Chapter 3

BCL11B EXPRESSION IN HEMATOPOIETIC LIENAGES

3.1. Introduction

3.1.1. Current knowledge of Bcl11b expression patterns

During evolution, a homolog of *Bcl11b* first appeared in cartilaginous fishes. In sea lampreys, a jawless vertebrate, expression of a *Bcl11b* ortholog is specifically detected in $VLRA^+$ cells that are similar to T lymphocytes in vertebrates, but not in $VLRB^+$ cells that are similar to B lymphocytes in vertebrates (Guo et al., 2009). In bony fish, the *Bcl11b* ortholog is expressed in the thymus and positively regulates *ccr9* expression, which encodes the receptor for ccl25, a novel chemokine expressed in thymic epithelium (Bajoghli et al., 2009). In both the mouse and human, *Bcl11b* is highly expressed in T cells (Bernard et al., 2001; Wakabayashi et al., 2003b). Gene expression studies indicate that expression of many genes important for T cell commitment starts to increase in the transition from DN1 to DN2, with Bcl11b being the most drastically upregulated transcription factor (Tydell et al., 2007).

3.1.2. Reporter Molecules in Genetically Engineered Mice

Reporter molecules are commonly used in transgenic mice to follow the in vivo gene expression patterns through all developmental stages of the life cycle in all

tissues. The most widely used reporter molecule is the β-galactosidease (β-gal) enzyme of *E. coli* encoded by the *lacZ* gene (Young et al., 1993). However, its staining process usually affects cell viability and sometimes introduces false positive signals in flow cytometry analysis (Abe et al., 1996). Green fluorescent protein (GFP) is another common reporter molecule used in transgenic mice. It is useful for the study of living cells since its expression can be assayed conveniently without any staining processes, though its sensitivity and potential toxicity need to be considered (Hadjantonakis et al., 1998; Huang et al., 2000). Tandem dimer Tomato (tdTomato) provides a useful alternative to enhanced green fluorescent protein (eGFP) for the simultaneous detection of fluorescent protein in histological sections together with fluorescence immunohistochemistry (IHC) (Morris et al., 2010; Shaner et al., 2004). TdTomato is a fluorescent protein with maximum excitation at 554 nm and maximum emission at 581 nm and was used to monitor metastatic progression in live animals (Shaner et al., 2004; Winnard et al., 2006).

3.1.3. Purposes of this chapter

Most Bcl11b expression profiles have been obtained from RT-PCR, northern blot analysis, or RNA anti-sense in situ hybridization. These methods are usually not sensitive enough to detect expression of *Bcl11b* at the single cell level. Using *E. coli lacZ* as a reporter in mice, expression of *Bcl11b* was detected in thymocytes at single cell level (Song Choon Lee, unpublished). However, *lacZ* staining process is time-consuming and can bring false positive signals into analysis by flow cytometry, especially for NK cells, because the substrate, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), are likely to react with enzymes in large granules that are abundant in NK cells. Additionally, Bcl11b expression in different hematopoietic lineages such as NK cell, NKT cell, thymic NK cells have not been characterized in

details using the lacZ mice. Therefore I investigated expression pattern of *Bcl11b* in hematopoietic lineage, mainly T cell subsets, using a *Bcl11b-tdTomato* knock-in reporter mouse line in this chapter. As a T cell gene, *Bcl11b* is highly expressed in all T cells throughout T cell development but absent in other hematopoietic cell lineages.

