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Transcriptional Control of Lineage Differentiation in Immune Cells

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Preface

Insights into the regulation of immune cell lineage differentiation and specification as well as into the control of lineage integrity, stability, and plasticity are of fundamental importance to understand innate and adaptive immune responses. In this volume, leading experts provide an up-to-date and comprehensive overview of recent advances in transcriptional control mechanisms and transcription factor networks that regulate these processes in a variety of different immune cell lineages.

Tomokatsu Ikawa addresses the genetic and epigenetic control of early lymphocyte development, in particular the B versus T cell lineage decision. Ellmeier and Taniuchi discuss the role of BTB-zinc finger transcription factors during T cell development and in the regulation of T cell-mediated immunity. Kronenberg and Engel focus on the transcriptional control of the development and function of Va14*i* NKT cells. Huehn, Delacher and colleagues focus on regulatory T cells and review the current knowledge about their transcriptional control, while Zhu and Christie discuss transcriptional regulatory networks essential for CD4 T cell differentiation. Brady and Male summarize the transcriptional control of NK cell differentiation and function. Nutt, Pang and Carotta highlight recent advances in the transcriptional control of pre-B cell development and leukemia prevention. Diefenbach and Klose provide an overview about transcriptional regulation of innate lymphoid cell development and function, and Kaisho and Sasaki focus on transcriptional control mechanisms of dendritic cell differentiation. Finally, O'Shea, Bonelli and colleagues discuss helper T cell plasticity and the impact of extrinsic and intrinsic signals on transcriptomes and epigenomes.

Together, the reviews illustrate key transcriptional control mechanisms that regulate the development and function of immune cells. The reviews demonstrate the impressive knowledge gained during the last decade but also remind us that there are still many open questions that have to be addressed in order to understand the transcriptional regulatory circuits underlying innate and adaptive immunity.

Wilfried Ellmeier
Ichiro Taniuchi

Contents

Genetic and Epigenetic Control of Early Lymphocyte Development

Tomokatsu Ikawa

Abstract T, B, and NK lymphocytes are generated from pluripotent hematopoietic stem cells through a successive series of lineage restriction processes. Many regulatory components, such as transcription factors, cytokines/cytokine receptors, and signal transduction molecules orchestrate cell fate specification and determination. In particular, transcription factors play a key role in regulating lineage-associated gene programs. Recent findings suggest the involvement of epigenetic factors in the maintenance of cell fate. Here, we review the early developmental events during lymphocyte lineage determination, focusing on the transcriptional networks and epigenetic regulation. Finally, we also discuss the developmental relationship between acquired and innate lymphoid cells.

Contents

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1 Introduction

Lymphocytes consist of the T and B cells of the adaptive immune system as well as natural killer (NK) cells, lymphoid tissue inducing (LTi) cells, and natural helper (NH) cells, which have recently been defined as a new subset termed innate lymphoid cells (ILCs). All of these lymphocytes are derived from hematopoietic stem cells (HSCs) found in fetal liver before birth and in bone marrow (BM) after birth, although the developmental pathways and the molecular mechanisms of their diversification processes are still not completely understood. The lymphocytes are generated through a gain of specific gene expression signatures mediated by a combinatorial network of transcription factors. This is accompanied by the gradual loss of differentiation potential for alternative cell lineages (Yang et al. 2010; Mandel and Grosschedl 2010) (Fig. 1). Lineage-specific transcription factors play pivotal roles in the lineage restriction process. These transcription factors activate lineage-specific genes to promote lineage development, while simultaneously restricting the developmental potential of multipotent progenitors to the lymphoid pathway by suppressing the lineage-inappropriate genes, which are associated with other differentiation pathways. Recent findings also suggest that the downregulation of stem cell-associated genes by transcription factors is also important for lineage restriction programs (Nutt and Kee 2007; Rothenberg et al. 2008). Many transcription factors have been shown to be critical for the generation of lymphoid cells. However, how these transcription factors work together through regulatory networks and how the specific combinations of transcription factors within the networks synergize or antagonize each other are yet to be defined. In this review, we focus on the transcriptional networks and the epigenetic mechanisms that orchestrate lymphoid cell genesis and provide an overview of current models of the lymphoid lineage commitment process during immune cell development.

2 Generation of T and B Cell Progenitors in BM

Many kinds of BM progenitors including HSCs and the downstream progenitors with T or B cell potential have been identified (Fig. 1). The earliest progenitors can be isolated based on the absence of lineage markers (Lin^-) in combination with high expression of c-kit and Sca-1 (LSK cells) (Ikuta and Weissman 1992;

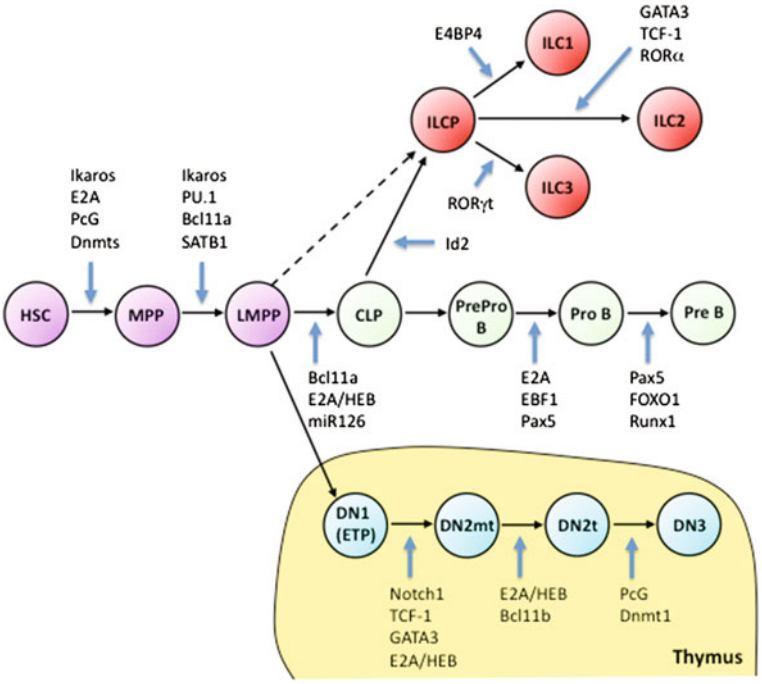

Fig. 1 Schematic model of early lymphocyte development. Multipotent progenitors (*purple*) give rise to T cell (*blue*), B cell (*green*), and ILC (*red*) progenitors during specification to each lymphoid lineage. Transcription factors and epigenetic factors which are thought to be involved in cell fate specification and determination are indicated. Common innate lymphoid progenitors (ILCP) are still speculative, although Id2 is a master regulator of ILC development

Spangrude et al. 1988). This population can be further subdivided into at least three populations based on surface expression of CD34 (Osawa et al. 1996; Yamamoto et al. 2013) and fms-like tyrosine kinase receptor-3 (Flt-3) (Christensen and Weissman 2001; Adolfsson et al. 2001). $CD34^{-}Flt\text{-}3^{-}$ LSK cells are defined as long-term repopulating HSCs that retain self-renewal activity and have the capacity to generate all blood lineages, including T and B cells. The immediate downstream progenitors of HSCs are the $CD34^{+}Flt\text{-}3^{-}$ multipotent progenitors (MPPs) that have lost the ability to self-renew, but retain multilineage differentiation capability (Yang et al. 2005). $CD34^{+}Flt\text{-}3^{+}$ LSK cells are classified as lymphoid-primed MPPs (LMPPs), because they have lost megakaryocyte /erythroid potential and are biased toward lymphoid and granulocyte /macrophage lineages (Adolfsson et al. 2005). LMPPs contain lymphoid restricted cells, early lymphoid progenitors (ELPs), defined using reporter mice by expression of Rag1-GFP (Igarashi et al. 2002; Schwarz et al. 2007). Further downstream progenitors that retain T and B cell potential are termed common lymphoid progenitors (CLPs) (Kondo et al. 1997). CLPs were originally thought to be exclusively committed to T and B lineages, but are now suggested to be a rather heterogeneous population in terms of their

differentiation potential. The Ly6D surface marker can separate the CLP population into two populations, the Ly6D$^-$ progenitors with B cell, T cell, NK cell, and dendritic cell (DC) potential and the Ly6D$^+$ progenitors, which are largely restricted to the B cell lineage (Inlay et al. 2009). Fate mapping analysis also suggests that CLPs with a history of λ5 and Rag-1 expression are largely restricted to the B cell lineage (Mansson et al. 2010). Furthermore, as described below, the earliest lymphoid progenitors in the thymus retain limited B cell potential compared to the T or myeloid lineages (Wada et al. 2008; Kawamoto et al. 2010). Taken together, these results suggest that the CLP may not be the main source of thymic immigrants that contribute to the generation of T cells.

The first identifiable B cell-specific progenitors in BM are the Pre-Pro B cells or fraction A, which have the cell surface phenotype IgM$^-$B220$^+$CD43$^+$CD19$^-$ HSA$^-$BP1$^-$ (Hardy et al. 1991; Li et al. 1996). Pre-Pro B cells are specified but not totally committed to the B cell lineage. Cells committed to this lineage are called Pro B cells, which are identified by the expression of B220, CD43, and CD19.

3 Transcriptional Regulation During T Cell Lineage Commitment

3.1 T and Myeloid Potential are Closely Associated in Early Thymic Progenitors

Thymocytes can be separated into four populations based on the expression of CD4 and CD8. The CD4$^-$CD8$^-$ double negative (DN) stage is the most immature stage among these four populations. DN cells develop into CD4$^+$CD8$^+$ double positive (DP) cells and finally mature into CD4$^+$CD8$^-$ single positive (CD4SP) or CD4$^-$CD8$^+$ (CD8SP) cells. DN cells can be further subdivided into four populations based on the surface expression of CD44 and CD25: CD44$^+$CD25$^+$ (DN1), CD44$^+$CD25$^+$ (DN2), CD44$^-$CD25$^+$ (DN3), and CD44$^-$CD25$^-$ (DN4) (Godfrey et al. 1993), and differentiation progresses in this order (Fig. 2). Previous work demonstrated that the c-kit$^+$ cells among DN1 cells, also called early thymic progenitors (ETPs), were the most immature progenitors in the thymus (Matsuzaki et al. 1993; Allman et al. 2003; Wada et al. 2008). ETPs are not committed to the T cell lineage but maintain the potential to generate T cells, NK cells, myeloid cells, DCs, and B cells. However, we and other groups demonstrated that most of the earliest T cell progenitors in the DN1 subset have already lost B cell potential (Wada et al. 2008; Bell and Bhandoola 2008; Lu et al. 2005), moreover, the overall frequency of cells with B cell potential is much lower than that of cells with T or myeloid potential. To simultaneously assess myeloid and T cell potential, we developed a new culture system using TSt-4/DLL1 stromal cells, which ectopically express a Notch ligand DLL1. By culturing individual DN1 cells, we found that a substantial proportion of these thymic T cell progenitors retain myeloid

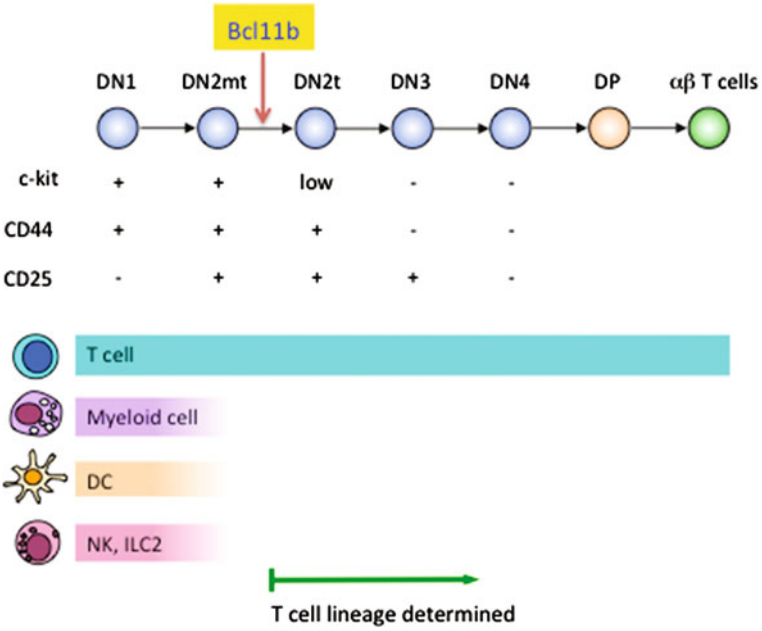

Fig. 2 Schematic model of T cell lineage commitment in the thymus. Non-T lineage potential including myeloid, DC, NK, and ILC2 lineages is terminated at the DN2mt to DN2t stage. Bcl11b is required for this checkpoint

potential. This observation was confirmed by other groups (Bell and Bhandoola 2008; Porritt et al. 2004; Balciunaite et al. 2005), who also reported that the T and myeloid lineages, but not the B cell lineage, are closely related to each other in the earliest T cell progenitors in adult thymus. Myeloid potential in the T cell progenitors was challenged by studies using fate mapping analysis with an IL7Rα-Cre driven reporter mice (Schlenner et al. 2010). These authors suggested a distinct origin of T and myeloid lineages in vivo based on the observation of a lower frequency of labeled myeloid cells than T cells in the thymus. However, 10–20 % of the macrophages and neutrophils in the thymus were marked by the history of IL-7R expression in this assay system. This observation is consistent with the previous report using a different in vivo system demonstrating that approximately 30 % of the macrophages are derived from early thymic T lineage progenitors in the steady state (Wada et al. 2008). Taken together, these results demonstrated that the T cell progenitors retain myeloid potential after terminating B cell potential, indicating that the CLPs are not the intermediate progenitors during T cell development.

3.2 Termination of Myeloid Potential in T Cell Progenitors

Since the earliest T cell progenitors in the thymus retain substantial myeloid potential, we wished to know when the myeloid potential is terminated. Using plckGFP Tg mice, in which GFP expression is regulated by the lck promoter, we have previously shown that DN2 cells can be separated into GFP^- and GFP^+ population (Masuda et al. 2007). GFP^-DN2 cells retain myeloid cell, DC, and NK cell potential, while GFP^+DN2 cells are restricted to the T lineage. Consistent with this observation, the expression of T lineage-associated genes, GATA3, CD3ε, and pre-Tα was upregulated in cells at the GFP^+DN2 stage and, conversely, the myeloid transcription factor PU.1 was downregulated in these cells. We named GFP^-DN2 cells as DN2mt (myeloid-T) and GFP^+DN2 cells as DN2t (T lineage-committed) cells. Rothenberg et al. proposed a similar separation of DN2 cells based on the expression level of c-kit into $ckit^{high}CD25^+$DN2a and $ckit^{low}CD25^+$DN2b cells (Rothenberg et al. 2008). Global gene expression analysis comparing these two populations suggests that DN2a precedes DN2b. These results indicate that the non-T lineage potential, myeloid and NK potential, of T cell progenitors is lost at the DN2 stage (Fig. 2).

To investigate the mechanism underlying termination of myeloid and NK potential, we have developed a novel T cell differentiation culture system (Ikawa et al. 2010). When murine hematopoietic progenitors were cultured on immobilized Notch ligand DLL4 in the presence of cytokines including IL-7, progenitors became arrested at the DN2mt stage. The arrested cells maintained an enormous proliferation capacity and differentiation potential to generate T cells as well as NK and myeloid lineage cells. Interestingly, withdrawal of IL-7 allowed the cells to commit to the T lineage, which could be recognized by the expression of plckGFP. Moreover, the committed T cell progenitors differentiated into DP cells under the same culture conditions. These results indicate that T cell differentiation can be induced in a stromal-free culture system by simply reducing the concentration of IL-7 in the middle of the culture period. Similar developmental arrest was observed in the thymus of mice lacking the transcription factor Bcl11b. The DN2 cells in Bcl11b-deficient mice can be propagated on TSt-4/DLL1 cells, retaining the potential to generate myeloid and NK lineage cells even after 1 month. Overexpression of Bcl11b in these cultured DN2 cells promoted the generation of DN3 cells, indicating the Bcl11b plays pivotal roles in T lineage determination (Fig. 2). Critical roles of Bcl11b in this T lineage checkpoint have also been demonstrated by other groups (Li et al. 2010a, b).

