Chapter 1

INTRODUCTION

1.1. Mouse as a genetic tool

1.1.1. A brief history

Lab mice are invaluable tools in modern biomedical research. Compared to other mammals, the mouse is currently the most commonly used mammalian model organism because of its short generation time, small size and prolificacy in breeding. As a pioneer mouse geneticist, William Ernest Castle carried out the first systematic analysis of Mendelian inheritance and genetic variation in mice (Snell and Reed, 1993). Another important milestone for the study of the mouse is the establishment of inbred mouse strains. A pure genetic background improves the reproducibility of experiments using different individuals from the same mouse strain because they are genetically identical. Currently, all the common inbred strains in the labs have been inbred for at least 80 generations since their original isolation; thus the genomes of all siblings are essentially identical (Silver, 1985). The inbred mouse strains provide invaluable platforms for studying the immune system and modeling human immune disease.

1.1.2. Genetic manipulation of the mouse genome

The biggest advantage of using the mouse to study the immune system and to model human disease is the availability of a range of genetic technologies. In 1981, several groups produced transgenic mice by injecting transgenic DNA into mouse

pronuclei (Brinster et al., 1981; Wagner et al., 1981a; Wagner et al., 1981b). DNA introduced into the mouse genome by this method results in stable integration of the transgene into the germline. This technology offers scientists opportunities to perform gain-of-function studies for specific genes in the mouse model. However, there are drawbacks in this technology. Firstly, the injected transgene usually inserts randomly into mouse genome, raising the possibility of disrupting other genes nearby. Secondly, because the nature of this technology is to overexpress injected DNA, it is not suitable to silence a particular gene for loss-of-function studies, which are the gold standard methods for studying gene function, although overexpression of dominant negative forms of some genes can sometimes achieve this purpose. In addition, the copy number of the transgene that integrates into the mouse genome varies, so it is difficult to control the expression level of the transgene (Hickman-Davis and Davis, 2006). Finally, except bacterial artificial chromosome (BAC) transgenes (Antoch et al., 1997), introduced transgenes usually are not large enough to contain all of the *cis*-acting elements that are required for fully regulated expression (Kleinjan and van Heyningen, 2005).

In 1981, pluripotent mouse embryonic stem cells (ES) were isolated from the inner cell mass of 3.5 days post-coitum (dpc) wild type mouse embryos (Evans and Kaufman, 1981; Martin, 1981). Later, it was demonstrated that these ES cells were able to contribute to the germline in chimera mice derived from ES cells, even after genome modification by retrovirus (Bradley et al., 1984; Robertson et al., 1986). Precise manipulation of the mouse genome was achieved by demonstrating that homologous recombination works efficiently in mouse ES cells (Capecchi, 1989; Koller and Smithies, 1989; Smithies et al., 1985; Thomas and Capecchi, 1986). Thus a combination of mouse ES cell manipulation and homologous recombination

technologies gave birth to ‘gene targeting’ and revolutionized mouse genetics. In the near future, it is feasible that all mouse genes will be inactivated individually and phenotypes can be studied in these knockout mice (Guan et al., 2010). These mutant mouse resources will likely shift the current biomedical research paradigm such that comprehensive analyses of genes in many biological processes, including immunity, can be performed.

1.1.3. Using the mouse to study immunity

Because of the similarity between human and the mouse genomes and their development and physiology, the mouse has been instrumental in elucidating key processes in the immune system and revealing molecular mechanisms of immune diseases (Lander et al., 2001; Venter et al., 2001; Waterston et al., 2002). In 1948, George D. Snell began defining and naming the H2 haplotypes, using inbred mouse strain such as DBP/2 and C57BL (Gorer, 1948; Snell, 1951). Later, Marianne and her colleagues derived an inbred mouse strain with spontaneous autoimmune haemolytic anemia and termed it as New Zealand black (NZB) (M. Bielschowsky, 1959). Mice from this strain died with hepatosplenomegaly and jaundice that were seen in human autoimmune haemolytic anemia (Casey, 1966). We learnt precious lessons about the development of the immune system from strains such as *nude*, *scid* (severe combined immune deficiency) and *lpr* with spontaneous mutations, besides NZB (Mak et al., 2001). To accelerate the mutation rate and increase the mutation spectrum, Russell and colleagues at Oak Ridege Laboratories introduced the ethylating chemical N-ethyl-N-nitrosourea (ENU) to produce spontaneous genetic mutations in mouse strains (Russell et al., 1979). These ENU-induced genetic mutant strains were used to reveal many genes regulating the immune system (Nelms and Goodnow, 2001). A drawback with this approach is that it is usually difficult to identify the mutations

causing the defects in these strains because the mutations are random and the mouse genome was, until recently, complicated to sequence. However, this problem will be solved in the future due to the development of next generation sequencing technology (Mardis, 2008).