3.2. Results

3.2.1. Bcl11b expression in thymocytes

To determine Bcl11b expression in T cells at a single cell level, I analyzed *Bcl11b-tdTomato* knock-in mice ($Bcl11b^{TOM/+}$), which were generated by Juexuan Wang in our lab. In this mouse strain, the *tdTomato* cassette was inserted into the 3' untranslated region (UTR) of *Bcl11b*. Hence, the expression of *Bcl11b* can be conveniently studied by detecting fluorescent signals from tdTomato in flow cytometry (Fig. 3.1). The haematopoiesis in $Bcl11b^{TOM/+}$ mice was normal, as percentages of B cells (Fig. 3.2A), T cells (Fig. 3.2B) and myeloid lineage cells (Fig. 3.2C) were similar to that of wild type mice. In $Bcl11b^{TOM/+}$ mice, flow cytometry analysis showed that *Bcl11b* was expressed in more than 95% of CD4 and CD8 double positive (DP) thymocytes (Fig. 3.3A) and about 85% of CD8 single positive (SP) (Fig. 3.3B) and CD4 SP T thymocytes (Fig. 3.3C). These results are consistent with expression of *Bcl11b* obtained using RT-PCR and direct Bcl11b antibody staining (Cismasiu et al., 2006; Tydell et al., 2007), and also confirm that tdTomato signals faithfully recapitulate Bcl11b expression.

After depletion of DP and SP T cells, early T cell subsets were defined by expression of CD117, CD44, and CD25 in the lineage negative (Lin^-) thymocytes (Fig. 3.4A) (Rothenberg et al., 2008). FACS analysis showed that Bcl11b was absent in ETP (Fig. 3.4B), which are defined as $CD117^{++}$DN1 and have non-T-cell potentials

including NK, dendritic, and myeloid cell lineages (Rothenberg et al., 2008). Bcl11b was only expressed in about 50% of DN2a ($CD117^+$DN2) T thymocytes (Fig. 3.4D) which have lost dendritic cell potential, suggesting DN2a population is heterogeneous (Masuda et al., 2007). *Bcl11b* was expressed at relatively lower levels in $CD117^-$DN1 thymocytes (Fig. 3.4C), compared to its high expression in DN2b ($CD117^-$DN2) (Fig. 3.4E), DN3 (Fig. 3.4F) and DN4 T cells (Fig. 3.4G). The upregulation of Bcl11b during transition from ETP to DN2b suggests a critical role of Bcl11b in early T cell development (Masuda et al., 2007) (Tydell et al., 2007).

3.2.2. Bcl11b expression in mature T cells

T cells migrate from the thymus to secondary lymphoid organs such as the spleen and lymph nodes after maturation. *Bcl11b* was expressed in about 95% of $CD8^+$ and $CD4^+$ splenic T cells (Fig. 3.5A). In mature T cells, 20% of peripheral activated ($CD44^-CD62L^+$) $CD8^+$ T cells (Fig. 3.6A and 3.5B), and 11% of activated ($CD44^-CD62L^+$) $CD4^+$ T cells (Fig. 3.6A and 3.5C) had very low levels of *Bcl11b* expression. In contrast, more than 99% of naïve ($CD44^+CD62L^-$) $CD8^+$ and $CD4^+$ splenic T cells highly expressed Bcl11b (Fig. 3.5B and 3.5C). Consistent with FACS analysis, qRT-PCR using RNA from sorted T cells showed that Bcl11b expression in activated T cells was two folds lower than that in naïve T cells (Fig. 3.6B). Collectively, the results from both flow cytometry and qRT-PCR suggest that Bcl11b may participate in the regulation of T cell activation.

Unlike αβ-T cells, γδ-T cells are still found in the Bcl11b knockout fetal thymus, indicating that Bcl11b is dispensable for fetal γδ-T cells (Wakabayashi et al., 2003a). However, no studies have been reported about the expression and function of Bcl11b in adult γδ-T cells. Here, using $Bcl11b^{TOM/+}$ mice, *Bcl11b* expression was

detected in 90% of adult γδ-T thymocytes (Fig. 3.7A and 3.7B), suggesting that Bcl11b may also be important for adult γδ-T cells.

Natural killer T (NKT) cells are a subset of T cells that express NK cell surface markers such as NK1.1 and DX5 (Godfrey et al., 2004). The majority of NKT cells are CD1d-restricted and are stained positive for CD3 and CD1d dimer (Fig. 3.7C). 95% of CD1d-restricted NKT cells expressed Bcl11b (Fig. 3.7D), indicating that Bcl11b could play a role in NKT cells.