3.3 Transcriptional Regulation that Determines T Cell Fate

Many transcription factors including Bcl11b have been shown to be important for the T cell fate choice during the lineage determination process (Yang et al. 2010; Rothenberg 2012). These transcription factors function at different stages of T cell

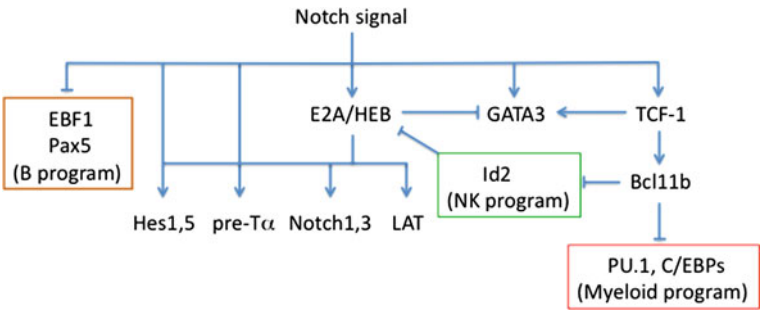

Fig. 3 Transcriptional network during T cell lineage commitment. Notch signals from the thymic environment initiate the T lineage program. E2A/HEB act in synergy with Notch to promote T cell commitment. TCF-1 activates Bcl11b, which in turn suppresses the other lineage programs, such as myeloid and NK lineages

development and play different roles during T lineage commitment by coordinating the gene expression program (Fig. 3).

T cell factor 1 (TCF-1, encoded by the transcription factor 7 gene, *Tcf7*) is an essential transcription factor in early T cell differentiation (Verbeek et al. 1995; Hattori et al. 1996; Schilham et al. 1998). TCF-1 and its partner LEF-1 act as a transducer of Wnt pathway signals (Staal et al. 2008; Staal and Sen 2008). TCF1-deficient mice were originally shown to have a reduced number of thymocytes and a partial block of T cell development at the immature single positive (ISP) to DP stage (Schilham et al. 1998). However, recent findings suggest that TCF-1 is essential at the earliest stage of T cell specification in the thymus (Weber et al. 2011; Yu et al. 2012). These studies show that the expression of *Tcf7* is directly activated by Notch signals and that TCF-1 in turn activates T lineage gene program including Bcl11b and GATA3. Forced expression of TCF-1 can bypass most of the requirement for Notch signaling and promote T lineage specification even without Notch signals in vitro. Interestingly, TCF-1 is also shown to be required for group 2 innate lymphoid cell (ILC2) development through GATA3-dependent and -independent pathways (Yang et al. 2013). ILC2 cells are innate lymphocytes that produce T helper type 2 (Th2) cell-associated cytokines interleukin (IL)-5 and IL-13 and mediate airway inflammation and helminth infection. TCF1-deficient mice lack ILC2 in the lung and BM. By using retroviral dominant-negative Mastermind (dnMAML), a pan Notch inhibitor, these investigators confirmed that the Notch signaling is required for ILC2 generation. Transduction of *Tcf7* retrovirus partially restored the generation of ILC2 from dnMAML–expressing BM progenitors, indicating the TCF-1 acts downstream of Notch signaling during ILC2 development, similar to early T cell development. Thus, transcriptional regulatory elements that underlie early T cell development also induce the generation of ILC2, suggesting a close developmental relationship between T cells and innate lymphoid cells.

GATA3 is a zinc finger transcription factor that has also long been recognized as an important factor for the development of T cells at multiple checkpoints (Ting et al. 1996). GATA3 is indispensable for T cell development during the DN3 stage and for the generation of CD4SP cells (Pai et al. 2003). In addition, GATA3 is a master regulator of Th2 cell function (Zheng and Flavell 1997; Zhu et al. 2004). Several findings have demonstrated the critical importance of GATA3 for early T cell development (Hattori et al. 1996; Taghon et al. 2007). The expression of GATA3 initiates at the DN1 stage and is sustained throughout T cell development. Hosoya et al. have recently shown that GATA3 plays an essential role in DN1 cell generation (Hosoya et al. 2009). By contrast, normal numbers of prethymic progenitors, LMPPs and CLPs, were observed in GATA3-deficient fetal livers, indicating the critical role of GATA3 for the development of early thymic T cell progenitors.

E proteins are helix-loop-helix transcription factors that are essential for T and B lymphocyte development (de Pooter and Kee 2010). There are four E proteins in mammals, E2A (E12 and E47, encoded by the *Tcf3* gene), HEB (*Tcf12*), and E2-2 (*Tcf4*) (Massari and Murre 2000). They function as homodimers or heterodimers in regulating differentiation at various stages of lymphocyte development. E2A/HEB heterodimers especially are required for early T cell development. Loss of E2A or HEB results in a partial block of early T cell development at the DN1 or ISP stages. Deletion of both E2A and HEB leads to a severe block at the DN stage, prior to pre-TCR expression, indicating the cooperation of these proteins for normal T cell development in the thymus. Association of E2A with other critical factors has also been shown. E2A is required to limit GATA3 expression specifically at the DN2 stage (Xu et al. 2013), thus elevated expression of GATA3 is seen in E2A-deficient DN2 cells. Ectopic GATA3 siRNA expression restores the DN3-like cell development by E2A-deficient MPPs, indicating that suppression of GATA3 by E2A is required for proper T cell development. E2A has also been shown to cooperate with Notch signaling to promote early T lineage commitment (Ikawa et al. 2006). Expression of several Notch-related genes, Notch1, Notch3, Hes1, and Deltex1 are directly regulated by E2A and once the Notch genes are activated, E2A and Notch act in synergy to promote T cell development. E2A continuously regulates the expression of Notch1 until the DN3 stage (Yashiro-Ohtani et al. 2009). E2A also plays critical roles in T cell receptor (TCR) β gene rearrangement and allelic exclusion (Agata et al. 2007). In addition to its essential role in intrathymic development, E2A is also known to be important for normal development of prethymic progenitors. LMPPs, which can be identified in the LSK compartment by surface expression of Flt3, are severely reduced in the absence of E2A (Dias et al. 2008; Semerad et al. 2009). E2A-deficient LMPPs show reduced expression of lymphoid-related genes, Notch1, Rag1, and CCR9. The combined activities of E2A and HEB are required for the induction of forkhead box O1 (FOXO1) expression at the CLP stage (Welinder et al. 2011). Together, these results suggest that E proteins play critical roles in both prethymic and intrathymic T cell development by inducing different combinatorial targets.

4 Transcriptional Regulation of B Cell Lineage Commitment

The process of commitment to the B cell lineage is also dependent on a network of transcription factors (Nutt and Kee 2007; Mandel and Grosschedl 2010). Gene targeting studies have demonstrated that several transcription factors, including Ikaros, PU.1, LRF, Bcl11a, Runx1, E2A, EBF1, and Pax5 play critical roles in inducing B lineage specification and determination, although most of the transcription factors except EBF1 and Pax5 are also essential for the development of hematopoietic stem/progenitors, T cells or myeloid cells. Moreover, while it was initially thought that these factors play different functions at different developmental stages, it has recently become clearer that these transcription factors act in a complex network to regulate the cell fate choices in a synergistic or antagonistic manner.

PU.1 is one of the key players in the gene regulatory networks controlling cell fate determination of lymphoid versus myeloid lineages, probably at the LMPP stage (de Koter and Singh 2000; Kueh et al. 2013). It has been suggested that the cell fate choice critically depends on PU.1 levels. The downregulation of PU.1 is required for B cell development, whereas higher levels favor the development of myeloid lineage cells. Recent findings suggest that the cell cycle status plays a critical role for inducing positive PU.1 feedback loops, which may lead to the myeloid fate choice (Kueh et al. 2013).

LRF (leukemia/lymphoma-related factor) has been suggested to act upstream of E2A and EBF1. LRF is a POZ and Kruppel (POK)-type transcription factor with multiple functions in hematopoietic/immune systems (Lunardi et al. 2013). Notably, inactivation of LRF promotes T cell fate at the expense of B cell development, suggesting that LRF regulates the Notch-mediated lineage decision (Maeda et al. 2007). However, a recent study demonstrated that the promotion of T lineage cells in BM of LRF-deficient mice was due to the aberrant expression of DLL4 in erythroblasts, suggesting that LRF does not directly regulate T or B lineage fate choices under physiological conditions (Lee et al. 2013).

Bcl11a also functions upstream of E2A and EBF1 and is known to regulate lymphoid and erythroid development (Durum 2003). Loss of Bcl11a results in a lack of B cells and impaired thymocyte maturation (Liu et al. 2003). Recent findings also demonstrated a critical role of Bcl11a for pDC development by regulating Flt3 expression. The numbers of LSK cells and CLPs were greatly reduced in both fetal liver and BM of Bcl11a-deficient mice. In addition, expression of Flt3 and IL-7R was reduced in MPPs in BM of Bcl11a-deficient mice, suggesting a critical role of Bcl11a in the development and maintenance of LMPPs and CLPs (Wu et al. 2013). This observation is intriguing in that Bcl11a shares many features with another Kruppel-like transcription factor, Bcl11b, which is critical for T lineage commitment as described above. Bcl11a and Bcl11b may function in an antagonistic manner to regulate T or B lineage development. It would also be interesting to

clarify the relationship between Bcl11a and other transcription factors, Ikaros and PU.1, which play critical roles at similar developmental stages.

As noted above, E proteins (E2A, HEB, and E2-2) are required for maintaining HSC pools and promoting the development of lymphoid progenitors (Dias et al. 2008; Semerad et al. 2009). The E2A proteins act upstream and in concert with EBF1 and Pax5 to promote specification and determination to the B cell lineage. E2A and HEB cooperatively induce the expression of FOXO1 at the CLP stage (Welinder et al. 2011). A requirement of E2A for commitment to the B lineage was also shown by establishment of long-term cultured multipotent progenitors derived from E2A-deficient BM (Ikawa et al. 2004). E2A-deficient hematopoietic progenitors (HPCs) can efficiently grow on S17 stromal cells in the presence of SCF, IL-7, and Flt3-Ligand, and the cells maintain the potential to generate T cells, NK cells, myeloid cells, DCs, and erythroid cells. Enforced expression of E47 in the E2A-deficient HPCs directly activates the transcription of a subset of B cell-specific genes, including $\lambda 5$, mb-1, and Pax5. By contrast, E47 inhibits the expression of genes involved in the differentiation of other lineages, TCF-1 and GATA1. These results indicate that E2A plays a critical role in B lineage commitment.

Once E2A activates EBF1, E2A and EBF1 act in concert to promote B cell development. EBF1 is a crucial factor in the regulatory circuitry that underlies B cell specification. EBF1 can restore the ability of PU.1-, Ikaros-, E2A-, and Pax5-deficient HPCs to differentiate into pro B cells that are committed to the B lineage and carry rearrangements of their immunoglobulin heavy-chain loci (IgH) (Seet et al. 2004; Kikuchi et al. 2005; Medina et al. 2004; Reynaud et al. 2008; Pongubala et al. 2008). Inactivation of EBF1 results in the complete block of B cell differentiation at the pre-pro B cell stage. Interestingly, EBF1-deficient HPCs also retain alternative lineage potential. The committed pro B cells with conditionally deleted Ebf1 acquire the potential to generate T cells and ILCs (Nechanitzky et al. 2013). Derepression of genes regulating T cell and ILC development, such as Id2 and TCF-1, was observed in the EBF1-deficient HPCs, suggesting an essential role of EBF1 in preventing the conversion to ILC or T cell fates by directly suppressing the expression of these target genes.

Pax5 has been also recognized as a master regulator of B cell commitment, as Pax5-deficient Pro B cells gain alternative lineage potential and dedifferentiate into non-B lineage cells (Nutt et al. 1999; Busslinger 2004). In addition, conditional deletion of Pax5 leads to reprogramming of mature B cells into T lineage cells (Cobaleda et al. 2007). Expression of non-B cell-associated genes, such as M-CFSR and Notch1, in Pax5-deficient Pro B cells suggests an essential role of Pax5 in suppressing genes involved in alternative lineage differentiation. Genome-wide analysis has identified the many Pax5 target genes in early and later stages of B cell development (Schebesta et al. 2007; Revilla et al. 2012).

5 Epigenetic Regulation in Lymphocyte Development

5.1 *Polycomb Group Proteins and Lymphoid Lineage Commitment*

Increasing evidence suggests that epigenetic factors play a crucial role in controlling the hematopoietic system. Among chromatin modifiers, Polycomb group (PcG) proteins are key regulators of lymphocyte cell fate (Zandi et al. 2010; Aloia et al. 2013). Several studies have elucidated the role of PcG proteins in HSC maintenance. One of the Polycomb repressive complexes (PRC) 1 components, Bmi1 has been reported to suppress the *Ink4a/Arf* locus, which encodes the cell cycle inhibitors p16 and p19 (Park et al. 2003). Moreover, Bmi1 is also implicated in repression of transcription factors essential for B cell determination, such as EBF1 and Pax5. Deletion of Bmi1 results in aberrant expression of EBF1 and Pax5, which leads to premature B lymphoid lineage specification (Oguro et al. 2010). In addition, Bmi1 was demonstrated to regulate thymocyte proliferation by suppressing the *Ink4a/Arf* locus (Miyazaki et al. 2008). Another PRC1 component, the Cbx family members, is also implicated in regulating self-renewal activities of HSCs, although the precise function seems to be different among the different Cbx genes (Klauke et al. 2013; van den Boom et al. 2013). Interestingly, Cbx4 has been reported to specifically regulate the proliferation and maintenance of thymic epithelial cells (Liu et al. 2013). Ring1B is a RING finger E3 ligase that monoubiquitylates H2A. Conditional deletion of Ring1B impairs B cell generation in BM, whereas the proliferation of immature progenitor cells and myeloid cells is enhanced (Cales et al. 2008). In addition of PRC1, PRC2 components have also been suggested to play an important role for proper HSC identity. Loss of Ezh2 severely impaired the self-renewal of fetal and adult HSCs (Mochizuki-Kashio et al. 2011; Hidalgo et al. 2012), and Ezh1-deficient mice have reduced number of HSCs in BM, suggesting some overlap, but not exactly same function of Ezh genes in the maintenance of HSC pools. Critical roles of Ezh2 in HSC self-renewal and early B cell development were also shown by inactivation of Ezh2 (Su et al. 2003; Kamminga et al. 2006). Taken together, these observations suggest that PcG proteins play a critical role at multiple stages in hematopoietic and lymphoid developmental system.

5.2 *DNA Methylation and Other Chromatin Modifiers in Lymphoid Cell Fates*

DNA methylation is an epigenetic mechanism essential for normal development, including transcriptional regulation, genomic imprinting, and genomic instability. DNA methyltransferases (Dnmts) are the group of enzymes responsible for

establishment and maintenance of genomic DNA methylation. They include the de novo methyltransferases Dnmt3a and Dnmt3b and the maintenance methyltransferase Dnmt1. Conditional deletion of Dnmt1 in the hematopoietic system impairs the self-renewal of HSCs (Broske et al. 2009; Trowbridge et al. 2009). Dnmt3a$^{-/-}$Dnmt3b$^{-/-}$ HSCs also have defects in their self-renewal activity, however, they can undergo normal differentiation to all hematopoietic lineages, indicating that de novo DNA methylation is not required for the differentiation of hematopoietic lineage cells (Tadokoro et al. 2007). Dnmt1 is also essential for proper development, survival, and function of T cells (Lee et al. 2001; Makar et al. 2003).

Special AT-Rich Sequence Binding protein 1 (SATB1) is a gene silencer that recruits chromatin-remodeling factors to regulate chromatin structure and gene expression. Expression of SATB1 increases during lymphoid lineage specification. Deletion of SATB1 reduces lymphopoietic activity of hematopoietic stem/progenitor cells, while the generation of myeloid lineage cells is unaffected. Ectopic expression of SATB1 promotes the differentiation toward T and B lymphoid lineages, suggesting a critical role of SATB1 in lymphoid lineage specification (Satoh et al. 2013).

Micro RNAs (miRNAs) are a recently discovered class of small (18-24 nt), noncoding RNAs that can downregulate target genes at the posttranscriptional level (Yuan and Muljo 2013). One miRNA, miR126, has recently been suggested to control B lineage specification independently of transcription factor activities. B cell but not myeloid development was promoted by the ectopic expression of miR126 in hematopoietic progenitors in fetal liver, both in vivo and in vitro. In addition, overexpression of miR126 in multipotent EBF1-deficient progenitors resulted in a partial induction of the B cell lineage program without upregulating E2A, EBF1, and Pax5 at the transcriptional level. These results indicate a critical role of miR126 in lymphoid versus myeloid lineage fates that is independent of canonical transcriptional cascades (Okuyama et al. 2013).