Thanks to the advent of genetically engineered 'knockout' mice, targeted mutations have been introduced to many loci to study functions of these genes in the immunity. In 1990, Smithies' and Jaenisch's groups independently generated β2-microglobulin knockout mice in which expression of major histocompatibility complex (MHC) class I molecules was abolished. The mutant mice developed normally but had severely reduced numbers of $CD8^+$ cytotoxic T lymphocytes (CTLs), indicating that MHC class I molecules are required for the selection of MHC-class-I-restricted $CD8^+$ T cells and for antigen recognition by these cells, but not necessary for T cell development (Koller et al., 1990; Zijlstra et al., 1990). In 1991, mice deficient for the T-cell co-receptor, CD8, were generated in Mak's lab. This study demonstrated that CD8 is essential for the development of $CD8^+$ CTLs but not $CD4^+$ helper T cells (Fung-Leung et al., 1991). Moreover, mice that lack the heavy chain of IgM (μ chain) do not have B cells, showing the importance of this gene and B cell receptor (BCR) for B cell development (Kitamura et al., 1991).

1.2. Conditional knockout (CKO) mice

1.2.1. CKO technology and its application

Analysis of knockout mouse strains has provided fundamental insights into functions of the mouse genome. However, knockout lines of certain genes die *in utero* due to the important roles of these genes in embryos. Consequently, functions of these genes in adult mice cannot be investigated. To overcome this limitation, CKO

approaches have been developed to overcome the embryonic lethality problem and to investigate gene function temporally and spatially using the Cre-loxP system, special type of site-specific recombination (Betz et al., 1996; Glaser et al., 2005; Rajewsky et al., 1996). For example, germline deletion of tumour-suppressor genes *Brca1* and *Pten* leads to early embryonic lethality, the functions of which in T cells could only be demonstrated in CKO strains (Mak et al., 2000; Suzuki et al., 2001). Similarly, *Notch1* and *Gata3* were ablated in adult CKO mice, where T cell development was arrested in early stages, demonstrating the importance of these two genes for T cell development (Pai et al., 2003; Radtke et al., 1999). This could not have been possible in germline knockout mouse strains since *Notch1*- and *Gata3*-deficient mice die at embryonic stages.

Due to the significant differences between the mouse and human genomes, especially in the MHC, it is important to develop mouse models of human disease by replacing large segments of the mouse genome with the wile-type or mutant syntenic region of the human DNA sequence (Wallace et al., 2007). To this end, the Cre-loxP site-specific recombination system has also been used to engineer chromosome segments in mouse to produce humanized mice. For example, the humanized mouse strain where the mouse α globin locus has been replaced with human sequence encoding a mutated α globin locus recapitulates α thalassemia more accurately than the corresponding knock-in mutation in the mouse gene (Wallace et al., 2007).

The procedure for generating CKO mice is essentially the same regardless of the specific targeting strategy (Chan et al., 2007) (Fig. 1.1). Briefly, targeting vectors that contain DNA fragments homologous to the target locus, loxP sites and selectable markers, such as antibiotics resistant genes, are generated in *E. coli* at first. Subsequently, these vectors are transfected into mouse ES cells for homologous

recombination. After drug selection and PCR confirmation, correctly targeted ES cell colonies are identified by either Southern blot analysis or long-range PCR genotyping. These ES cells are expanded and injected into 3.5 dpc blastocysts, which are then re-implanted into the uteri of pseudopregnant surrogate females. Chimeras are subsequently selected from the resulting pups and mated with wild-type mice to check for germ-line transmission of the targeted allele in the F1 offspring. From the F1 offspring, both male and female heterozygotes are selected and mated to produce homozygotes, which are subsequently crossed to different Cre-expressing transgenic mice for temporal and spatial gene deletion. Depending on the nature of the promoter that drives Cre recombinase expression, deletion of targeted gene can occur in specific cells/tissue or in all cells, or at a specific developmental stage (Nagy, 2000).

1.2.2. Inducible Cre systems

It is critical to choose a well-characterized Cre transgenic line that allows the Cre-mediated excision of targeted genes. Consequently, promoters that are used to drive Cre expression need to be carefully validated for its expression levels and specificity to fulfill research purposes (Nagy, 2000). An important improvement to Cre transgenic technology was the development of inducible Cre systems that permit the control of Cre activation, thus allowing temporal deletion of the targeted genes. Currently, there are two widely used inducible Cre systems. One is the tetracycline (Tet)-dependent regulatory system (Tet-off or Tet-on) (Gossen and Bujard, 1992). In the Tet-off system, the tetracycline-controlled transactivator (tTA), a fusion protein binds to the tetracycline response element (TRE) and activates transcription of the target gene, in the absence of the inducer, doxycyclin (Dox) (Takahashi et al., 1986). In contrast, a reverse version of the Tet repressor protein (TetR) known as rtTA binds

the TRE and activates transcription of the target gene only in the presence of Dox in the Tet-on system (Triezenberg et al., 1988).

