles of ITNKs were similar to that of LAKs, with upregulation of NK cell-associated genes and downregulation of T cell genes. Also, I reported that in vivo reprogrammed ITNK cells had more features of NK cell surface markers expression than in vitro derived ones. ITNKs that were derived from DP thymocytes in vivo showed more potent killing ability than LAKs and eliminated MHC-I positive tumour cells. Moreover, these ITNKs were capable of preventing progression of B16 melanoma tumour cell in vivo.

5.3.1. Potential targets of Bcl11b

The microarray analysis showed that ITNKs shared similar gene expression profiles with LAKs instead of their parental DN3 T cells. For example, NK-associated genes such as *Id2*, *Zfp105* and *Nfil3* were highly expressed while key T cell genes like *Notch1*, *Hes1* and *Gata3* were suppressed in ITNK cells. Among these genes, some are critical for T-cell or NK-cell transcription program. Thus expression changes of these genes (drivers) might result in the reprogramming from T cells to ITNKs. In contrast, expression changes of other genes (passengers) may simply be the outcome of the reprogramming. We do not know which genes are the drivers of this reprogramming from T cells to ITNKs, nor which are the passengers. Nfil3, which promotes the expressions of other NK-associated genes thus drives T cells to NK lineage (Gascoyne et al., 2009), might be one of the drivers of reprogramming after Bcl11b ablation. In contrast, the decrease of CD8 and CD3 expressions in ITNKs shown in qRT-PCR results might simply be caused by the loss of the T cell identity. To identify the drivers of the reprogramming from T cells to ITNKs here, we need to

probe the direct targets of Bcl11b by ChIP-seq experiments in the future (Kastner et al., 2010).

5.3.2. Differences between ITNKs derived in vitro and in vivo

There are at least three key differences between ITNK cells derived from in vitro and in vivo. Firstly, in vivo derived ITNK cells expressed several NK cell-associated surface markers such as Ly49C/I and Ly49G2, which were absent in ITNK cells reprogrammed from the same type of T cells in vitro. A lack of Ly49 family expression is also observed in regular NK cells that are derived from BM or thymic progenitors in OP9 stromal cell co-cultured system (Rolink et al., 2006; Vegh et al., 2010). Interestingly, NK cells produced from the same progenitors in vivo do express Ly49 family (Carotta et al., 2006). It was possible that in vivo microenvironment provided some cytokines and cell-cell interactions that are required for the induction of Ly49 family expression in NK cells to ITNKs derived in vivo and made them express members of Ly49 family.

In addition, in vivo ITNKs efficiently killed targets expressing MHC-I molecules, while in vitro derived ones did not. These results demonstrate that in vitro ITNKs lacking inhibitory Ly49 family could use other inhibitory receptors to recognize MHC-I molecules. In contrast, in vivo derived ITNKs killed target regardless to MHC-I molecules expression, though the killing activities for RMA line was lower than that for RMA-s. We thus predict that inhibitory receptors were functional but was overshadowed by activating signals triggered by tumour cells in the in vivo derived ITNK cells. Alternatively, the retained T cell recognition elements in these ITNKs might trigger the killing machinery to eliminate MHC-I positive tumor targets. In this case, however, we do not know whether T cell and NK cell

recognition elements triggered T cell and NK cell killing machinery separately or integrate the signals simultaneously in ITNKs at first.

The third difference is that in vivo derived ITNK cells had stronger killing ability than in vitro derived ITNKs did, possibly due to more activating receptors expression or stronger killing machinery in ITNKs derived in vivo.

Overall, in vivo derived ITNK did not lose self-tolerance, as *Rag2*$^{-/-}$*Il2rγ*$^{-/-}$ host mice that had been transplanted with *Bcl11b*-deficient DP T thymocytes did not show any abnormality such as autoimmune syndrome or inflammation, suggesting that reprogrammed ITNK cells were able to recognize host cells. Taken together, the in vivo microenvironment might condition ITNK cells to express functional inhibitory receptors and activating receptors, shaping them into potent killers with 'self-recognition' ability.

5.3.3. Application of ITNKs for tumor killing

We showed that ITNK cells could efficiently kill B16 melanoma cells and prevent cancer progression *in vivo*. However, several preliminary experiments must be performed before claiming that ITNKs have clinical potential to treat cancer. Firstly, due to the limitation of our current animal license, *Rag2*$^{-/-}$*Il2rγ*$^{-/-}$ mice with ITNK cells had to be killed within 14 days post injection of tumour cells in this study. Therefore we did not have the opportunity to know how long these ITNKs can protect hosts from tumour cells, which depends on two factors. One is the persistence of ITNKs in the host. The data presented in this study indicate that ITNKs derived from different types of T cells under different microenvironment had various lifetimes and proliferation potentials. The longer ITNKs can live in host, the better they can protect host from tumour attacks. The other factor is the ratio of ITNKs to tumour cells. The

killing assays show that effective clearance of tumour cells could be achieved when this ratio was high enough.

To demonstrate the clinical potential of ITNKs for cancer immunotherapy, we also need to better mimic the reality of cancer treatment. Unlike cancer vaccines, ITNKs will be transferred to cancer patients and be expected to kill tumour cells that have grown in situ. In this thesis, ITNK were already present before injection and establishment of B16 tumour cells, making them kill tumor cells more easily than in a therapeutic situation. Therefore we need to inject tumour cells into mice before transferring ITNKs into host mice.

Thirdly, we need to study whether ITNKs are able to kill other tumour cell lines in vivo besides B16 melanoma cells, to further explore the clinical application of ITNKs for different types of cancer.

Finally, to apply ITNKs to treat human cancer, we need to develop a platform to modulate BCL11B or its downstream targets in human T cells to enable the production of human ITNKs. These ITNKs have to be carefully characterized in vitro and in vivo for its safety and efficiency in different animal models before entering clinical trials. In summary, although the application of ITNK cells for cancer therapy is very promising, a lot of basic research is required.