6 Transcriptional Regulation of ILC Development and the Relationship Between Acquired and Innate Lymphocytes

For many years, NK cells have been considered to be the only innate lymphocyte. However, it has recently become clear that innate lymphocytes are a more diverse group than previously anticipated. Innate lymphocytes, now called ILCs, are categorized into three groups, ILC1, ILC2, and ILC3 based on their functional characteristics, such as the cytokines that they produce and the transcription factors that are required for their development and function (Spits and Cupedo 2012; Walker et al. 2013). The generation of all ILCs depends on the transcriptional inhibitor Id2 (Klose et al. 2012). Id2 is one of a family of Id proteins that function

as negative regulators of E proteins. Since E proteins, such as E2A and HEB normally promote T and B lymphocyte development, Id2 may function to induce ILC development by inhibiting adaptive lymphoid cell fate determination. ILCs also rely on the common cytokine receptor γ-chain (γ_c) and IL-7Rα, suggesting a critical role for IL-7 signaling in their development, similar to the situation with T and B lymphocyte development.

NK cells are a major component of ILC1 and are defined by the production of the IFNγ. $CD122^{+}NK1.1^{-}$ NK cell-committed progenitors have been identified in fetal thymus and BM (Ikawa et al. 1999, 2001; Rosmaraki et al. 2001). Mice deficient for IL-2Rβ, IL-15Rα or IL-15 lack NK cells and detectable NK cell-mediated cytotoxicity, indicating a critical role of IL-15 signaling in the development and function of NK cells (Kennedy et al. 2000; Suzuki et al. 1997). The basic leucine zipper transcription factor E4BP4 was specifically shown to be required for the generation of NK cells. E4BP4-deficient mice lack NK cells. Real time RT-PCR analysis showed reduced expression of GATA3 and Id2 in $E4BP4^{-/-}$HPCs in BM. Overexpression of Id2 in $E4BP4^{-/-}$HPCs restored the production of NK cells, indicating that Id2 acts downstream of E4BP4 to promote NK cell development (Gascoyne et al. 2009).

ILCs that produce Th2-type cytokines IL-5 and IL-13 in response to stimulation by IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) are classified as ILC2. ILC2 includes NH cells (Moro et al. 2010), nuocytes (Neill et al. 2010), type 2 innate helper (Ih2) cells, and multipotent progenitor type 2 (MPP^{type2}) cells (Price et al. 2010). Although their phenotypic markers are slightly different, the functional differences and relationships among these ILC2 populations remain to be determined. ILC2 requires the transcription factor retinoic acid receptor-related orphan receptor-α (RORα) (Halim et al. 2012; Wong et al. 2012) and GATA3 (Hoyler et al. 2012; Mjosberg et al. 2012; Furusawa et al. 2013) for their functions, such as antihelminth responses and allergic lung inflammation. About half of NH cells are marked with a history of Rag1 expression, detected using RAG1Cre/ROSA26YFP mice, implying a lymphoid developmental origin (Yang et al. 2011). This notion is also supported by the observation that the CLPs but not myeloid–erythroid progenitors gave rise to NH cells. In addition, Notch signaling has been shown to be essential for the generation of nuocytes from CLPs in vitro. As described above, TCF-1, which plays a critical role for T cell development, is also required for the development of ILC2 (Yang et al. 2013). Taken together, these observations indicate that T cells and ILC2 may share similar gene regulatory networks for their proper development and function.

ILC3 are defined by the capability to produce IL-17A and/or IL-22. The generation and function of ILC3 are totally dependent on the activity of transcription factor RORγt. On the basis of functional characteristics, ILC3 are categorized into three populations, LTi cells, IL-22-producing cells, and IL-17-producing cells. LTi cells were discovered almost two decades ago and represent the prototypic ILC3. LTi cells are crucial for the formation of secondary lymphoid organs during embryogenesis (van de Pavert and Mebius 2010). Although LTi cells appear to be closely related to other ILC3s, the relationship between them is still unclear. It also

remains to be determined how the expression of RORγt is regulated during ILC3 development. More detailed characterization of each ILC3 subset and the relevant molecular mechanisms are needed for better understanding of the roles of these ILC populations.

7 Conclusions

Although many of the details regarding the mechanistic basis of lymphoid lineage determination by transcription factors and epigenetic factors have been elucidated, the exact structure of the network of these factors and the relationship among them remain largely unknown. Further dissection of the process between HSCs and committed T or B cells will be needed to draw an exact map of the network during lymphoid lineage specification. Establishment of in vitro models of lymphocyte development will also facilitate our understanding of the exact mechanisms of how these transcriptional networks operate. Combinations of genome-wide analysis, such as RNA-seq, ChIP-seq, and other epigenetic experimental systems will help us to fully clarify how cell-type specific gene programs are established and maintained to promote lymphocyte development. The development of the newly classified lymphoid cells, the ILCs, and the relationship between ILCs and T and B lymphocytes are intriguing issues to understand the overall development of the immune system.

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The Role of BTB-Zinc Finger Transcription Factors During T Cell Development and in the Regulation of T Cell-mediated Immunity

Wilfried Ellmeier and Ichiro Taniuchi

Abstract The proper regulation of the development and function of peripheral helper and cytotoxic T cell lineages is essential for T cell-mediated adaptive immunity. Progress made during the last 10–15 years led to the identification of several transcription factors and transcription factor networks that control the development and function of T cell subsets. Among the transcription factors identified are also several members of the so-called BTB/POZ domain containing zinc finger (ZF) transcription factor family (BTB-ZF), and important roles of BTB-ZF factors have been described. In this review, we will provide an up-to-date overview about the role of BTB-ZF factors during T cell development and in peripheral T cells.

Contents

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1 Introduction

1.1 A Brief Summary of αβT Cell Development

T-lineage lymphocytes, which are defined as a cell population expressing either the $\alpha\beta$TCR or the $\gamma\delta$TCR chains, develop in the thymus. After immigration into the thymus, early T cell progenitors that still retain the developmental potency to develop into other hematopoietic lineages undergo several regulatory processes to be fully committed to the T lymphocyte lineage. These early immature T cells neither express CD4 nor CD8 and therefore are called double-negative (DN) thymocytes. The early development of thymocytes is accompanied with the initiation of the recombination of either the *Tcrb* or *Tcrd* gene loci that encode for the TCRβ or TCRδ chains, respectively. The successful generation of a functional TCRβ chain results in the formation of the pre-TCR complex, which is a heterodimeric complex on the surface of DN thymocytes formed by the newly generated TCRβ chain with the invariant pre-Tα protein. The expression of a functional pre-TCR, a checkpoint known as β-selection, results in the inhibition of a further rearrangement of the *Tcrb* locus as well as in a rapid proliferation and leads to the induction of *Cd4*, and *Cd8a* and *Cd8b1* (*Cd8*) gene expression. Hence, DN thymocytes progress to the CD4 and CD8 expressing double-positive (DP) stage of T cell development. The transition to the DP stage is accompanied with the functional rearrangement of the *Tcra* locus and DP thymocytes express a mature $\alpha\beta$TCR formed by the TCRα and TCRβ chains. $CD4^+CD8^+$ DP phenotype cells are subjected to another selection process, known as a positive/negative selection, during which reactivity of $\alpha\beta$TCR to self-peptide/MHC is evaluated and CD4 and CD8 proteins serve as coreceptors for peptide/MHC recognition during this process. As a consequence of positive and negative selection, only limited numbers of DP thymocytes are allowed to further differentiate and to face the cell fate decision to become either helper or cytotoxic T cells. It has been known that both TCR specificity to MHC types and CD4/CD8 coreceptor expression perfectly correlates with outcome of helper/cytotoxic lineage choice. Thymocytes selected via MHC class I molecules differentiate into cytotoxic T cells and shut-off *Cd4* gene expression, thereby acquiring a $CD4^-CD8^+$ single-positive (CD8SP) surface phenotype. On the contrary, those DP cells selected by MHC class II develop toward the helper T lineage and become $CD4^+CD8^-$ single-positive (CD4SP)

thymocytes by loosing *Cd8* gene expression. Thus, the CD4/CD8 coreceptors expression profile is a good marker to define distinct developmental stages of thymocytes (Carpenter and Bosselut 2010; Singer et al. 2008). Progress made during the last 10–15 years led to the identification of several transcription factors and the characterization of a transcription factor network that is essential for *Cd4* and *Cd8* gene regulation and for helper/cytotoxic lineage choice during T cell development (Taniuchi and Ellmeier 2011; Ellmeier et al. 2013). Among the transcription factors identified are ThPOK and MAZR, two members of the so-called BTB/POZ domain containing zinc finger (ZF) transcription factor family (BTB-ZF) (Stogios et al. 2005). Moreover, members of the BTB-ZF family play also important roles at other stages of T cell development and BTB-ZF factors have been identified as crucial regulators of peripheral T cell function (Bilic and Ellmeier 2007; Siggs and Beutler 2012; Beaulieu and Sant'Angelo 2011). In this review, we will provide an up-to-date overview about the role of BTB-ZF factors during T cell development and in peripheral T cells.

1.2 The BTB/POZ Domain Containing Family of Zinc Finger Transcription Factors

The BTB (broad-complex, tramtrack, and bric-a-brac) domain, also known as POZ (Pox virus and ZF) domain, is an eukaryotic protein–protein interaction motif. The BTB domain, which is approximately 90–120 amino acids long, can mediate homo-oligomerization, hetero-oligomerization, and facilitates also interactions with other proteins that lack BTB domains (Bardwell and Treisman 1994; Stogios et al. 2005; Collins et al. 2001). It has been reported that there are approximately 200 genes in the human genome that contain a BTB domain (Stogios et al. 2005). These BTB domain containing factors can be divided into several subgroups dependent on the presence of additional domains, such as C_2H_2 ZF motifs (BTB-ZF), factors containing a so-called Kelch motif (Stogios and Prive 2004) and proteins with a potassium channel tetramerization T1 domain (KCTD proteins; Liu et al. 2013) among others (Stogios et al. 2005). Although there is little amino acid sequence homology in the BTB domain between members of different subgroups, a structural analysis reveals conservation in the tertiary protein structure (Stogios et al. 2005). The various BTB proteins have a broad range of biological functions and regulate a variety of different cellular and molecular processes. These include the transcriptional control of development, differentiation, cancer (Kelly and Daniel 2006; Bilic and Ellmeier 2007; Beaulieu and Sant'Angelo 2011; Lee and Maeda 2012; Siggs and Beutler 2012; Lunardi et al. 2013), the regulation of actin and cytoskeleton dynamics (Perez-Torrado et al. 2006; Albagli et al. 1995), protein targeting for ubiquitination (Pintard et al. 2004; Genschik et al. 2013) and others (Stogios et al. 2005).

BTB-ZF factors form one large subgroup of BTB domain containing proteins. There are 49 BTB-ZF genes in mammalian genomes (Gray et al. 2013) and all members have their BTB domain, like many other BTB factors, located at the N-terminus, while the ZF DNA binding domain is located at the C-terminal end (Stogios et al. 2005). Several studies have shown that the BTB domains of some BTB-ZF proteins mediate homo- as well as hetero-oligomerization of BTB-ZF proteins (Hoatlin et al. 1999; Takenaga et al. 2003; Kobayashi et al. 2000). In addition, BTB-ZF factors interact with via their BTB domain nuclear corepressors such as NCoR1, SMRT, and BCoR (Huynh and Bardwell 1998; Ahmad et al. 2003; Melnick et al. 2000, 2002; Polo et al. 2004; Huynh et al. 2000; Bilic et al. 2006), which are part of large, multi-subunit complexes that can contain various chromatin-modifying enzymes like members of the BAF complex, methyl-DNA binding proteins, and histone deacetylases (HDACs) (Jepsen and Rosenfeld 2002; Mottis et al. 2013). Thus, it is likely that BTB-ZF factors might serve as site-specific recruitment factors for chromatin-modifying complexes to their target genes. Moreover, BTB-ZF factors such as PLZF and Bcl6 interact with the E3 ubiquitin ligase Cullin 3 (Mathew et al. 2012), which influences the ubiquitination status of several components of chromatin-remodeling complexes (Lydeard et al. 2013). BTB-ZF proteins have been linked with transcriptional repression, although they can also activate target genes. It is likely that the protein composition as well as posttranslational modifications of such multi-subunit complexes recruited via BTB-ZF proteins will determine whether a BTB-ZF factor will act as a repressor or activator of its target gene.

2 BTB-ZF Proteins and the Regulation of T Cell Development and Function

So far, nine BTB-ZF proteins have been implicated in the regulation of various aspects of T cell development and function (Fig. 1 and Table 1). All these factors show a similar domain-like structure with an N-terminal BTB domain and the C-terminal Zn finger motifs; however, the number of ZFs and the spacing between consecutive ZFs within the C-terminal ZF domain differs greatly (Fig. 2).

2.1 Zbtb1: A Determinant of Lymphocyte Development

Zbtb1 (ZF and BTB domain containing 1), encoded by the *Zbtb1* gene, has been identified by Butcher and colleagues in an ENU screen as an important regulator of lymphocyte development, in particular the T cell lineage (Siggs et al. 2012). Mice homozygous for a missense mutation (C47R) in Zbtb1 (designated as *scanT* mutant strain) were developmentally normal and fertile; however, mutant mice were devoid

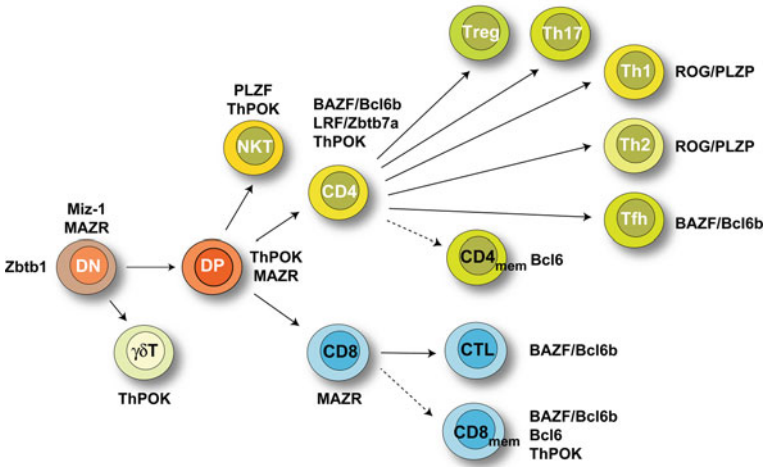

Fig. 1 BTB-ZF factors regulating T cell development and the differentiation/function of peripheral T cell subsets. The drawing shows an overview about the various developmental stages of thymocyte development and different subsets of peripheral $CD4^+$ and $CD8^+$ T cells. The name of the BTB-ZF factors implicated in the regulation of a particular thymocyte and T cell subset are indicated. See text for more details. *DN* double-negative, *DP* double-positive, *NKT* natural killer T cells, *Th1* T-helper 1, *Th2* T-helper 2, *Th17* IL-17-producing Th cells, *Treg* regulatory T cells, *Tfh* follicular helper T cells, *CTL* cytotoxic T lymphocytes. The *dotted lines with arrowheads* indicate less well-defined differentiation pathways leading to the generation of memory CD4 (CD4mem) and CD8 (CD8mem) T cells

of T cells, while NK cell numbers and to a lesser extent also B cell numbers were reduced in comparison to wild-type mice. The generation of BM chimeras reveled that the phenotype was intrinsic to the hematopoietic system, although hematopoietic cell development was not affected before lymphoid specification. In a competitive environment in mixed BM chimeras, all lymphoid lineages were absent, while the myeloid compartment was not affected (Siggs et al. 2012). Puck and colleagues independently identified Zbtb1 as an important regulator of the generation of T cells in a homozygous transgenic strain that lacked T cells due to an insertion of the transgene in the *Zbtb1* locus (Punwani et al. 2012). The generation of Zbtb1-null mice confirmed that Zbtb1 is essential for the generation of T cells due to hematopoietic cell-intrinsic defects, while NK cells were less affected in comparison to T cells. B cell numbers were almost normal in *Zbtb1*$^{-/-}$ mice. The analysis of fetal thymi showed that already decreased numbers of early DN1 thymocytes and a failure to progress beyond this stage. *Zbtb1* is also expressed in the spleen and in lymph nodes. In developing thymocytes, Zbtb1 is upregulated during the DN to DP transition. Two splicing isoforms of *Zbtb1* have been identified, one encoding for a full-length Zbtb1 protein with eight ZF motifs, while a shorter *Zbtb1* isoform expressed at lower levels encodes for a protein with only five ZF motifs (Punwani et al. 2012). Although not demonstrated in T cells, Zbtb1 can function as a transcriptional repressor (Liu et al. 2011; Matic et al. 2010) and the repressor activity of

Table 1 This table shows the murine and human gene names encoding for BTB-ZF factors implicated in T cell development and the regulation of peripheral T cell function. The function of BTB-ZF factors and the reference reporting the activity is shown at the right