The other inducible Cre system is the Cre-ERT system, in which the chemical compounds, Tamoxifen (TAM) or its derivative 4-hydroxytamoxifen (OHT) are used to induce Cre expression (Metzger and Chambon, 2001). In the Cre-ERT system, the Cre recombinase is fused to a mutant form of ligand-binding domain of the human estrogen receptor (ER). Once this fusion protein binds to TAM or OHT, but not endogenous estrogen or progesterone, it translocates from cytoplasm to nucleus, activating Cre (Metzger and Chambon, 2001). This Cre-ERT system is widely used in immunology research. For example, using the Cre-ERT system, *Myc* (c-Myc) was also selectively deleted in B cells. The proliferation in response to anti-CD40 plus IL-4 in these *Myc*-deficient B cells was severely impaired, demonstrating the important function of Myc in proliferation and apoptosis in mature B cells (de Alboran et al., 2001). However, the induction of Cre expression by Tamoxifen or OHT is not efficient in some tissues such as the brain (Brocard et al., 1997). In this thesis, the Cre-ERT system was used to induce deletion of *Bcl11b* in T cells.

1.3. Lymphopoiesis

1.3.1. Haematopoiesis

Haematopoiesis is the production of blood cellular components, in which hematopoietic cells, including the red blood cells, platelets and white blood cells, are derived from the hematopoietic stem cells (HSCs) (Harrison, 1976). In the mouse, definitive haematopoiesis occurs at 8.0 days postconception (dpc 8) in the yolk sac blood islands, followed by the aorta-gonand-mesonephros (AGM) region, and then migrates to the fetal liver in the mouse embryos (Godin et al., 1995). After birth, the

place of haematopoiesis changes from the fetal liver to the bone marrow (BM), where a specialized microenvironment, the endosteal niche, forms to support haematopoiesis (Godin and Cumano, 2002).

HSCs are composed of long-term HSCs that self-renew for the life of the host, and short-term HSCs that retain self-renewal capacity for approximately 8 weeks (Morrison and Weissman, 1994). Short-term HSCs proliferate and differentiate into multipotent progenitors (MPP): common myeloid progenitors (CMP) and common lymphoid progenitors (CLP). CMP, the progenitors for the myeloerythroid lineages, give rise to granulocyte macrophage progenitors (GMPs) and megakaryocyte erythroid progenitors (MEPs) (Akashi et al., 1999) (Fig. 1.2). GMPs can differentiate towards neutrophils, monocytes, macrophages, eosinophils, basophils, and mast cells, while MEPs generate megakaryocytes and erythrocytes (Iwasaki and Akashi, 2007). In contrast, CLPs are restricted to give rise to T cells, B cells, natural killer (NK) cells, and some dendritic cells, though recent studies suggest that some progenitors derived from CLPs retain myeloid potentials (Bell and Bhandoola, 2008; Kondo et al., 1997; Manz et al., 2001; Wada et al., 2008). However, cell fate can be changed by overexpression or ablation of certain transcription factors in hematopoietic lineages (Graf and Enver, 2009).

1.3.2. B cell development

In the 1950s, the lymphocyte was identified as the cell responsible for both cellular and humoral immunity. Lymphocytes are mainly composed of B cells, T cells and NK cells. B cells and T cells are two arms of the adaptive immune system, which recognizes and has memory for specific pathogens, and subsequently mounts strong attacks each time the pathogen is encountered. B cells produce antibodies against antigens and perform the role of antigen-presenting cells.

In the fetal liver and the bone marrow, CLPs differentiate to B cells under regulation of external cytokines such as Fms-like tyrosine kinase receptor-3 (Flt-3) ligand and Interleukin-7 (IL-7) and endogenous transcription factors including E2A, Ebf1, Bcl11a, and Pax5 (Busslinger, 2004; Liu et al., 2003; Northrup and Allman, 2008). B220 and CD43 are chiefly used to identify pre-pro B cells, the earliest B cell lineage restricted progenitors, although B220 is also expressed in some NK cells (Hardy and Hayakawa, 2001). No, or very few, $B220^{+}CD43^{+}$ pre-pro B cells are detected in E2A, EBF1 or Bcl11a mutant mice (Bain et al., 1994; Lin and Grosschedl, 1995; Liu et al., 2003; Zhuang et al., 1994). B cell precursors commit to the B cell lineage by acquiring expression of *Pax5*, which drives CD19 expression in pro-B cells (Busslinger, 2004). Indeed B cell development is arrested at an early pro-B cells stage in *Pax5*-deficient mice (Urbanek et al., 1994). Furthermore, acute loss of Pax5 in pro-B cells causes B cells to lose B cell identity and reprogram to T cells (Mikkola et al., 2002; Rolink et al., 1999). Pro-B cells differentiate to pre-B cells when they start to express pre-B cell receptors (pre-BCR), which enables BCR signaling (Karasuyama et al., 1990; Karasuyama et al., 1993). Pre-B cells finally become mature B cells after they pass the BCR checkpoints (Hardy and Hayakawa, 2001) (Fig. 1.3).