3.2.3. Bcl11b expression in other hematopoietic cells

NK cell development can be operationally divided into four different stages, which are usually defined using CD122, NK1.1, CD27 and CD11b (Fig. 3.8A) (Di Santo, 2006). During NK cell development, Bcl11b was only transiently expressed at low levels in some less mature ($NK1.1^+CD27^+CD11b^-$) NK cells but not in NK progenitors ($CD3^-CD122^+NK1.1^-$) or mature NK cells ($NK1.1^+CD27^-CD11b^+$) (Fig. 3.8B). In contrast, the majority of thymic NK cells, identified by CD127 and Gata3 expression (Vosshenrich et al., 2006), expressed Bcl11b (Fig. 3.9A and 3.9B). Therefore Bcl11b can be considered as an additional marker to distinguish thymic NK cells from regular NK cells that develop in BM.

In other hematopoietic lineages, Bc11b expression was not detected in B ($B220^+CD19^+$) or myeloid cells ($CD11b^+Gr\text{-}1^+$) (Fig. 3.10). Taken together, the T cell restricted expression of *Bcl11b* further suggests its importance in T cell lineage.

3.3. Discussion

In this chapter, I presented studies on the pattern and dynamics of *Bcl11b* expression in T cell, NK cell and other hematopoietic lineages using a *Bcl11b-tdTomato* knock-in reporter mouse. Without intracellular antibody staining, this

mouse strain accurately recapitulated Bcl11b expression at a single cell level. *Bcl11b* expression profiles in hematopoietic lineages showed that Bcl11b was a T cell specific transcription factor. In T cells, *Bcl11b* expression increased during T cell commitment while decreased after T cell activation, suggesting it may have multiple functions in T cell development and T cell-mediated immunity.

3.3.1. Bcl11b is T-cell specific

In the hematopoietic system, Bcl11b was absent in B cells, myeloid cells and most NK cells, but highly expressed in T cell lineages. Developmentally, its expression is tightly associated with T cell commitment. Early thymocytes, for example, start to express Bcl11b during the transition from ETP to DN2b, at a time when early thymocytes gradually lose non-T-cell potentials, implicating that Bcl11b plays an important role in this process. Bcl11b expression is maintained at high levels in T cells beyond DN2b stages (Tydell et al., 2007). Therefore, further experiments are required to study function of Bcl11b in T cells at different developmental stages. Additionally, using fluorescent tdTomato to mark Bcl11b, the *Bcl11b-tdTomato* knock-in reporter mouse is very useful to monitor T cell activities in vivo.

3.3.2. Advantages and pitfalls of Bcl11b-tdTomato knock-in mice

It is very helpful to use fluorescent protein knock-in mice to study expression profiles of transcription factors for the two reasons (Nolan et al., 1988). Firstly, the resolution of the gene expression profiles obtained from fluorescent protein reporter mice is at a single cell level. Secondly, the gene expression can be monitored real-time in live cells both in vitro and in vivo, as no fixation or antibody staining is

required. For example, cells that express the tagged gene can be conveniently observed by confocal microscope or sorted in flow cytometry.

Nevertheless, it is important to note that this approach has several pitfalls too. Firstly, it takes enormous time and resources to generate a fluorescent protein knock-in mouse line. Additionally, it is important to decide where to insert the fluorescent gene cassette because the insertion may affect the expression of the tagged gene or genes nearby. For example, in the *Bcl11b-tdTomato* knock-in mouse strain, the tdTomato cassette was inserted into the 3'UTR, where transcriptional and post-transcriptional regulatory machineries may bind, thus might affect *Bcl11b* expression. Moreover, genes encoding fluorescent proteins are separated with targeted genes by IRES in most cases which may interfere with the endogenous gene expression or protein translation (Mohrs et al., 2001). Finally, in the case of extensive post-transcriptional or post-translational regulation of Bcl11b, the tdTomato reporter might not recapitulate the true expression pattern of Bcl11b protein. To solve this problem, the endogenous gene locus maybe engineered so that it produces a fusion protein between the gene product and the fluorescent protein (Feltri et al., 1999; Telling et al., 1997). This approach, however, has its own shortcomings as the fusion protein may lose the activities of one or both proteins or change the original cellular localization of the tagged protein.