Murine gene	Human gene	Synonym (alternative names)	Functions during T cell development and in peripheral T cell
Bcl6	*BCL6*	(BCL5, BCL6A, LAZ3, ZBTB27, ZNF51)	Key factor for Tfh differentiation and Tfh survival (Johnston et al. 2009; Nurieva et al. 2009; Yu et al. 2009; Hollister et al. 2013) Bcl6$^{-/-}$ Tregs failed to suppress Th2-type immune responses in vivo leading to strong lung inflammation in an allergic airway inflammation model (Dent et al. 1997; Ye et al. 1997; Sawant et al. 2012) Bcl6 is an important regulator of both CD4^{+} and CD8^{+} memory T cell generation and homeostasis (Ichii et al. 2002, 2004, 2007)
Bcl6b	*BCL6b*	**BAZF** (ZBTB28, ZNF62)	Regulates activation of naïve CD4^{+} T cells (Takamori et al. 2004) Modulates secondary response of CD8^{+} memory T cells (Manders et al. 2005) BAZF/Bcl6b-null CD8^{+} T cells influence the number of cycling hematopoietic progenitor cells in the spleen (Broxmeyer et al. 2007)
Patz1	*PATZ1*	**MAZR** (RIAZ, ZBTB19, ZNF278, ZSG)	Represses CD8 expression in DN thymocytes (Bilic et al. 2006) Part of the transcription factor network controlling CD4/CD8 cell fate choice (Sakaguchi et al. 2010)
Zbtb1	*ZBTB1*		Regulates the generation of the lymphoid lineage, in particular T cells and NK cells (Siggs et al. 2012; Punwani et al. 2012)
Zbtb7a	*ZBTB7A*	**LRF** (FBI1, FBI-1, pokemon, ZBTB7, ZNF857A)	Indirectly affects B versus T cell choice via upregulation of Notch ligand Delta-4 on erythroblasts (Lee et al. 2013a) Controls Th cell-specific gene expression (Carpenter et al. 2012)
Zbtb7b	*ZBTB7B*	**ThPOK** (cKrox)	Key commitment factor for CD4 lineage specification (He et al. 2005; Sun et al. 2005) Represses CD8 lineage genes in CD4^{+} T cells (Wang et al. 2008a; Egawa 2009; Rui et al. 2012) Regulates expansion of CD8^{+} memory T cells (Setoguchi et al. 2009) Important for $\gamma\delta$T cell maturation (Park et al. 2010) Regulates functional differentiation of invariant NKT cell subsets (Engel et al. 2010, 2012; Enders et al. 2012)

(continued)

Table 1 (continued)

Murine gene	Human gene	Synonym (alternative names)	Functions during T cell development and in peripheral T cell
Zbtb16	*ZBTB16*	**PLZF** (green's luxoid, ZNF145)	Essential for the development and function of invariant NKT (Kovalovsky et al. 2008; Savage et al. 2008) Important for the regulation of an effector program and effector function in innate-like T cells (Savage et al. 2008; Raberger et al. 2008; Kovalovsky et al. 2010) Important for the development of innate-like $CD8^+$ T cells (Weinreich et al. 2010; Verykokakis et al. 2010)
Zbtb17	*ZBTB17*	**Miz-1** (pHZ-67, ZNF151, ZNF60)	Essential for early T cell lineage development at ETP/DN1 stage (Saba et al. 2011b) Ensures proper pre-TCR expression and the regulation of P53 target genes in DN3 thymocytes (Saba et al. 2011a)
Zbtb32	*ZBTB32*	**Rog, PLZP** (FAXF, FAZF, TZFP, ZNF538)	Important regulator of Th2-type immune responses in vitro (Miaw et al. 2000, 2004) and in vivo (Hirahara et al. 2008; Hirasaki et al. 2011)

Zbtb1 is regulated by SUMOylation (Matic et al. 2010). Together, these data indicate an essential role for Zbtb1 in the generation of the T cell lineage and also for the generation of NK and B cells.

Of note, *Zbtb1*$^{-/-}$ mice show increased numbers of short-term HSC, multipotent progenitors, and common lymphoid progenitor cells (Punwani et al. 2012), while *scanT* mice do not (Siggs et al. 2012). This might indicate that the mutant Zbtb1 generated from the C47R *Zbtb1* allele might still have some residual function. Further studies are required to reveal the molecular mechanisms of how Zbtb1 regulate lymphocyte development and the differentiation of the hematopoietic system.

2.2 *Miz-1: A Regulator of Early T Cell Differentiation*

Miz-1 (Myc-interacting ZF protein 1), encoded by the *Zbtb17* gene, is a transcription factor that has been initially identified as a c-myc interacting factor (Peukert et al. 1997). Like many other BTB-ZF factors, Miz-1 can both positively and negatively regulate its target genes, dependent on interaction with other factors (Moroy et al. 2011). Germline deletion of Miz-1 results in embryonic lethality due to defects during gastrulation (Adhikary et al. 2003). Therefore, Möröy and colleagues generated a conditional *Miz-1* allele that lacks the BTB domain upon Cre/loxP-mediated recombination (Kosan et al. 2010). Initially, studies using *Vav-Cre*-mediated deletion of Miz-1 focused on B cells and revealed a crucial role for Miz-1 in IL-7

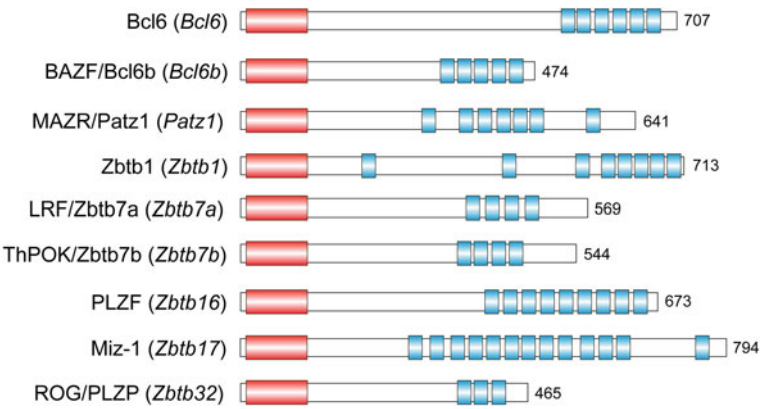

Fig. 2 Domain structure of BTB-ZF proteins. The drawing show the location of the BTB domain (in *red*) and the C_2H_2 zinc finger motifs (in *blue*) in BTB-ZF factors implicated in the regulation of T cells. The name of the protein and the gene name (in parenthesis) are indicated at the *left*. The numbers at the *right* indicate the length in amino acids. For MAZR/Patz1, an alternative splice variant encoding for a 537 amino acids long BTB-ZF protein that contains six zinc finger motifs has been described (Kobayashi et al. 2000)

receptor signaling during early B cell development (Kosan et al. 2010). Subsequent studies showed that Miz-1 is also essential for the T cell lineage. Loss of Miz-1 (using the *Vav-Cre*-deleter strain) led to a severe reduction (>100 fold) of thymocyte numbers accompanied also by a severe reduction of DN subsets, in particular ETP/DN1 and DN2 stages (Saba et al. 2011b). By performing a comprehensive in vitro analysis using the OP9-DL1 system, the drop in ETP/DN1 cells could be linked to extensive cell death in the absence of Miz-1. A further analysis showed that Miz-1 regulates the expression of SOCS1, most likely by a direct regulation since Miz-1 binds to the Socs1 promoter region and loss of Miz-1 leads to an upregulation of Socs1 expression. As a consequence, STAT5 activation and Bcl-2 expression in response to IL-7 signaling is impaired. The functional importance of Bcl-2 upregulation was confirmed genetically, since transgenic overexpression of Bcl-2 rescues the survival defect of Miz-1-null ETP/DN1 cells, indicating a crucial role for Miz-1 for the survival of ETP/DN1 cells (Saba et al. 2011b).

Although the transgenic expression of Bcl-2 restored in part thymocyte numbers in the absence of Miz-1, there was still a reduction of DP thymocytes due to a developmental block at the DN3 stage, indicating another role for Miz-1 during early T cell development (Saba et al. 2011a). Despite normal expression of many genes required for the generation of a pre-TCR including *Rag1*, *Rag2*, *Cd3e*, *pTa*, and intact VDJ recombination, only a few Miz-1-null cells expressed a surface pre-TCR. Miz-1-null DN3 cells do not proliferate and display increased cell death and this correlated with the enhanced expression of p53 target genes such as Cdkn1a, Puma, and Noxa. However, transgenic TCR expression together with transgenic Bcl-2 rescued partially the developmental block at the DN3 stage, suggesting that the role of Miz-1 in DN3 cells is in part to ensure proper pre-TCR expression and

the regulation of P53 target genes (Saba et al. 2011a). Of note, thymocyte development and the appearance of mature CD4SP and CD8SP cells was normal when Miz-1 was deleted at DN2/3 stage by *Lck-Cre* (Saba et al. 2011b), indicating that Miz-1 mainly controls early T cell development but has rather a minor role at later stages of thymocyte maturation beyond the DN stage. Alternatively, other factors can compensate for loss of Miz-1 thymocyte differentiation after β-selection.

2.3 ThPOK/Zbtb7b: A Master Commitment Factor for CD4 Lineage Differentiation

ThPOK (T-helper-inducing POZ/Krueppel-like factor, initially known as cKrox), encoded by the *Zbtb7b* gene, was first identified as a binding protein to the promoter regions of collagen genes (Widom et al. 1997), indicating that the expression in this protein is not restricted to hematopoietic cells. Indeed human Zbtb7b gene expression was detected in foreskin and fibroblast (Widom et al. 2001). During T cell differentiation, *Zbtb7b* gene exhibits a quite unique expression pattern, since it is induced in MHC class II-signaled DP thymocytes and expressed in $CD4^+$ helper T cells, while developing CD8 lineage T cells remain ThPOK negative (He et al. 2005; Sun et al. 2005).

Two groups independently identified that ThPOK is a key regulator of CD4 lineage development. Kappes and colleagues unraveled the role of ThPOK during T cell development by using a combination of classical positional cloning approaches to identify the responsible gene locus causing the helper-deficient (HD) phenotype (Dave et al. 1998) and transgenesis to rescue the gene defect identified in HD mice (He et al. 2005). A spontaneous missense mutation in the *Thpok* gene, which alters an arginine residue in the second zinc finger domain to a glycine, resulted in a severe reduction of $CD4^+$ T cell in the periphery through redirected differentiation of MHC class II-restricted thymocytes toward $CD4^-CD8^+$ T cells (He et al. 2005). Bosselut and colleagues identified ThPOK in a screen for genes induced in DP cells during CD4 lineage differentiation (Sun et al. 2005). Both groups showed that enforced expression of ThPOK from $CD4^+CD8^+$ DP preselection thymocytes and onward prevents generation of $CD8^+$ cells through directing MHC class I-restricted cells to become $CD4^+CD8^-$ T cells (He et al. 2005; Sun et al. 2005). Subsequent loss of function studies of ThPOK during thymocyte differentiation by gene targeting confirmed the important role for ThPOK in the regulation of CD4/CD8 cell fate choice (Egawa and Littman 2008; Muroi et al. 2008; Wang et al. 2008b). Together, these results indicate that ThPOK expression is not only essential but also sufficient to endow $CD4^+CD8^-$ phenotype during thymocyte maturation beyond a MHC restriction of cells (Kappes et al. 2006).

A key mechanism by which ThPOK endows CD4 expression to the helper T cells is the antagonistic function of ThPOK against the *Cd4* silencer (Wildt et al. 2007; Muroi et al. 2008). Moreover, ThPOK represses CD8 lineage genes such as Runx3 and cytotoxic effector genes such as *Granzyme B* and *Perforin* (Egawa and Littman 2008; Wang et al. 2008a) and might also directly repress the *Cd8* gene complex via binding to *Cd8* enhancers (Rui et al. 2012). However, it remains uncharacterized how ThPOK contributes to confer total helper function to MHC class II-restricted T cells. A recent study indicated that MHC class II-restricted cells retained some helper-related functions in the absence of ThPOK (Carpenter et al. 2012). As we discussed later in detail, LRF/Zbtb7a, which is the most-related BTB-ZF family member to ThPOK, is shown to compensate for loss of ThPOK function in some helper T cell subsets (Carpenter et al. 2012).

The findings that revealed an essential role of ThPOK for CD4 lineage development stimulated studies addressing the mechanism that restricts ThPOK expression only to MHC class II-restricted cells. These studies led to the identification of a transcriptional silencer element, hereafter referred to as a *Thpok* silencer, in the *Thpok* gene locus (He et al. 2008; Setoguchi et al. 2008). Kappes and colleagues utilized transgenic reporter expression assays and identified two *cis*-regulatory regions, designated as distal and proximal regulatory elements (DRE and PRE, respectively). Interestingly, DRE was shown to function both as a transcriptional enhancer and as a silencer, while only an enhancer function was associated with PRE (He et al. 2008). The *Thpok* silencer was independently identified by Taniuchi and colleagues (Setoguchi et al. 2008). The silencer is essential to repress *Thpok* expression during differentiation of MHC class I-restricted cells (He et al. 2008; Setoguchi et al. 2008), thereby preventing an aberrant differentiation pathway toward $CD4^+$ cells in those cells. Along with a *Thpok* derepression by lack of Runx complex function (Setoguchi et al. 2008), "knock-in" mutagenesis approaches within the *Thpok* silencer showed that *Thpok* silencer activity requires binding of Runx complexes (Tanaka et al. 2013). However, Runx binding to the silencer was also detected in cells expressing *Thpok* gene (Setoguchi et al. 2008), indicating that Runx binding alone is not sufficient to activate the *Thpok* silencer. Thus, it is possible that an uncharacterized mechanism in addition to Runx binding is involved in a control of the *Thpok* silencer activity. As we will discuss below, MAZR, another member of the BTB-ZF gene family, was shown to be necessary for full *Thpok* repression in MHC class I-signaled cells through regulation of the *Thpok* silencer function (Sakaguchi et al. 2010). A further characterization of protein complexes bound to the *Thpok* silencer will be important to unravel the mechanism(s) that facilitates the switch in *Thpok* silencer activity between CD4 and CD8 lineage cells.

In addition to the inactivation of the *Thpok* silencer, several positive *cis*-regulatory elements (i.e., enhancers) are also necessary for appropriate ThPOK expression in CD4 lineage T cells. It was recently shown that distinct sequences within DRE are responsible for enhancer and silencer activity of DRE (He et al. 2008; Muroi et al. 2013). While the enhancer activity in DRE is responsible for the initiation of *Thpok* gene, the enhancer within PRE functions later to upregulate and

maintain ThPOK expression (Muroi et al. 2008). It is likely that these two enhancers, which display a distinct stage-specificity, cooperatively regulate *Thpok* expression.

Interestingly, ThPOK also regulates $CD8^+$ T cells function. In vitro stimulation of $CD8^+$ T cells leads to the derepression of ThPOK in a fraction of $CD8^+$ T cells (Setoguchi et al. 2009). Although the loss of ThPOK does not affect CD8 T cell differentiation into effector T cells and the long-term persistence of Ag-specific memory T cells, the clonal expansion is significantly less in both primary and secondary $CD8^+$ T cell responses in the absence of ThPOK (Setoguchi et al. 2009), indicating an unexpected role for ThPOK in CD8 lineage T cells in vivo.

In addition to its function in conventional $\alpha\beta$T cells, ThPOK also plays a role in the regulation of innate-like T cells such as $\gamma\delta$T cells and invariant NKT (iNKT) cells. ThPOK expression is upregulated during the developmental transition from $CD24^+$ immature to $CD24^-$ mature $\gamma\delta$ thymocytes and the maturation of $\gamma\delta$T cells in the thymus, in particular $NK1.1^+$ $\gamma\delta$ thymocytes subsets, is impaired in ThPOK-deficient mice (Park et al. 2010). iNKT cells are another innate-type T cells expressing invariant Vα14 chain (Constantinides and Bendelac 2013; Rossjohn et al. 2012) and are recently characterized to be composed of several functionally different subsets (Lee et al. 2013b). The emergence of $CD8^+$ iNKT cells in ThPOK-deficient mice indicates that ThPOK also represses *Cd8* gene expression in this innate cell subset as observed in conventional $\alpha\beta$T cell differentiation pathways (Engel et al. 2010). ThPOK also contributes to confer IL4 producing property to iNKT cells during maturation of iNKT cells (Engel et al. 2010). In addition, ThPOK-deficient mice contain a higher proportion of IL-17-producing cells (NKT17) (Enders et al. 2012; Engel et al. 2012), indicating that ThPOK negatively regulate differentiation of this NKT17 subset. Thus, ThPOK is involved in the specification of distinct NKT cell subsets.