1.3.3. T cell development

T cells were identified as a population of lymphocytes that are derived from the thymus and mediate cellular immunity by Glick (Glick, 1979). T cells recognize antigen peptides that are bound to MHC molecules via their T cell receptors. There are two main subpopulations of T cells: $CD4^{+}$ helper T and $CD8^{+}$ cytotoxic T cells. $CD4^{+}$ T cells recognize MHC II molecule complexes and secrete various cytokines after being activated. Conversely, $CD8^{+}$ T cells recognize MHC class I molecules and exhibit cell-killing activity after activation. AIDS provides a vivid and tragic

illustration of the importance of T cells in immunity. The human immunodeficiency virus (HIV), the causative agent of AIDS, binds to the CD4 molecules. And this infection causes depletion of $CD4^+$ T cells in Acquired immune deficiency syndrome (AIDS) patients. Without $CD4^+$ T cells, AIDS patients become hypersusceptible to pathogens that inhabit in tissues without much harming, and die of the opportunistic infections (Trono et al., 2010).

T cell development, which happens in the thymus, involves progenitor homing and lineage specification and commitment (Ciofani and Zuniga-Pflucker, 2007; Rothenberg et al., 2008). It also requires the intrathymic microenvironment and interactions among key transcription factors (Anderson et al., 2006; Ciofani and Zuniga-Pflucker, 2007; Rothenberg et al., 2008). In adult mice, CLPs migrate from the bone marrow to the thymus and initiate the program of T cell differentiation (Adolfsson et al., 2005; Yoshida et al., 2006). Progenitors in the thymus lack the T cell receptor (TCR) co-receptors CD4 and CD8, and are therefore refereed as double negative (DN) cells (Anderson et al., 1996). The DN population can be further subdivided by the cell surface markers CD117 (c-Kit), CD44, and CD25 (Godfrey et al., 1993). The $CD117^+$DN1 ($CD44^+CD25^-$) subsets, also known as early T cell precursors (ETP), are thought to contain multipotent progenitors (Allman et al., 2003; Bell and Bhandoola, 2008; Ikawa et al., 1999; Michie et al., 2000; Wada et al., 2008). T cell specification happens during the transition from ETP to DN2 ($CD44^+CD25^+$) with up-regulation of some key T cell genes, such as *Bcl11b*, *Tcf12* (HEBAlt), *Gata3* and *Notch1*, although NK and myeloid potentials still persist in DN2 cells (Bell and Bhandoola, 2008; Wada et al., 2008) (Taghon et al., 2006; Taghon et al., 2005; Yui and Rothenberg, 2004). ETPs and DN2 thymocytes initiate TCR gene rearrangements, however, they do not exhibit full V(D)J rearrangements or express

any TCRβ or TCRγδ on their cell surface, which are symbols of committed T cells. The non-T cell potentials are lost in the DN3 ($CD44^-CD25^+$) thymocytes. Some DN3 T cells successfully rearrange TCR γ- and δ-chains instead of β-chain and differentiate into γδ-T cells. The majority of T cells at DN3 stage commit to the αβ-T cell lineage by further upregulating key T cell genes and shutting down expression of genes that are important for non-T cell lineages (Rothenberg et al., 2008). DN4 ($CD44^-CD25^-$) thymocytes have undergone β-selection after successful TCRβ gene rearrangement (Dudley et al., 1994) and have already initiated the process of differentiating to the $CD4^+CD8^+$ double positive (DP) stage (Nikolic-Zugic and Moore, 1989; Petrie et al., 1990). Positive selection of the developing T-cell receptor repertoire occurs in the thymic cortex, whereas events in the medulla purge the system of self-reactive cells. Thymic epithelial-cell microenvironments are crucial for production of T cells and their selection and maintenance during immune responses (Anderson et al., 2006). After positive and negative selection processes, surviving thymocytes migrate to the peripheral lymphoid tissues where the cytokine IL-7 and the constant interaction of T cells with self-peptide MHC play a critical role in T cell maintenance (Takada and Jameson, 2009) (Fig. 1.4).

1.3.4. TCR signaling

TCR is expressed in T cells and is responsible for recognizing antigens bound to MHC molecules. Upon stimulation by antigens, TCR signaling is activated, promoting a number of signaling cascades that ultimately determine cell fate through the regulation of cytokine production, cell survival, proliferation, and differentiation (Marsland and Kopf, 2008). At the beginning of TCR activation, lymphocyte protein-tyrosine kinase (LCK) modulates the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) on the cytosolic side of the TCR/CD3 complex. ζ-

chain associated protein kinase (Zap-70) is next recruited to the TCR/CD3 complex and becomes activated. Following activation of Zap-70, its downstream scaffold proteins, including SLP-76, are recruited and phosphorylated. Phosphorylation of SLP-76 promotes recruitment of Vav, the adaptor proteins NCK and GADS, and an inducible T cell kinase (ITK) (Qi and August, 2007). ITK then modulate the phosphorylation of phospholipase C γ1 (PLCγ1), leading the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to produce the second messengers diacylglycerol (DAG) and inositol trisphosphate (IP3) (Burbach et al., 2007). IP3 triggers the release of $Ca2^{+}$ from endoplasmic reticulum, resulting in the entry of extracellular $Ca2^{+}$ into cells through calcium release-activated $Ca2^{+}$ (CRAC) channels (Cronin and Penninger, 2007). Thus calcium flux assays are commonly used to measure TCR activation.

1.3.5. NK cell development

The NK cell was first discovered by Rolf Kiessling in the 1970s, when he found a small population of large granular lymphocytes display cytotoxic activity against a wide range of tumor cells in the absence of any previous immunization with the tumor (Herberman et al., 1975; Kiessling et al., 1975). This population of cells were named "natural killer" because of the initial notion that they did not require prior activation to kill cells. It was later shown that NK cells preferably targeted cells, which expressed low levels of MHC class I molecules, a concept termed as "missing-self" recognition (Kiessling et al., 1978; Kiessling et al., 1976; Oldham, 1983). NK cells constitute an essential component of the innate immune system, which, unlike the adaptive immune system, recognizes and responds to pathogens without requiring prior priming through clonal antigen receptors. Mice with deficiencies in NK stimulatory immunoreceptors such as NKG2D and DNAM-1 are defective in tumor

surveillance in models of spontaneous malignancy (Guerra et al., 2008; Iguchi-Manaka et al., 2008).

NK cell development occurs mainly in BM, although a bipotent T/NK progenitor (TNKPs) containing T- and NK-cell potentials has been identified in mouse fetal liver and fetal thymus (Michie et al., 2000). In BM, CLPs commits to natural killer cell precursors (NKPs), which are defined as Lin^- (lineage)$CD122^+NK1.1^-DX5^-$ (Di Santo and Vosshenrich, 2006). Upon CD122 expression, NKPs lose B-, T- or myeloid-cell potentials and respond to IL-15 stimulation, which promotes NK cell development (Puzanov et al., 1996). Then, NKPs further differentiate into immature NK cells that express NK1.1 in bone marrow and liver (Kim et al., 2002; Takeda et al., 2005). CD94 (KLRD), a membrane protein, covalently associates with five different members of the NKG2 family, except NKG2D (Borrego et al., 2006; Chang et al., 1995). By acquiring expression of inhibitory NK-cell receptors including members of the Ly49 and CD94-NKG2 family, immature NK cells start to obtain NK-cell self-tolerance. and become mature NK cells that are positively stained for DX5 and CD11b. Mature NK cells shape their weapons by producing Fasl, Trail, Interferon-γ (IFN-γ), perforin and granzymes and circulate in the body as defenders through homeostasis (Colucci et al., 2003). Recently, a small population of NK cells that originate in the thymus was identified. These thymic NK cells express both GATA3 and CD127 (IL-7Rα) and have compromised cytotoxicity but considerable cytokine production (Vosshenrich et al., 2006).

1.3.6. NKT cell development

Natural killer T (NKT) cells are a subset of T cells that share some features with NK cells. The majority of NKT cells are defined as CD1d-restricted T cells that

express an invariant TCR combined with a limited, but not invariant, TCR β-chain repertoire (Godfrey et al., 2004). Different subsets of NKT cells have different functions in regulating immune responses. For example, $NK1.1^-$ NKT cells produce large amounts of IL-4 and little IFN-γ, while $NK1.1^+$ NKT cells produce less IL-4 and more IFN-γ (Benlagha et al., 2002; Pellicci et al., 2002). NKT cells arise in the thymus from a population of DP thymocytes that express a TCR which binds to CD1d plus self-lipid or glycolipid antigen (Bendelac, 1995). CD24, CD44 and NK1.1 are used to define NKT cells at different developmental stages (Benlagha et al., 2005). The earliest NKT cell precursors ($CD24^+CD44^{lo}NK1.1^{lo}$) differentiate to highly proliferative NKT progenitors, in which CD24 is later downregulated. These NKT progenitors subsequently expand the NKT cell pool and further differentiate to $CD24^{lo}CD44^{hi}NK1.1^{lo}$ population. Then, this population upregulates NK cell surface markers, such as NK1.1, and become potent producers of IFN-γ (Gadue and Stein, 2002).