It has been proposed that separation of CD4 helper and CD8 cytotoxic lineage in the thymus is stably inherited after activation of cells. Indeed, the findings of an involvement of epigenetic mechanism in the repression of the *Cd4* and *Thpok* genes provided supportive mechanistic insight into how *Cd4* and *Thpok* genes are kept silenced in $CD8^+$ cytotoxic T cells (Zou et al. 2001; Tanaka et al. 2013). However, little is known about how *Cd8* gene expression is repressed and conversely how *Cd4* and *Thpok* expression is stably maintained in helper T cells. Current studies have unraveled an unappreciated developmental plasticity retained in $CD4^+$ T cells, which allow them to reactivate the program to become cytotoxic-related property. When $CD4^+$ T cells are transferred into lympho-deficient host mice, a proportion of these cells exposed to gut-specific environmental cues reexpress CD8α chain and become $CD4^+CD8\alpha\alpha^+$ cells that also express other cytotoxic-related genes (Mucida et al. 2013; Reis et al. 2013). Prior to such a dressing up with cytotoxic features, ThPOK expression is vanishing, while Runx3 expression is induced. Continuous expression of ThPOK using retroviral expression vectors prevents this reprogramming, confirming that ThPOK downregulation is essential for the acquisition of cytotoxic features. Similar to the initial lineage selection in the thymus, the *Thpok* silencer is also involved in erasing *Thpok*

expression in the gut (Mucida et al. 2013). Thus, an antagonistic cross-regulation between ThPOK and Runx3 also regulates the maintenance of CD4 lineage identity in peripheral lymphoid organs.

2.4 MAZR/Patz1: Part of the Transcription Factor Network that Controls CD4/CD8 Lineage Choice

MAZR (Myc-associated Zn finger related)/Patz1 [POZ (BTB) and AT hook containing ZF 1], encoded by the *Patz1* gene, has been first described as an interacting partner of Bach2 (Kobayashi et al. 2000), which is a B cell-specific transcriptional repressor involved in antibody class switching (Muto et al. 2004) and that has also been shown to function in T cells (Hu and Chen 2013; Roychoudhuri et al. 2013; Tsukumo et al. 2013). In humans, PATZ1 has been identified as a factor interacting with the Ring finger protein RNF4, a mediator of androgen receptor activity (Fedele et al. 2000). It was described that MAZR/Patz1 activates several promoters (Kobayashi et al. 2000; Morii et al. 2002); however, it also functions as a transcriptional repressor (Fedele et al. 2000; Bilic et al. 2006; Sakaguchi et al. 2010; Abramova et al. 2013). This indicates context- and gene loci-dependent transcriptional activation and repression functions of MAZR/Patz1. Mice with a germline deletion of MAZR/Patz1 are embryonic lethal on a C57BL/6 background due to defects in the CNS and in the cardiac outflow tract. The MAZR/Patz1-null mice on a mixed 129 Sv/C57BL/6 background are born at reduced Mendelian ratio and are smaller in size (Sakaguchi et al. 2010; Valentino et al. 2013). This indicates important functions for MAZR/Patz1 during embryonic development, differentiation, and proliferation. Moreover, MAZR/Patz1 is linked with oncogenesis, since MAZR/Patz1-deficient mice develop Bcl6-dependent lymphomas (Pero et al. 2012). Moreover, MAZR/Patz1 is also involved in the regulation of embryonic stem cell identify (Ow et al. 2013). During T cell development it was shown that MAZR/Patz1 interacts in DN thymocytes with several *Cd8 cis*-regulatory elements. MAZR/Patz1 is expressed at high levels in DN thymocytes and downregulated at later stages of developing T cells. MAZR/Patz1, like other BTB-ZF proteins, interacts with the nuclear coreceptor NCoR1 and enforced expression of MAZR/Patz1 during T cell development impairs the activation of CD8 expression in a proportion of DP thymocytes, resulting in a variegated expression of CD8 (Bilic et al. 2006). This indicates that MAZR/Patz1 is part of a transcriptional complex that represses CD8 in DN cells and that downregulation of MAZR/Patz1 is necessary for the proper activation of the *Cd8ab* gene complex during the DN to DP transition.

The generation of MAZR/Patz1-deficient mice combined with $Mazr^{+/+}$ and $Mazr^{-/-}$ fetal liver transfer experiments into recipient mice revealed that MAZR/Patz1 is also part of the transcription factor network that controls CD4/CD8 cell fate choice of DP thymocytes (Sakaguchi et al. 2010). In the absence of MAZR/Patz1,

a fraction of MHC class I-signaled DP thymocytes redirects into CD4 lineage T cells instead of developing into the CD8 lineage. A detailed molecular and genetic analysis of MAZR/Patz1-deficient mice revealed that MAZR/Patz1 represses ThPOK, the master commitment factor of CD4 lineage differentiation. As described above, ThPOK expression leads to the development of CD4 lineage cells, while repression of ThPOK is essential for CD8 lineage differentiation. In the absence of MAZR/Patz1, ThPOK is derepressed in a fraction of MHC class I-signaled $CD4^+CD8^{lo}$ thymocytes, leading to the redirection of $CD8^+$ T cells into the CD4 lineage. Interestingly, MAZR/Patz1 interacts with Runx complexes (Sakaguchi et al. 2010), which are essential for the repression of ThPOK (Setoguchi et al. 2008). This suggests that MAZR/Patz1 and Runx complexes together are required to repress ThPOK expression during CD8 lineage differentiation. Moreover, a fraction of peripheral MAZR/Patz1-null $CD8^+$ T cells derepressed ThPOK (Sakaguchi et al. 2010), suggesting that MAZR/Patz1 has also a role in regulating peripheral $CD8^+$ T cell function.

2.5 *PLZF: A Key Regulator of NKT Cells and Other Innate-like T Cells*

PLZF (promyelocytic leukemia ZF), encoded by the *Zbtb16* gene, was initially identified as target of chromosomal translocations that lead to the development of acute promyelocytic leukemia (Chen et al. 1993; Suliman et al. 2012). Subsequent studies following the generation of PLZF-deficient mice (Costoya et al. 2004) revealed important functions for PLZF in many biological processes such as renewal of germ stem cell and spermatogenesis, skeletal patterning, and also in the hematopoietic system (Costoya et al. 2004; Suliman et al. 2012). Moreover, a nonsense mutation in the *Zbtb16* gene is the molecular cause of the luxoid phenotype in mice (Buaas et al. 2004).

Several studies have shown that PLZF is important for the development of innate-like T cells, a subset of T cells that is characterized by having a $CD44^{hi}$ expression phenotype and by displaying immediate effector functions such as the rapid release of cytokines upon activation (Lee et al. 2011). In contrast to "classical" $CD44^{hi}$ effector/memory T cells, innate-like T cells acquired their effector phenotype during their development and not in response to antigen stimulation. Certain innate-like T cells are derived from DP thymocytes and at least some of these cells can be selected on nonclassical MHC class Ib molecules (Rodgers and Cook 2005) via interaction with hematopoietic cells rather than with thymic epithelial cells (Urdahl et al. 2002). Innate-like T cell subsets include, among others, iNKT cells, H2-M3-specific T cells (Colmone and Wang 2006; Mir and Sharma 2013), mucosal-associated invariant T (MAIT) cells (Treiner and Lantz 2006; Le Bourhis et al. 2013), and certain $\gamma\delta TCR^+$ T cell subsets. PLZF received a lot of attention in the field of T cell biology when it was shown that PLZF plays an

important role for the development and function of iNKT cells (Kovalovsky et al. 2008; Savage et al. 2008). Although loss of PLZF did not change the development of conventional T cells, loss of PLZF led to severely reduced numbers of iNKT cells in the thymus, spleen, and liver. The PLZF-null iNKT cells that emerged showed impaired effector function and were preferentially redistributed to lymph nodes (Kovalovsky et al. 2008; Savage et al. 2008). In contrast, enforced transgenic expression of PLZF in conventional T cells (which do not express PLZF) induced an effector phenotype (Raberger et al. 2008; Savage et al. 2008; Kovalovsky et al. 2010) and led to a migration of T cells into peripheral tissues such as lung and liver (Savage et al. 2008). This links PLZF expression with the regulation of an effector program and effector function, a process regulated by PLZF in association with the E3 ligase cullin 3 (Mathew et al. 2012), although the molecular details about how PLZF regulates target genes are largely unknown.

PLZF expression has been observed in other innate-like T cell subsets such as $V\gamma1.1^+V\delta6.3^+$ TCR expressing T cells in the mouse (Alonzo et al. 2010; Felices et al. 2009; Kreslavsky et al. 2009) and MAIT cells (Savage et al. 2008) and unconventional $CD4^+$ T cells that are selected on MHC class II-dependent thymocyte–thymocyte interactions in the human (Lee et al. 2010). A surprising role for PLZF and PLZF-expressing T cell subsets for the proper regulation of T cell development was demonstrated in studies that analyzed several mutant mouse mice that have increased numbers of $CD8^+$ T cells with innate-like T cell characteristics (Alonzo and Sant'Angelo 2011; Lee et al. 2011). Mice deficient for Itk (Atherly et al. 2006; Broussard et al. 2006), Klf2 (Weinreich et al. 2009; Weinreich et al. 2010), CBP (Fukuyama et al. 2009) or Id3 (Verykokakis et al. 2010) develop large numbers of innate-like $CD8^+$ T cells. However, these developmental alterations are not intrinsic to the developing innate-like $CD8^+$ T cells but are caused due to IL-4-producing $PLFZ^+$ T cell subsets including NKT cells and $\gamma\delta TCR^+$ T cells that are enhanced in the absence of Itk, Klf2, CBP, and Id3 (Weinreich et al. 2010; Verykokakis et al. 2010). In the absence of PLZF or IL-4 signaling, the development of the innate-like T cell phenotype is reverted and Itk-, KLF2-, CBP-null mice (Weinreich et al. 2010), or $Id3^{-/-}$ mice (Verykokakis et al. 2010) have a normal appearance of naïve $CD8^+$ T cells.

2.6 ROG/PLZP: Regulating T Cell Activation and Th2 Cytokine Production

The transcription factor ROG (repressor of GATA)/PLZP (PLZF-like zinc finger protein), which is encoded by the *Zbtb32* gene, was isolated in a search for factors that interact with GATA-3 (Miaw et al. 2000), which is a key regulator of early T cell development, the specification of the CD4 T cell lineage and for Th2 cell differentiation (Hosoya et al. 2010). ROG/PLZP, also known as TFZP (Lin et al. 1999) and FAZF (Hoatlin et al. 1999) is expressed, if at all, at very low levels in

the thymus, spleen and in non-stimulated T cells. However, ROG/PLZP is transiently induced within 24 h under Th1 and Th2 polarizing conditions and reinduced upon anti-CD3 restimulation (Miaw et al. 2000, 2004). Early studies showed that enforced expression of ROG/PLZP in established Th2 cell clones inhibits cytokine expression, further indicating a repressive role via GATA-3 inhibition. ROG/PLZP inhibited also IFNγ expression in Th1 cell clones (Miaw et al. 2000), thus ROG/PLZP might regulate other transcription factors in addition to GATA-3. Subsequent studies revealed that ROG/PLZP regulates also T cell proliferation upon TCR triggering independent of GATA-3 activity (Kang et al. 2005; Miaw et al. 2004; Piazza et al. 2004). ROG/PLZP has been shown to be a target gene of NFATc2 (Miaw et al. 2004). NFATc2-deficient $CD4^+$ T cells display a hyperproliferative phenotype with increased production of IL-4 (Xanthoudakis et al. 1996; Hodge et al. 1996). NFATc2-null T cells failed to fully upregulate ROG/PLZP and transgenic expression of ROG/PLZP attenuated the hyperproliferation observed in NFATc2-deficient $CD4^+$ T cells, while expression of ROG/PLZP in wild-type $CD4^+$ T cells only modestly interfered with the proliferation upon anti-CD3/anti-CD28 stimulation (Miaw et al. 2004). Thus, ROG/PLZP might be part of a NFATc2-mediated negative feedback mechanism that controls the extent of T cell activation.

The proposed role of ROG/PLZP in T cell proliferation and cytokine production has been confirmed by the generation and analysis of *Zbtb32*$^{-/-}$ mice. ROG/PLZP-deficient mice are born at normal Mendelian ratio and show no gross developmental or pathological alterations. ROG/PLZP-null T cells have an increased proliferative response to anti-CD3 stimulation and produce increased levels of IL-2 due to enhanced NF-κB activity (Kang et al. 2005). Surprisingly, ROG/PLZP-deficient T cells differentiated normally into Th1 or Th2 cells in vitro with only a modest elevation of cytokine expression. However, Nakayama and colleagues showed that ROG/PLZP-deficient mice have enhanced allergic airway inflammation accompanied with an increase in Th2 cytokines in the bronchoalveolar lavage, while transgenic mice expressing exogenously ROG in the T cell lineage showed reduced allergic airway inflammation (Hirahara et al. 2008). The effect was intrinsic to T cells, since adoptive transfer of OVA-primed ROG/PLZP-null $CD4^+$ T cells or of OVA-primed transgenic ROG/PLZP-expressing $CD4^+$ T cells into OVA-primed wild-type mice enhanced or attenuated eosinophil numbers in the inflamed lung, respectively (Hirahara et al. 2008). Moreover, ROG/PLZP inhibits also type-2 allergic responses in a contact hypersensitivity model (Hirasaki et al. 2011). Together, these studies demonstrate a crucial in vivo role for ROG/PLZP in Th2-type-mediated diseases. Whether ROG/PLZP is also important for Th1 and Th17-type immune responses in vivo is not clear. ROG/PLZP-deficient mice have a similar incidence and clinical score in EAE, indicating that Th1/Th17-mediated immune responses are not severely affected by loss of ROG/PLZP (Kang et al. 2005). Further studies are needed to reveal potential functions of ROG/PLZP beyond Th2-type immune responses.

Of note, ROG/PLZP represses GATA-3-mediated transactivation in Th2 cells by preventing, at least in part, GATA-3 binding to DNA (Miaw et al. 2000). However, ROG/PLZP might also use different cell-type specific mechanisms to repress target genes independently of GATA-3. $CD8^+$ T cells that are activated under Th2-polarizing conditions (Tc2 cells; Kelso and Groves 1997; Seder et al. 1992) produce Th2-type cytokines such as IL-4, IL-5, and IL-13, although IL-4 expression is lower than in Th2 cells (Croft et al. 1994) and cannot be enhanced by overexpression of GATA-3 (Miaw et al. 2004). ROG/PLZP is expressed at much higher levels in Tc2 cells compared to Th2 cells. ROG/PLZP binds to a so-called ROG responsive element within the 3′-UTR of the *Il13* gene, which is in close proximity upstream to the *Il4* gene locus. This *cis*-regulatory region is also bound by HDAC1 and HDAC2, and binding of ROG/PLZP, HDAC1, and HDAC2 correlated with diminished acetylation of the *Il4* gene locus in Tc2 cells in comparison to Th2 cells (Miaw et al. 2004). This suggests that the recruitment of ROG/PLZP together with HDACs and potentially other components of corepressor complexes in Tc2 cells leads to a weakened expression of IL-4 in Tc2 cells.

Taken together, ROG/PLZP is a crucial factor in the regulation of Th2 immune responses in vivo.

2.7 LRF/Zbtb7a: Controlling ThPOK-Independent Helper Functions

The transcription factor LRF/Zbtb7a (Leukemia/lymphoma Related Factor; known previously as Pokemon), encoded by the *Zbtb7a* gene, regulates many lineage decisions during hematopoiesis and plays a role in the maturation and differentiation of peripheral B cells. Moreover, LRF/Zbtb7a has important functions during oncogenic transformation (Lunardi et al. 2013). $Zbtb7a^{-/-}$ mice are embryonic lethal and die around day 16 due to severe anemia (Maeda et al. 2007). The analysis of conditional $Zbtb7a^{fl/fl}$ mice in which *Zbtb7a* was inducible deleted by Mx-Cre implicated LRF/Zbtb7a in the regulation of B versus T cells choice, since DP thymocytes developed in the BM at the expense of B cells upon deletion of LRF/Zbtb7a (Maeda et al. 2007). This phenotype is reminiscent to the phenotype of mice that expresses a constitutively active form of Notch (Pui et al. 1999), suggesting that LRF/Zbtb7a might antagonize Notch signaling. A later study demonstrated that aberrant T cell development in the BM in the absence of LRF/Zbtb7a is due to the upregulation of Notch ligand Delta-4 on erythroblasts, which leads to a premature differentiation of hematopoietic stem cells toward T cells (Lee et al. 2013a). This indicates that loss of LRF/Zbtb7a indirectly affects B versus T cell choice.