1.4. Function of Bcl11b

1.4.1. Bcl11a

The B-cell lymphoma/leukemia 11 (*Bcl11*) family has two members, Bcl11a and Bcl11b, both being Kruppel-like C2H2 type zinc finger transcription factors (Satterwhite et al., 2001). *Bcl11a* was first discovered as a retroviral insertion site (*Evi9*) in myeloid leukemia tumours in the BXH-2 mouse (Nakamura et al., 1996) (Nakamura et al., 2000). *Evi9* was later re-named as *Bcl11a* since it was found ectopically expressed in some B cell lymphomas caused by chromosomal translocations (Satterwhite et al., 2001). Bcl11a is required for normal lymphoid development. Germline deletion of *Bcl11a* causes neonatal lethality and an absence of

B cells at the earliest B cell development stages (Liu et al., 2003). *Bcl11a* is also expressed in early T cell progenitors and is also important for T cell development (Li et al., 2010) (Tydell et al., 2007). Recipient mice of *Bcl11a*-deficient fetal liver cells develop T cell leukemia (Liu et al., 2003). Recent genome-wide association studies in human have revealed association of the *BCL11A* locus with persistent fetal hemoglobin in the adult (Menzel et al., 2007) (Uda et al., 2008). In the subsequent validation assays, knocking down BCL11A in human primary adult erythroid cells indeed leads to robust HbF expression (Sankaran et al., 2008). Further characterization of Bcl11a mutant mice also uncovers the key role of Bcl11a in the fetal to adult expression switch of hemoglobin (Sankaran et al., 2008); (Sankaran et al., 2009).

1.4.2. Bcl11b in leukemia

Bcl11b is the other member of the *Bcl11* family in the mouse and human genomes. The *Bcl11b* was initially identified as a tumour suppressor gene in T cells was thus named *Rit1* (*radiation-induced tumor suppressor gene 1*) because homozygous deletions and point mutations were located to this locus in a genome-wide allelic loss analysis of γ–ray induced mouse thymic lymphomas (Wakabayashi et al., 2003a) (Matsumoto et al., 1998) (Shinbo et al., 1999).

BCL11B is also found to be involved in human T cell leukemia. A specific cryptic translocation, t(5;14)(q35.1;32.2), present in about one fifth of T-cell adult leukemia/lymphoma (T-ALL) patients, serves to activate expression of HOX11L2 by juxtaposition with strong T cell enhancer elements at the 3' of the BCL11B locus (Bernard et al., 2001; MacLeod et al., 2003; Nagel et al., 2003). Additionally, a novel chromosomal aberration, inv(14)(q11.2q32.31) was reported in T-ALL samples. In this inversion, the 5' part of BCL11B, including exons 1-3, was joined to the TRDD3

segment of the TCRδ locus. Consequently, in-frame transcripts with truncated BCL11B and TCRδ constant region were highly expressed in screened T-ALLs but not in normal T cells (Przybylski et al., 2005). Interestingly, though *BCL11B* is considered as a tumour suppressor gene, it is highly expressed in many human T cell tumour lines and is required for their survival. Suppression of *BCL11B* by RNA interference (RNAi) causes apoptosis of these tumour cells, possibly due to a decrease of a cell-cycle inhibitor, p27, and an anti-apoptotic protein, BCL-xL. This indicates involvement of the mitochondrial apoptotic pathway. In contrast, normal mature T cells remained unaffected within the experimental time period (Grabarczyk et al., 2007; Kamimura et al., 2007a) (Karanam et al., 2010). Therefore, BCL11B could be an attractive therapeutic RNAi target in T-cell malignancies.