In the T cell lineage, LRF/Zbtb7a is expressed at low levels in DP thymocytes and is upregulated to much higher levels in CD4SP and CD8SP thymocytes and in peripheral T cells (Carpenter et al. 2012). However, deletion of LRF/Zbtb7a using

Cd4-Cre did not lead to any alterations during T cell development (Carpenter et al. 2012). A surprising role for LRF/Zbtb7a in the control of Th cell-specific gene expression was identified by Bosselut and colleagues when T-helper immune responses were analyzed in ThPOK-deficient mice (Carpenter et al. 2012). As described above, ThPOK/Zbtbt7b, to which LRF/Zbtb7a is closely related, is a key commitment factor for the CD4 T cell lineage. In the absence of ThPOK, MHC class II-restricted thymocytes are redirected into $CD8^+$ T cells (Keefe et al. 1999) and a fraction of these cells reexpressed CD4 upon activation (Carpenter et al. 2012). Moreover, ThPOK-null mice are able to mount a Th1-type T-helper response upon *Leishmania major* infection (Carpenter et al. 2012). This indicates that ThPOK is not essential for the maintenance of some Th cell functions and that other factors might be responsible for compensating ThPOK function or controlling ThPOK-independent Th functions. The analysis of conditional ThPOK and LRF/Zbtbta double-deficient mice revealed that ThPOK-independent Th functions are dependent on LRF/Zbtb7a (Carpenter et al. 2012), suggesting that ThPOK/Zbtb7b and LRF/Zbtb7a have in part redundant functions in maintaining Th cell-specific gene expression.

2.8 BAZF/Bcl6b: Regulating Memory $CD8^+$ T Cells

BAZF (Bcl6-associated ZF protein, also known as Bcl6b), encoded by the *Bcl6b* gene, is a transcriptional repressor that was identified due to the very high homology to Bcl6 (Okabe et al. 1998). BAZF/Bcl6b and Bcl6 bind to the same target sequences that partially overlap with the STAT6 binding sites, suggesting that BAZF/Bcl6b and Bcl6 may repress some STAT-mediated transcription by binding to STAT binding sites (Hartatik et al. 2001). Bcl6 and BAZF/Bcl6b are able to bind to each other and this interaction with Bcl6 appears to be essential for the repressive function of BAZF/Bcl6b (Takenaga et al. 2003). BAZF/Bcl6b does not bind directly nuclear corepressor complexes, thereby BAZF/Bcl6b might recruit corepressor complexes via its association with Bcl6 (Takenaga et al. 2003). Whether other BTB-ZF proteins are able to interact with BAZF/Bcl6b has not been reported. BAZF/Bcl6b expression is restricted to heart, lung, and activated splenocytes (Okabe et al. 1998). Later studies showed that BAZF/Bcl6b is expressed in CD4SP and CD8SP thymocytes and in peripheral activated $CD4^+$ T cells (Takamori et al. 2004) and in memory $CD8^+$ T cells (Manders et al. 2005). BAZF/Bcl6b is implicated in the regulation of angiogenensis in wound healing (Ohnuki et al. 2012); however, not much is known about the function outside the hematopoietic system.

A hint about the role of BAZF/Bcl6b in T cells was provided with the analysis of BAZF/Bcl6b knockout mice, which were independently generated by three research groups. BAZF/Bcl6b-deficient mice are viable, fertile, and display no gross abnormalities (Takamori et al. 2004; Manders et al. 2005; Broxmeyer et al. 2007). Tokuhisa and colleagues focused on the analysis of $CD4^+$ T cells. T cell

development is normal in the absence of BAZF/Bcl6b and there is also a normal distribution of peripheral naïve and memory $CD4^+$ and $CD8^+$ T cell subsets. However, BAZF/Bcl6b-deficient $CD4^+$ T cells show reduced proliferation upon anti-CD3 stimulation and impaired IL-2 production. In contrast, transgenic Bcl6b-expressing $CD4^+$ T cells showed an increase proliferative response upon anti-CD3 stimulation (Takamori et al. 2004). The proliferation of $CD44^{hi}$ effector/memory $CD4^+$ T cells is not affected by gain or loss of BAZF/Bcl6b function, suggesting that BAZF/Bcl6b functions specifically during the activation of naïve T cells (Takamori et al. 2004). Fearon and colleagues analyzed the role of BAZF/Bcl6b during the secondary response of memory $CD8^+$ T cells, since $CD44^{hi}$ $CD8^+$ T cells expressed higher levels of BAZF/Bcl6b compared to $CD44^{lo}$ $CD8^+$ T cells (Manders et al. 2005). Enforced expression of BAZF/Bcl6b reduced the growth of $CD8^+$ T cells in response to IL-2 (Manders et al. 2005). Using a vaccinia virus and an influenza infection model it was shown that BAZF/Bcl6b-deficient mice have normal primary $CD8^+$ T cell responses. However, $CD8^+$ memory T cells were unable to induce IL-2 and to generate effector cells after in vitro restimulation and the magnitude of the memory response in vivo was reduced (Manders et al. 2005). These data suggest that BAZF/Bcl6b has a nonredundant role in controlling the secondary response of $CD8^+$ memory T cells. Dent and colleagues generated another strain of BAZF/Bcl6b-deficient mice (Broxmeyer et al. 2007). The authors focused on the role of BAZF/Bcl6b in hematopoiesis and found that the numbers of cycling hematopoietic progenitor cells (HPC) were reduced in the BM of BAZF/Bcl6b-null mice, while the numbers of cycling HPC in the spleen were increased upon loss of BAZF/Bcl6b. Depletion experiments revealed that the enhanced population of HPC in the spleen is due to the presence of BAZF/Bcl6b-null $CD8^+$ T cells. Thus, it is likely that BAZF/Bcl6b-null $CD8^+$ T cells produce a cytokine or other soluble factors that interfere with the function of HPC. Further studies are required to understand this in more detail.

2.9 Bcl6: Regulating B and T Lymphocytes

The proto-oncogene Bcl6 (B cell leukemia/lymphoma 6), encoded by the *Bcl6* gene, has been initially identified as a gene that is frequently translocated in B cell lymphomas (Ye et al. 1993, 1995). Bcl6 has important functions in B cells and is essential for germinal center (GC) B cell formation (Dent et al. 1997; Ye et al. 1997). Bcl6 is expressed at high levels in GC B cells and it represses target genes important for the terminal differentiation of B cells into plasma cells, such as Blimp-1 (Shaffer et al. 2000). Bcl6 represses also p53 in GC B lymphocytes and modulates DNA damage-induced apoptosis in GC B cells (Phan and Dalla-Favera 2004), suggesting that Bcl6 contributes to lymphomagenesis in part by the suppression of p53 (for reviews on the role of Bcl6 in B cells and in B cell lymphomas, see Ci et al. 2008; Basso and Dalla-Favera 2012; Bunting and Melnick 2013).

The analysis of *Bcl6*$^{-/-}$ mice indicated that Bcl6 has also important functions in the T cell lineage. Bcl6 is a crucial regulator of the differentiation of follicular helper T cells (Tfh), which are an important T cell subset essential for the generation and function of GC B cells (Vinuesa and Cyster 2011; Crotty 2011). Bcl6 is expressed at high levels in Tfh and Bcl-6-deficient T cells failed to differentiate into Tfh cells (Johnston et al. 2009; Nurieva et al. 2009; Yu et al. 2009; Liu et al. 2012). Further, Bcl6-null CD4$^+$ T cells failed to induce GC responses (Nurieva et al. 2009; Johnston et al. 2009) and Bcl6-null mice show enhanced differentiation of other Th subsets. In contrast, enforced expression of Bcl6 induces key Tfh molecules such as CXCR5 and PD-1 but inhibits the differentiation of Th1, Th2, and Th17 cells (Yu et al. 2009), although another study suggest that Bcl6 might have a positive role for the generation of Th17 cells (Mondal et al. 2010). More recent data using conditional targeting approaches confirmed the importance of Bcl6 for the generation of Tfh in vivo and indicated also a role for Bcl6 in the regulation of Tfh survival (Hollister et al. 2013).

Bcl6 has also been implicated in regulating Treg function. Bcl6-null Treg cells were able to suppress T cell responses in vitro and in a Th1-type colitis model in vivo. However, *Bcl6*$^{-/-}$ Tregs failed to suppress Th2-type immune responses in vivo leading to strong lung inflammation in an allergic airway inflammation model (Sawant et al. 2012). It has been shown that Bcl6 repressed Th2-type genes in Tregs by impairing the transcriptional activity of Gata3. In the absence of Bcl6, Treg acquires certain Th2 effector functions (Sawant et al. 2012). This might contribute to the enhanced Th2 responses and Th2-type inflammation observed in *Bcl6*$^{-/-}$ mice, which develop at a high frequency myocarditis and pulmonary vasculitis due to infiltration of eosinophils contributing to the early death of about 50 % of *Bcl6*$^{-/-}$ mice (Dent et al. 1997; Ye et al. 1997). However, increased differentiation of Th1, Th2, and Th17 cells as observed in Bcl6-null mice was not observed upon T cell-specific deletion of Bcl6 (Hollister et al. 2013). This indicates that T cell-extrinsic factors might regulate enhanced Th subset differentiation upon germline deletion of Bcl6. In addition, a subset of Bcl6-dependent follicular CXCR5-expressing Foxp3$^+$ regulatory T cells as well as CXCR5$^+$ follicular NKT cells have been described and implicated in the regulation of the GC reaction (Chung et al. 2011; Linterman et al. 2011).

Bcl6 also plays a role in the generation and maintenance of CD8$^+$ memory T cells, in particular central memory T cells (Ichii et al. 2002, 2004). *Bcl6*$^{-/-}$ mice display reduced numbers of central memory T cells, while transgenic expression of Bcl6 leads to increased numbers of central memory T cells and Bcl6 transgenic T cells display enhanced proliferation upon restimulation. Moreover, Bcl6 is important for the generation of long-term memory CD4$^+$ T cells, probably via regulating survival of memory precursor CD4$^+$ T cells (Ichii et al. 2007). Thus, Bcl6 is an important regulator of both CD4$^+$ and CD8$^+$ memory T cell generation and homeostasis.

3 Conclusion

Members of the BTB-ZF family have been implicated in the development of human diseases such as B cell lymphomas and promyelocytic leukemia for over 20 years and the crucial role of some of these factors in B cells was soon thereafter established. During the last 10 years, BTB-ZF factors received a lot of immunological attention also from T cell biologists and these studies revealed important roles for BTB-ZF factors in the T cell lineage from early stage T cell progenitors until the formation of memory T cells during an immune response. Several important questions about this gene family remain to be addressed. To comprehensively understand how BTB-ZF factors modulate the immune systems, novel tools such as reporter mice for BTB-ZF factors are required to identify immune cell subsets that potentially might be controlled by these factors. Moreover, at a molecular level, it will be important to characterize protein complexes that together with BTB-ZF factors regulate target gene expression. This will reveal whether some BZB-ZF factors share certain interacting partners and/or whether unique interacting partners exists and also how posttranslational modifications of BTB-ZF factors regulate their activity. A better description of these interacting networks will help to understand why BTB-ZF factors act at certain gene loci as transcriptional repressors, while other genes loci are activated by BZB-ZF factors. Finally, it can be expected that the identification of genome-wide target genes using ChIP-seq and RNA-seq approaches and the functional analysis of pathways regulated by the various members of the BTB-ZF family will provide novel insight into regulatory circuits that control T cell development and function. This will also help to better understand the role of BTB-ZF factors in hematopoietic cells beyond the T cell lineage. Since mutations in ZBTB24 were identified in patients suffering with immunodeficiency, centromeric instability, and facial anomalies syndrome type 2 (ICF2) (de Greef et al. 2011; Chouery et al. 2012; Nitta et al. 2013), this might also provide insight into the molecular cause of human immunodeficiencies.

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Transcriptional Control of the Development and Function of Vα14*i* NKT Cells

Isaac Engel and Mitchell Kronenberg

Abstract The majority of T lymphocytes, sometimes referred to as as mainstream or conventional T cells, are characterized by a diverse T cell antigen receptor (TCR) repertoire. They require antigen priming in order to become memory cells capable of mounting a rapid effector response. It has become established, however, that there are several distinct T cell lineages that exhibit a memory phenotype in the absence of antigen priming, even as they differentiate in the thymus. These lymphocytes typically express a markedly restricted TCR repertoire and their rapid response kinetics has led to their being described as innate-like T cells. In addition, several of these subsets typically express surface markers commonly found on natural killer cells, which has led to the moniker natural killer T cells (NKT cells). This review will describe our current understanding of the unique ways whereby transcription factors control the development and function of an abundant and widely studied lineage of NKT cells that recognizes glycolipid antigens.

Contents

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1 Introduction: What Are Invariant NKT Cells?

In the early 1990's, a population of lymphocytes was identified in the thymus and periphery of C57BL/6 mice that co-expresses an $\alpha\beta$ TCR and the NK receptor NK1.1 (Bendelac et al. 1994; Levitsky et al. 1991). Subsequent analyses revealed that the majority of these cells expressed an invariant TCR α chain, comprised of Vα14 (*Trav11*) joined to Jα18 (*Traj18*), together with a TCR β chain utilizing primarily either the Vβ8 (*Trbv13*), 7 (*Trbv29*) or 2 (*Trbv1*) families (Lantz and Bendelac 1994). These cells, later renamed Vα14 invariant natural killer T cells (Vα14*i* NKT cells), based on their TCR, α chain, or more concisely, *i*NKT cells, were found to recognize glycolipids bound to CD1d, a nonpolymorphic or non-classical MHC class I antigen presenting molecule (Bendelac et al. 1995; Burdin et al. 1998; Kawano et al. 1997). These T cells are typically detected using xCD1d tetramers loaded with the glycolipid α-galactosyl-ceramide (αGalCer), and in fact they have come to be defined by this tetramer staining (Benlagha et al. 2000; Matsuda et al. 2000). Indeed, mice that lack either CD1d or the Jα18 gene segment are essentially devoid of *i*NKT cells (Cui et al. 1997), although there are a very few cells that lack a Jα18 rearrangement that could be detected with αGalCer-loaded CD1d tetramers (Uldrich et al. 2011). In humans, an orthologous population exists that expresses a Vα14-Jα18 homolog, formed by Vα24-Jα18 (*Trav10-Traj18*) rearrangement (Lantz and Bendelac 1994; Exley et al. 1997; Porcelli et al. 1993). In fact, mouse and human *i*NKT cells can be stimulated by αGalCer loaded into CD1d molecules of either species, demonstrating that this specificity is highly conserved (Brossay et al. 1998).

Peripheral mouse Vα14*i* NKT cells were found in the largest numbers in the spleen and liver, where they can account for as much as 1 % (spleen) or 40 % (liver) of the resident lymphocyte pool (Benlagha et al. 2000; Matsuda et al. 2000). Although the numbers and tissue distribution of human Vα24-Jα18 *i*NKT cells are not as well defined, and there was great variability of their frequency in peripheral blood, they appeared to be considerably less common than their mouse counterparts (Ishihara et al. 1999; Lee et al. 2002). The majority of liver and spleen mouse Vα14*i* NKT cells responded with rapid or innate-like kinetics to in vivo challenge by αGalCer or other CD1d-dependent antigens, leading to the secretion of large amounts of both Th1 and Th2 cytokines within 90 min of exposure (Matsuda et al. 2000;

Mattner et al. 2005; Kinjo et al. 2005). Human *i*NKT cells also exhibited the ability to secrete significant amounts of Th1 and Th2 cytokines when stimulated in vitro in the absence of prior priming (Exley et al. 1997; Lee et al. 2002; Gumperz et al. 2002). However, as will be discussed in detail below, recent studies have revealed the presence of additional functional subsets of Vα14*i* NKT cells with different cytokine response profiles compared to those originally defined for the predominant populations in the mouse liver and spleen.

The generation of a mature Vα14*i* NKT cell compartment involves phases of positive selection analogous to those driving the maturation of MHC class I and class II reactive CD4 single positive (SP) and CD8SP thymocytes, together with a proliferative expansion and maturation phase resembling in some aspects the transition of mature peripheral T cells from a naïve to an effector/memory phenotype (Matsuda and Gapin 2005). This process is accompanied by the acquisition of markers associated with the natural killer (NK) cell lineage. Given such complexity, it is thus not surprising that a large number of transcription factors have been found to impact Vα14*i* NKT cell development, including some that have little or no role in the generation of conventional T cell subsets. This review will describe our current understanding of the developmental processes underlying *i*NKT cell differentiation, and the roles that have been defined for transcription factors in regulating these processes, which are summarized in Table 1. We will emphasize studies in the mouse, because the preponderance of the data derives from mouse Vα14*i* NKT cells. We will trace the roles of the relevant transcription factors approximately in order of their influence on the ontogeny of *i*NKT cell differentiation, from the initiating events committing the cells in the thymus through the differentiation of mature functional subsets.

2 Initiating Events in Vα14*i* NKT Cell Differentiation

Several lines of evidence have demonstrated that the development of Vα14*i* NKT cells is thymus-dependent, and does not diverge from that of conventional $\alpha\beta$ T cells until positive selection at the CD4, CD8 DP stage (Kronenberg and Gapin 2002; Matsuda and Gapin 2005; Dao and Nolta 1999; Egawa et al. 2005), although there is evidence that a subset of these cells may be committed to become *i*NKT cells at the double negative (DN) stage (Dashtsoodol et al. 2008). Thus a defining event that initiates Vα14*i* NKT cell development is the formation, through random rearrangement, to generate the invariant Vα14i-Jα18 exon, and its expression in conjunction with a TCR β chain permissive for positive selection by CD1d. However, unlike conventional $\alpha\beta$ T cells, which are selected via MHC-encoded, peptide antigen presenting molecules expressed by thymic epithelial cells, Vα14*i* NKT cell positive selection was found to depend on the recognition of CD1d-dependent determinants presented by DP thymocytes (Wei et al. 2005). Endogenous glycolipids with CD1d-dependent NKT stimulatory activity have been characterized, leading to the hypothesis that thymocytes expressing the

Table 1 The transcription factors discussed in this review, grouped according to their roles in supporting Vα14i NKT development and function

DP lifespan/ rearrangement efficiency	Extrinsic effects on selection	Intrinsic effects on selection	Effector/memory phenotype	Expansion	Thymic subset differentiation	Tissue localization	Functional response	Post-thymic subsets
RORγt	myb	Bcl11b	PLZF	Egr proteins	PLZF	Id2	Hobit	Bcl-6
E and Id proteins	Bcl11b	Elf-1	Egr proteins	BATF	T-bet		Th-POK	RORγT
myb	RelB, p52	Tox	Id proteins	Fra-2/Fosl2	RORγT		Fra-2/Fosl2	FoxP3
Ets-1				c-myc	Vdr		RelA	
				RelA	Med1		p50	
					Hobit		c-Rel	
					Egr proteins			
					SMAD4			
					Th-POK			
					E and Id proteins			
					CBP			
					Klf2			
					Klf13			
					Gata-3			
					E4BP4			

Vα14*i* NKT TCR are selected by agonistic interactions, as opposed to the partially agonistic peptide/MHC complexes that drive the positive selection of conventional αβ T cells. However, the nature of the determinants loaded into CD1d on DP thymocytes that promote Vα14*i*NKT cell positive selection is controversial (Gapin et al. 2013).

In addition to the possible role of agonists in their positive selection, additional or accessory signals are involved in the differentiation of Vα14*i* NKT cells. In particular, homotypic interactions between two members of the SLAM family of surface receptors expressed by DP thymocytes, SLAM and Ly108, were essential for Vα14*i* NKT cell differentiation (Griewank et al. 2007). The signaling cascade initiated by SLAM and Ly108 interactions is complex and as yet incompletely understood, but it is known to involve the adaptor protein SAP, and the Src family tyrosine kinase Fyn (Chung et al. 2005; Eberl et al. 1999; Gadue et al. 1999; Nichols et al. 2005; Pasquier et al. 2005). SAP, which is encoded by the X-linked *Sh2d1a* gene, binds to both SLAM family proteins and to Fyn through distinct domains, and Vα14*i* NKT cell development was profoundly inhibited in the absence of either SAP or Fyn, or the absence of a combination of both SLAM and Ly108 (Griewank et al. 2007; Chung et al. 2005 #2811; Eberl et al. 1999; Gadue et al. 1999; Nichols et al. 2005; Pasquier et al. 2005). Patients with X-linked lymphoproliferative (XLP) disease due to mutations in the *Sh2d1a* gene were highly deficient for Vα24*i* NKT cells, demonstrating that the pathways governing the differentiation of *i*NKT cells in different mammalian species are likely to be similar (Nichols et al. 2005; Pasquier et al. 2005).

2.1 Transcription Factors Acting Early

There is a group of transcription factors that can impact Vα14*i* NKT development indirectly through their effects on DP thymocyte homeostasis. This is because the Jα18 component of the invariant Vα14*i* chain is encoded by a gene segment that is located in the distal region of the Jα locus near to Cα, which only becomes accessible for rearrangement late in the lifespan of vs (Engel and Kronenberg 2012; Guo et al. 2002). As a result, mutations that shorten DP thymocyte lifespan have a severe effect on Vα14*i* NKT numbers. While the rearrangement of other distal Jα segments is similarly impaired under these circumstances, because of the presumed redundancy in the diverse TCR repertoires found in MHC class I and class II reactive T lymphocytes, the shortened DP thymocytes lifespan does not lead to any other reported defects in T cell antigen responses.

Transcription factors found to be required to support DP survival, and by extension, the α chain rearrangement required to form the *i*NKT cell TCR, include the RAR-related orphan receptor RORγT, the E protein HEB and the c-myb proto-oncogene (Bezbradica et al. 2005; D'Cruz et al. 2010; Egawa et al. 2005; Hu et al. 2010). Each of these transcription factors is necessary to drive expression of the

anti-apoptotic Bcl-x protein in DP thymocytes and rearrangements in the distal end of the Jα locus. Enforced expression of Bcl-x via transgenesis corrected the DP thymocyte lifespan defect in all three mutant strains, and in the case of RORγT and HEB-null mice, restored Vα14*i* NKT cell development. Interestingly, enforced Bcl-x expression did not correct the Vα14*i* NKT cell developmental defect in c-myb-deficient mice (Hu et al. 2010). Further examination revealed that *CD4cre/myb*$^{f/f}$ DP thymocytes were unable to support Vα14*i* NKT development of *CD1d*$^{-/-}$ thymocytes in mixed chimeric animals, and that c-myb-null DPs exhibited deficiencies in the expression of CD1d, SLAM, Ly108 and SAP. Thus c-myb plays both Vα14*i* NKT cell-extrinsic and cell-intrinsic roles in the initiation of Vα14*i* NKT cell development.

Two recent reports provided evidence that an excess of activity from the E protein family of transcription factors can greatly enhance Vα14*i* NKT cell lineage specification (Li et al. 2013; Verykokakis et al. 2013). Both groups studied the Id2 and Id3 proteins that act as dominant negative inhibitors of E protein DNA binding, and found that inactivation of both the Id2 and Id3 genes at or prior to the DP stage led to a profound increase in Vα14*i* NKT cell numbers. However, the two groups came to different conclusions as to the mechanism by which E proteins were promoting Vα14*i* NKT specification. One of the studies came to the surprising conclusion that an excess of E protein activity, beginning prior to the DP stage, led to a specific increase in cells with an invariant Vα14-Jα18 rearrangement, suggesting that Vα14*i* NKT cell fate may be specified before surface expression of the Vα14*i* NKT cell TCR (Li et al. 2013). In contrast, the other report presented evidence that a deficit in Id proteins, and in particular, Id3, led to a general increase in rearrangement events at the distal end of the Jα locus, thus increasing the frequency of formation of the rearrangement (Verykokakis et al. 2013). The findings of the latter study are consistent with the known roles of E proteins in promoting Rag gene expression and antigen receptor gene rearrangement (Jones and Zhuang 2009).

The zinc finger transcription factor Bcl11b, which is known to control many aspects of thymocyte maturation, has also been shown to act through both Vα14*i* NKT cell-intrinsic and extrinsic mechanisms to support the initiation of Vα14*i* NKT cell development (Albu et al. 2011). However, Bcl11b appears to regulate processes that are distinct from those controlled by c-myb. While a deficit in Bcl11b did not affect TCRα rearrangement, mixed BM chimera experiments demonstrated that *CD4cre/Bcl11b*$^{f/f}$ thymocytes exhibited a profound block in Vα14*i* NKT cell development, even in the presence of WT DPs. The mechanism(s) underlying the Vα14*i* NKT cell-intrinsic functions of Bcl11b are as yet undefined, but may be related to TCR signaling defects that have been defined in Bcl11b-deficient DP thymocytes (Albu et al. 2007). A second set of mixed chimera experiments using *CD4cre/Bcl11b*$^{f/f}$ and *β2m*$^{-/-}$ (and thus surface CD1d-null) bone marrow showed that Bcl11b-deficient DP thymocytes could not positively select Vα14*i* NKT cell precursors (Albu et al. 2011). While Bcl11b-deficient DP thymocytes expressed normal levels of surface CD1d, they exhibited defects in their ability to present variants of αGalCer to Vα14*i* NKT cell hybridomas that

require intracellular processing in order to be recognized by the TCR. Furthermore, *CD4cre/Bcl11b*$^{f/f}$ thymocytes displayed abnormal lipid metabolism. These data imply that Bcl11b may control the expression of genes necessary for processing or loading into CD1d the determinants that act to positively select Vα14*i* NKT cells. In support of this hypothesis, Bcl11b-deficient thymocytes exhibited deficits in the expression of several genes involved in either sphingolipid processing or glycolipid trafficking.

The Ets family of transcription factors also plays important roles at the early stages of Vα14*i* NKT development. Mice lacking Ets-1 function were essentially devoid of Vα14*i* NKT cells, which may be due to defects in DP lifespan or TCRα rearrangement, as Ets-1-null DP thymocytes exhibit severely reduced levels of Vα14*i* transcripts (Walunas et al. 2000; Choi et al. 2011). An absence of Ets family member Elf-1 also led to a deficit in Vα14*i* NKT cells, though less pronounced than that observed in *Ets*$^{-/-}$ mice (Choi et al. 2011). Vα14*i* rearrangement appeared normal in *Elf-1*$^{-/-}$ DP thymocytes, so it is unlikely that a deficiency for Elf-1 affects Vα14*i* NKT precursor frequency. This *Elf-1*$^{-/-}$ developmental defect was Vα14*i* NKT cell-intrinsic, and may be related to an increased rate of apoptosis at an early stage of Vα14*i* NKT thymocyte maturation. A third member of the Ets family, MEF, was also likely to be necessary for normal Vα14*i* NKT cell development (Lacorazza et al. 2002).

The HMG-box containing protein TOX is induced in DP thymocytes after positive selection, and it has been shown to be required for the development of all CD4 SP thymocytes (Aliahmad and Kaye 2008). Vα14*i* NKT cells also were virtually absent from both the thymus and periphery of TOX-null mice. Although the nature of this defect has not been directly addressed, the timing of TOX expression strongly implies that it acts very early after positive selection to promote Vα14*i* NKT cell differentiation in a cell-intrinsic manner, and the data further suggested that there is some overlap between the factors necessary to promote development of the MHC class II reactive CD4 SP and Vα14*i* NKT lineages.

2.2 PLZF, EGR and E Proteins

Following positive selection, Vα14*i* NKT thymocytes undergo proliferative expansion and begin to express many of the markers typical of mature *i*NKT cells. Although this process is regulated by a number of transcription factors that also influence the differentiation of conventional T cell subsets, a key role is played by the broad complex tramtrack bric-a-brac-zinc finger (BTB-POZ) family member VS (encoded by the *Zbtb16* gene), whose expression in the hematopoietic lineage is largely confined to , including a subset of $\gamma\delta$ T cells in addition to *i*NKT cells (Kovalovsky et al. 2008; Savage et al. 2008). Egr2 acts to promote PLZF expression, although as discussed below, it has additional functions in Vα14*i* NKT cell development. Although T cells expressing the Vα14*i* TCR could be detected in PLZF-null mice, they were found in much smaller numbers. Furthermore, in terms

of surface antigen phenotype, functional response characteristics, and tissue distribution, PLZF-deficient Vα14*i* NKT cells were more similar to naïve CD4 and CD8 T lymphocytes than to wild type Vα14*i* NKT cells. Additionally, enforced expression of PLZF throughout the T cell lineage resulted in the acquisition of memory/effector phenotypic properties in conventional CD4 and CD8 SP T cells, as well as an increase in cell homing to the liver (Kovalovsky et al. 2010; Savage et al. 2008, 2011). Furthermore, the memory/effector-like T cells in PLZF transgenic mice displayed an unpolarized response with respect to the production of Th1 and Th2 cytokines, again similar to the phenotype of the dominant population of Vα14*i* NKT cells (Kovalovsky et al. 2010; Raberger et al. 2008; Savage et al. 2008). These observations suggest that, while PLZF is dispensable for the initial positive selection of Vα14*i* NKT cells, it is essential for much of their developmental program, notably the acquisition of effector functions. It is also likely that PLZF plays an analogous role in the maturation of other innate-like T cell lineages.

The mechanism(s) by which PLZF exerts its control of Vα14*i* NKT cell maturation are not well understood. Examination of the effect of PLZF deficiency on global gene expression in thymocytes expressing an innate-like $\gamma\delta$ TCR transgene identified several genes whose expression is dysregulated in the absence of PLZF, many of which also were preferentially expressed in Vα14*i* NKT cells (Gleimer et al. 2012). However, direct targets of PLZF have yet to be defined. BTB-POZ proteins are known to associate with a co-repressor complex containing NCoR, Sin3a and HDAC1, and so may act primarily as transcriptional repressors, although examples of BTB-POZ family proteins acting to directly promote transcription are also known (McConnell and Licht 2007). Interestingly, PLZF and other BTB-ZF factors have also been shown to associate with the E3 ubiquitin ligase cullin 3 (CUL3) and to transport it to the nucleus (Mathew et al. 2012). As deletion of *CUL3* in the T cell compartment phenocopied the effect on the Vα14*i* NKT cell lineage observed in PLZF-deficient mice, it is likely that PLZF exerts its control of the Vα14*i* NKT cell developmental program through CUL3-dependent modification of transcription complexes (Mathew et al. 2012).

Thymocyte development is largely directed by signaling events transmitted from the TCR complex that trigger calcium influx and calcineurin-dependent activation of NFAT proteins, as well as the activation of the Ras-MEK-Erk pathway. Both of these pathways act to drive expression of the Egr family of transcription factors, and genetic alterations that impair signaling through either of these pathways are inhibitory for conventional T cell development (Bueno et al. 2002; Neilson et al. 2004; Swan et al. 1995; Alberola-Ila et al. 1995). Similarly, disruption of either Ras-MEK-Erk or calcineurin/NFAT signaling resulted in an early block of Vα14*i* NKT cell development (Hu et al. 2011; Lazarevic et al. 2009). However, Vα14*i* NKT thymocytes express higher and more sustained levels of Egr proteins, and were found to be more dependent upon Egr2 than CD4 and CD8 SP thymocytes (Lazarevic et al. 2009; Seiler et al. 2012). These relatively high Egr levels play a critical and direct role in activating expression of PLZF (Seiler et al. 2012). Egr2 has been shown to bind directly to a site in the *Zbtb16* promoter, and Egr-deficient Vα14*i* NKT thymocytes do not upregulate PLZF. It is

possible that accessory signaling via the SLAM/SAP pathway acts to promote the increased levels of Egr necessary to promote PLZF expression, as agonistic antibodies against Ly108 have been shown to synergize with TCR cross-linking to prolong proximal TCR signaling events and increase expression of Egr2 and PLZF (Dutta et al. 2013). However, it may be that the increased levels of Egr proteins in Vα14*i* NKT thymocytes are largely a consequence of more potent TCR signaling due to the presumably agonistic nature of the selecting ligands for the Vα14*i* NKT TCR. This is supported by the fact that treatment with anti-TCRβ antibodies in vivo induced high levels of Egr2 and expression of PLZF in the majority of thymocytes (Seiler et al. 2012).

It is also evident that Egr proteins function to promote Vα14*i* NKT cell differentiation independently of their role in promoting PLZF expression. PLZF-deficiency did not reduce the cell cycle activity of Vα14*i* NKT thymocytes, and enforced expression of PLZF in T cells conferred an effector-like phenotype in the absence of increased cell division or agonist signaling (Kovalovsky et al. 2008, 2010; Savage et al. 2008, 2011). Because both Egr2 and PLZF-null Vα14*i* NKT thymocytes were defective in expansion, these data imply that PLZF acts to promote survival, while the proliferation of the Vα14*i* NKT pool is driven by mitotic signals that may be dependent upon Egr activity. The proliferative expansion of Vα14*i* NKT cells has also been shown to require the proto-oncogene c-myc, a transcription factor known to promote mitotic activity in a variety of cell types (Dose et al. 2009; Mycko et al. 2009). It is not been determined if Egr proteins drive c-myc expression; however, c-myc deficient Vα14*i* NKT cells did not exhibit defects in PLZF expression, which likely places c-myc downstream of Egr or other more immediate effectors of Vα14*i* NKT cell differentiation.