1.4.3. Bcl11b in early T cell development

Due to the involvement of Bcl11b in mouse thymic lymphoma development (Wakabayashi et al., 2003a), the Bcl11b germline knockout mouse strain was generated in 2003. Loss-of-function studies on Bcl11b in the mouse demonstrated that Bcl11b is required for early T cell development and the survival of T cells. The fact that Bcl11b homozygous mutant knockout mice die in the first few days after birth is likely due to neurological or other uncharacterized defects (Arlotta et al., 2005; Wakabayashi et al., 2003b) (Arlotta et al., 2008). In this *Bcl11b*-deficient strain, T cell development is blocked at the DN2-DN3 stage without obvious defects in other hematopoietic lineages (Wakabayashi et al., 2003b). In *Bcl11b*-deficient thymocytes, Vβ to Dβ rearrangements is impaired and contribute the lack of expression of pre-T cell receptors (pre-TCR) complex, which, in turn, leads to the profound apoptosis in the thymocytes. Apoptosis is unlikely to be the main reason for the failure of T cell development upon loss of Bcl11b, because inactivation of p53 in mutant thymocytes

is not sufficient to fully restore the T cell development, even though some immature single-positive (ISP) T cells are indeed detected in *Bcl11b*$^{-/-}$*p53*$^{-/-}$ embryos (Okazuka et al., 2005). The exact cause of T cell defects in *Bcl11b*-deficient mice thus remained unresolved.

Interestingly, Bcl11b haploinsufficiency was demonstrated in thymocyte development. Heterozygous Bcl11b mutant mice have fewer thymocytes compared to wild type mice. And these *Bcl11b*$^{+/-}$ thymocytes are much more prone to lymphomagenesis (Kamimura et al., 2007b). Moreover, in γ-irradiated mice, loss of one copy of *Bcl11b* is proposed to promote clonal expansion and differentiation arrest of thymocytes (Go et al., 2010). Nevertheless, it was puzzling that no tumour development has ever been reported in mice transplanted with *Bcl11b* homozygous knockout progenitors from fetal live.

1.4.4. Bcl11b in mature T cells

Bcl11b plays a critical role in DP thymocytes by controlling positive selection of both CD4 and CD8 lineages. *Bcl11b*-deficient DP thymocytes are prone to spontaneous apoptosis, possibly due to impaired proximal TCR signaling and attenuated extracellular signal-regulated kinase phosphorylation and calcium flux that are required for initiation of positive selection (Albu et al., 2007). Recently, it was reported that Bcl11b represses a gene expression program associated with mature CD4$^+$ and CD8$^+$ thymocytes, including *Zbtb7b* (Th-POK) and *Runx3* that are important for the development of mature CD4$^+$ and CD8$^+$ T cells, respectively (Dave et al., 1998; Kastner et al., 2010; Taniuchi et al., 2002). In human CD4$^+$ T cells, following activation through TCR, BCL11B promotes *IL2* expression by binding and activating the *IL2* promoter through the US1 site and by enhancing NFKB1 (NF-κB) activity (Cismasiu et al., 2009; Cismasiu et al., 2006). Downregulation of endogenous

BCL11B reduces the level of expression of IL-2, while overexpression of BCL11B augments IL-2 expression (Cismasiu et al., 2006). In $CD8^+$ T cells, Bcl11b plays a role in the antigen-specific clonal expansion and cytolytic effector function of (Zhang et al., 2010).

1.4.5. Bcl11b in other tissues

Besides the immune system, Bcl11b is also required in skin, neuron and tooth development (Arlotta et al., 2005; Arlotta et al., 2008; Golonzhka et al., 2007). *Bcl11b* is highly expressed in mouse skin during embryogenesis. In the developing epidermis at late stage of fetal development and in the adult skin, Bcl11b expression decreases and becomes restricted to the proliferating cells of the basal cell layer (Arlotta et al., 2005; Arlotta et al., 2008; Golonzhka et al., 2007). Further analysis indicates that a subset of skin stem cells may express Bcl11b (Golonzhka et al., 2007; Golonzhka et al., 2009a). Analysis of mice with gremline deletion of *Bcl11b* shows that Bcl11b is required in skin during development, particularly in keratinocyte proliferation and late differentiation events (Golonzhka et al., 2007; Golonzhka et al., 2009b). Similarly, BCL11B is expressed in human epidermis, and is linked to disease progression and/or maintenance in atopic dermatitis and allergic contact dermatitis patients (Ganguli-Indra et al., 2009).

Bcl11b is crucial for the development of corticospinal motor neurons axonal projections to the spinal cord in vivo (Arlotta et al., 2005). Bcl11b is also indispensable for striatal medium spiny neurons differentiation, striatal patch development, and the establishment of the cellular architecture of the striatum (Arlotta et al., 2008). In humans, BCL11B expression is maintained at high levels in normal adult striatum but significantly decreased in huntington disease (HD) cells. Furthermore, mutant huntington striatal neurons is sensitive to overexpression of

BCL11B, suggesting that sequestration and/or decreased expression of BCL11B is responsible for the deregulation of striatal gene expression and the specificity of pathology that are observed in HD (Desplats et al., 2008).

Bcl11b also participates in the regulation of epithelial cell differentiation during tooth morphogenesis and is highly expressed in ectodermic components of the developing tooth. *Bcl11b*-deficient mice show multiple defects at the bell stage and have abnormal incisors and molars (Golonzhka et al., 2009b).

Bcl11b is among the earliest known genes to be expressed specifically in the embryonic mammary placodes in the mouse. In mammary glands of adult mice, Bcl11b expression is predominantly restricted to basal cells and a small number of luminal progenitors. Deletion of *Bcl11b* in the virgin gland leads to precocious aleologenesis and a basal-to-luminal lineage switch in the basal cells. In contrast, transient overexpression of *Bcl11b* is sufficient to induce expression of basal cell specific genes in luminal cells. Thus, Bcl11b promotes and maintains basal identity, and suppresses the luminal lineage in the mammary gland (unpublished, Song Choon Lee).

1.4.6. Binding sites of Bcl11b

Bcl11b, also termed Ctip2 (COUP-TF interacting protein), was independently isolated for its ability to interact with all members of the chicken ovalbumin upstream promoter transcription factor (COUP-TF) subfamily of orphan nuclear receptors (Avram et al., 2000). COUP-TFs usually mediate transcriptional repression by recruiting nuclear receptor co-repressor (NCoR) and/or silencing mediator for retinoid and thyroid hormone receptor (SMRT) to the template. COUP-TF family members play important roles in pattern formation in the developing nervous systems of Xenopus and Drosophila (Avram et al., 2000; Mlodzik et al., 1990; van der Wees et

al., 1996). As a COUP-TF-interacting protein, Bcl11b mediates transcriptional repression when tethered to a promoter by interacting with a DNA binding protein, such as ARP1, which is a member of COUP-TF subfamily of orphan nuclear receptors (Avram et al., 2000). BCL11B also directly interact with two metastasis-associated proteins MTA1 and MTA2 (Cismasiu et al., 2005). In HEK293 cells, BCL11B recruits sirtuin 1 (SIRT1), a trichostatin-insensitive, nicotinamide-sensitive class III histone deacetylase, to the promoter region of a reporter gene template (Senawong et al., 2003; Senawong et al., 2005). In addition, BCL11B recruits histone deacetylase (HDAC)1 and HDAC2 to promote local histone H3 deacetylation at the HIV-1 promoter region (Marban et al., 2007).

Bcl11b regulates cell cycle by suppressing cyclin-dependent kinase inhibitors. Bcl11b cooperates with SUV39H1 and histone methylation to silence *Cdkn1a* (p21) (Cherrier et al., 2009), a major cell cycle regulator of the response to DNA damage senescence and tumor suppression (Bunz et al., 1998). Bcl11b also appears to repress another cyclin-dependent kinase inhibitor, *Cdkn1c* (p57KIP2) (Topark-Ngarm et al., 2006), which has the ability to associate with and inhibit the catalytic activity of a number of cylin-cdk complexes (Cunningham and Roussel, 2001).

1.5. Thesis projects

The work of this thesis is based on the following question: “What will happen to T cells after *Bcl11b* is acutely ablated?” To answer this question, I planned first to identify Bcl11b expression in the T cell compartment in the mouse, and then to characterize its functions at various stages of early T cell development, and in committed and mature T cells.

Currently, Bcl11b expression in T cells is derived primarily from RT-PCR analysis. Whilst being informative, it only measures Bcl11b expression in a

population of T cells at transcription levels but not protein levels. Therefore, the first goal of this project was to use a Bcl11b reporter mouse to probe Bcl11b expression in hematopoietic lineages, especially in different T cell subsets.

The second goal of this thesis was to study the functions of Bcl11b in T cells at different developmental stages and its role during the homeostasis of different T cell subpopulations. Transcription factors involving in T cell development have been extensively studied in the mouse (Carpenter and Bosselut, 2010; Rothenberg et al., 2008; Rothenberg and Taghon, 2005). However, unlike the requirement of Pax5 in B cells (Cobaleda et al., 2007; Mikkola et al., 2002), none of the characterized transcription factors had been found to be required to guard T cell identity against other hematopoietic lineage potentials. These earlier studies, and the available mouse genetic resources, in particular, the Bcl11b conditional knockout mouse and the Bcl11b reporter mouse strains prompted me to investigate the roles of Bcl11b in T cell.

This thesis is divided into two parts. In the first part, I have characterized the expression of Bcl11b in different T cell subsets and in other hematopoietic lineages using the *Bcl11b-tdTomato* knock-in reporter mouse. Then I will present data and analysis on Bcl11b loss-of-function studies in T cells at various developmental stages and of different T cell subsets. These functional studies also include generation and characterization of a novel type of cell derived from *Bcl11b*-deficient T cells.