Egr proteins are also known to activate transcription of the Id2 and Id3 genes, in turn leading to the inhibition of E proteins that is critical for maturation at multiple stages of thymocyte development (Bain et al. 2001). It has now been shown that in the absence of both Id2 and Id3, although Vα14*i* NKT thymocyte numbers were greatly increased, due to the effects on rearrangement to form the Vα14*i* TCR that were described above, the resulting Vα14*i* NKT cells expressed reduced levels of PLZF and were blocked at a very early stage of maturation (Verykokakis et al. 2013). These data imply that Egr proteins are only able to promote expression of PLZF after an E protein-dependent inhibitory mechanism is attenuated by Id proteins. In contrast, another group has now reported that E proteins positively regulate PLZF expression (D'Cruz et al. 2014). In this study, mice were examined that had CD4cre-driven deletion of both E protein genes, *E2A* and *HEB*, and also carried a Vα14*i* transgene to bypass the aforementioned rearrangement-dependent block in *i*NKT development observed in HEB-deficient thymocytes. The Vα14*i* NKT cells from these E protein-deficient mice were also blocked at a very early maturation stage and exhibited reduced expression of PLZF. Furthermore, it was demonstrated that E2A and HEB bound to two regions within the *Zbtb16* promoter. Taken together, these studies raise the paradoxical notion that E proteins can act both to promote as well as inhibit PLZF expression, at apparently the same

stage of Vα14*i* NKT maturation. It should be noted that the possibility that E proteins support Vα14*i* NKT positive selection by promoting DP thymocyte CD1d antigen presentation has not yet been formally excluded. An extrinsic defect in Vα14*i* NKT positive selection observed in c-Myb deficient thymocytes is thought to explain the elevated fractions of immature iNKT cells seen in *CD4cre/Myb*$^{f/f}$ mice carrying a Vα14*i* transgene (Hu et al. 2010). A similar extrinsic defect in Vα14*i* NKT selection could thus potentially underlie the defects in iNKT maturation and PLZF expression found in E protein deficient, Vα14*i* transgenic thymocytes. Clearly, additional experiments will be necessary to resolve how E and Id proteins function to regulate the early stages of Vα14*i* NKT development, and in particular the expression of PLZF.

2.3 AP-1 Transcription Factors

AP-1 transcription factor complexes, which are dimers of members of the Jun, Fos, ATF or MAF families of basic leucine zipper (bZIP) proteins, are prominent regulatory factors in a number of cell types, and are activated by a number of signaling cascades that operate in the T cell lineage. Manipulation of two AP-1 proteins has been shown to impact Vα14*i* NKT development, but in opposing directions, suggesting that different members of this family may either support or inhibit Vα14*i* NKT cell differentiation. Transgene-enforced overexpression of BATF, which is a negative regulator of AP-1 activity, resulted in a substantial decrease in Vα14*i* NKT cells in the thymus and periphery (Williams et al. 2003; Zullo et al. 2007). Deletion of *BATF* did not lead to changes in Vα14*i* NKT thymocyte numbers, although peripheral Vα14*i* NKT cells were increased in BATF-deficient animals (Betz et al. 2010; Jordan-Williams et al. 2013). However, a T-cell specific deletion of the Fos family member *Fra-2/Fosl2* led to significantly increased numbers of Vα14*i* NKT thymocytes (Lawson et al. 2009). It is has not been determined what stages of Vα14*i* NKT cell development were impacted by these perturbations of AP-1 activity, however, the known roles of these factors in regulating cell proliferation and survival suggest the possibility that AP-1 factors are important for the expansion of the Vα14*i* NKT thymocyte pool.

2.4 The NF-κB Family

The NF-κB family of transcription factors influences a vast number of processes in inflammation, cell survival and differentiation (Vallabhapurapu and Karin 2009). This family includes five members, p50-NF-κB1, p52-NF-κB2, c-Rel, RelB and RelA, which can form heterodimers, or in some cases, homodimers. The inhibitor of NF-κB (I-κB) complex retains these transcription factors in the cytoplasm in the absence of stimulatory signals. In the classical NF-κB pathway, the trimeric I-κB

kinase (IKK) complex is activated by stimulatory signals and it induces the phosphorylation and proteosomal degradation of I-κBα, which in turn allows NF-κB transcription factors to enter the nucleus. The alternative pathway involves the NF-κB inducing kinase (NIK) and IKKα, which phosphorylate the p100 precursor that facilitates its processing to generate p52-NF-κB2, and subsequent translocation of p52-RelB heterodimers to the nucleus (Dejardin et al. 2002).

Genetic manipulation of expression of members of the NF-κB pathway showed that these molecules play several roles in the differentiation and function of Vα14*i* NKT cells. A global diminution of NF-κB activity has been obtained by deleting the IKKβ subunit of the IKK complex, or alternatively, this has been achieved by creating a mouse strain expressing a degradation resistant form of the α chain of I-κB (I-κBα). Either strategy led to an almost complete loss of Vα14*i* NKT cells (Schmidt-Supprian et al. 2004; Stanic et al. 2004), although in one study the effect of inhibiting degradation of I-κBα was less pronounced (Sivakumar et al. 2003). However, deletion of individual members of the NF-κB family had more subtle, diverse, and generally later acting effects.

Deletion of *RelB*, or mutation of its upstream activator NIK, had effects on stromal cells that impaired Vα14*i* NKT cell differentiation. This effect was not related directly to TCR specificity for CD1d, as $Cd1d^{-/-}$ stromal cells were also capable of providing this accessory function (Elewaut et al. 2003; Sivakumar et al. 2003). RelB deficiency likely acted through reduced production of IL-15 mRNA by thymic stromal cells, as this transcript was decreased three-fold in the thymus tissue from $RelB^{-/-}$ mice (Sivakumar et al. 2003). IL-15 is needed for the survival and expansion of Vα14*i* NKT cell precursors (Matsuda et al. 2002; Ranson et al. 2003), and for their differentiation to form the most mature Vα14*i* NKT cells that express NK receptors, such as NK1.1 (Townsend et al. 2004). Deletion of *Nfκb2* in mice caused a reduction in Vα14*i* NKT cells that also was dependent on p52-NF-κB2 expression in an irradiation-resistant cell (Sivakumar et al. 2003). This is consistent with an activity of RelB-p52 heterodimers in the thymic stroma, but the effect of *Nfκb2* deletion was modest compared to the effect of *RelB* gene deficiency, suggesting that RelB must also be acting through other partners.

All the other effects of removal of single NF-κB subunits were cell autonomous, affecting either the precursor or the more mature Vα14*i* NKT cell itself. A defect in RelA caused the most profound decrease in the number of Vα14*i* NKT cells, with the $NK1.1^+$ cells being particularly reduced, especially in the periphery (Stankovic et al. 2011; Vallabhapurapu et al. 2008). The NK1.1 negative subset was not decreased in the thymus in one study (Stankovic et al. 2011), but was diminished in another (Vallabhapurapu et al. 2008). When RelA was deleted, not only was there a reduction in $NK1.1^+$ Vα14*i* NKT cells in the thymus, but also the NK1.1 thymic Vα14*i* NKT cells were impaired in their ability to expand in vitro in response to IL-15 (Vallabhapurapu et al. 2008). Furthermore, RelA-p50 heterodimers were shown to bind to an intronic sequence in the IL15Rα gene. Overall, the data are consistent with a model proposing that RelA is important for inducing expression of the IL-15 receptor, which is important for the expansion of maturing Vα14*i* NKT cells and/or survival of their mature, NK1.1 expressing counterparts.

Deficiency of NF-kB1 was reported to cause either a mild deficiency, on the C57BL/6 background (Stankovic et al. 2011), or a severe deficiency due to impaired survival of maturing Vα14*i* NKT cells, when mice on a mixed genetic background were analyzed (Stanic et al. 2004). By contrast, c-Rel deficiency had no effect, either on its own or in combination with NF-κB1 deficiency (Stankovic et al. 2011; Vallabhapurapu et al. 2008).

3 Vα14*i* NKT Thymic Maturation

3.1 Stages Versus Subsets

The differentiation of Vα14*i* NKT thymocytes has typically been characterized by the down regulation of CD24 expression, followed by the successive acquisition of CD44, and as noted above, NK1.1 surface antigen expression. This has led to a developmental staging scheme outlined in Fig. 1a, in which CD1d tetramer$^+$ thymocytes that are CD24$^+$, CD44$^-$, NK1.1$^-$ have been termed Stage 0, cells that are CD24$^-$, CD44$^-$, NK1.1$^-$ correspond to Stage 1, and CD24$^-$CD44$^+$ cells that are NK1.1$^-$ or NK1.1$^+$ correspond to Stages 2 and 3, respectively (Benlagha et al. 2005).

Recent studies, however, have helped to overturn the idea of a linear developmental pathway leading to a Vα14*i* NKT cell population that is relatively homogenous in function. The revision in thinking is focused in part on the Stage 2 population, which was thought to represent solely immature Vα14*i* NKT cells mostly destined to acquire NK receptor expression. However, evidence is accumulating that the population with the Stage 2 phenotype (CD24$^-$CD44$^+$,NK1.1$^-$) also contains two different mature subsets. The largest population with a Stage 2 phenotype in most mouse strains is biased to producing Th2 cytokines and can respond to IL-25 in a TCR-independent fashion, via expression of its receptor, also known as IL-17RB, while a smaller subset has been characterized that primarily produces IL-17 and in some cases IL-22 (Lee et al. 2013; Watarai et al. 2012; Michel et al. 2008). These findings have led to a new model emphasizing the functional heterogeneity of Vα14*i* NKT cells, in which the Th2 and Th17-biased populations are designated as NKT2 and NKT17, respectively (Fig. 1b). The remaining mature Vα14*i* NKT thymocytes, which produce both Th1 and Th2 cytokines, are referred to as NKT1. The NKT1 fraction represents essentially the entire Stage 3 population, but also comprises a small fraction of Stage 2 or NK1.1 negative cells, distinguished from NKT2 and NKT17 cells by the absence of expression of IL17RB. IL17RB is more than just a surface marker, however, as in its absence NKT2 and NKT17 development was largely abrogated, while NKT1 development appeared unaffected (Watarai et al. 2012). Furthermore, IL17RB expression can be detected even within the Stage 1 population, suggesting the possibility of an early bifurcation of the NKT1 population from NKT2 and NKT17

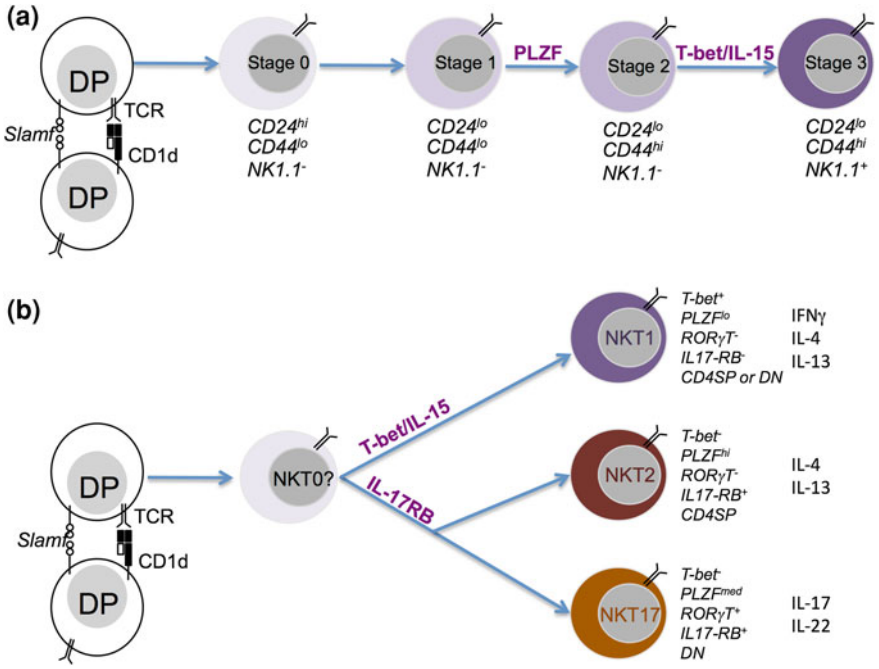

Fig. 1 Models of Vα14*i* NKT development in the thymus. **a** The original model, based largely on the observation that after positive selection Vα14*i* NKT cells downregulate CD24 and then successively elevate expression of CD44 and NK1.1. Expression of high levels of CD44 and other activation markers is dependent upon PLZF, while acquisition of NK1.1 and other NK antigens requires T-bet and IL-15 signaling. **b** Model based on the identification of three distinct subsets of mature Vα14*i* NKT cells in the thymus. These subsets are designated as NKT1, NKT2 and NKT17, as their cytokine expression profiles are respectively similar to that of the Th1, Th2 and Th17 subsets of mainstream CD4SP T cells (although NKT1 cells can produce Th2 as well as Th1 cytokines in response to TCR-dependent activation). The subsets can be defined on the basis of the differential expression of the transcription factors PLZF, T-bet and RORγT, or the surface markers CD4 and IL-17RB. The NKT2 and NKT17 subsets are found largely within Stage 2 ($CD44^{high}$, $NK1.1^{low}$), while NKT1 cells predominate in Stage 3 ($CD44^{high}$, $NK1.1^{high}$). IL-17RB is necessary for the development of both NKT2 and NKT17 cells, while NKT1 development is dependent upon T-bet and IL-15 signaling

cells (Watarai et al. 2012). It should also be noted that the ratios of NKT1, NKT2 and NKT17 cells vary greatly between different mouse strains, and that the most commonly used strain, C57BL/6 mice, has a relatively high fraction of (Lee et al. 2013). Also, we emphasize that the developmental stages versus functional subset models are not mutually exclusive. The consensus remains that Stages 0 and 1 are precursors and the IL-17RB$^-$ Stage 2 cells are likely precursors of the NK1.1$^+$ Stage 3 population (Watarai et al. 2012).

Much of the work that has led to the elucidation of the NKT1, 2 and 17 subsets has been through studies of their differential expression of transcription factors, in particular, T-bet, PLZF and RORγT. NKT1 cells express high levels of T-bet and

relatively low levels of PLZF, NKT2 cells express the highest amounts of PLZF and are mostly T-bet$^{low/-}$, and NKT17 cells are T-bet^{-}, exhibit intermediate levels of PLZF, and are the only subset that expresses RORγT (Lee et al. 2013). T-bet, PLZF and RORγT levels can all be determined at a single cell level by flow cytometry, and thus are now being used as markers to define the three mature Vα14*i* NKT thymic subsets.

3.2 NKT1 Promoting Transcription Factors

The functional importance of T-bet for the NKT1 subset is evident from analyses of T-bet-deficient animals, which exhibited profound deficits in NKT1/Stage 3 cells (Townsend et al. 2004). T-bet, which is encoded by the *Tbx21* gene, is necessary for expression of the IL2Rβ receptor (CD122), which forms part of the IL-15R complex, and deficiencies in either CD122 or IL-15 largely phenocopied the NKT1/Stage 3 defect observed in -null mice (Gordy et al. 2011; Townsend et al. 2004). Furthermore, T-bet is known to be required for expression of a variety of NK receptors, IFNγ, perforin and FasL in both Vα14*i* NKT cells and NK cells (Townsend et al. 2004). A number of other transcription factors also acted to promote NKT1/Stage 3 development, including the Vitamin D receptor (VDR), the Mediator complex member Med1, which acts to recruit RNA polymerase II to steroid hormone receptor/DNA complexes, Egr proteins and the Blimp-1 homolog Hobit. VDR and Med1-deficient Vα14*i* NKT cells exhibited defects in T-bet and CD122 expression, suggesting that these factors may lie upstream of the T-bet/IL-15 axis (Yu and Cantorna 2008; Yue et al. 2011). In addition to their role in driving the initial phases of Vα14*i* NKT cell differentiation, Egr proteins may also support the later maturation of NKT1/Stage 3 cells, at least in part through their binding and direct activation of the CD122 promoter (Seiler et al. 2012). Hobit, on the other hand, does not control T-bet levels, and thus may promote NKT1/Stage 3 thymocyte numbers through different mechanisms (van Gisbergen et al. 2012).

3.3 Transcription Factors that Regulate the NKT17 Subset

As RORγt is recognized as the signature transcription factor of lymphocytes that produce IL-17, it is not surprising that it drives the differentiation of NKT17 cells as well. It is difficult to directly examine the effect of an absence of RORγT expression on the NKT17 subset, however, because of the requirement for RORγT for optimal DP thymocyte survival, and when absent, the resulting rearrangement defect that cause an early block in Vα14*i* NKT cell development. Despite this, there has been progress in defining factors that are likely to act upstream of RORγT to support NKT17 development. One of these factors is activated through

TGF-β signaling. Deletion of the *TGRβRII* gene in DP thymocytes markedly reduced the number of RORγT$^+$ Vα14*i* NKT cells in the thymus and periphery, without affecting total Vα14*i* NKT cell numbers (Havenar-Daughton et al. 2012). TGF-β signaling induces the phosphorylation and nuclear translocation of the SMAD family of transcription factors, consisting of heterodimers of SMAD2 or SMAD3 with SMAD4. Deletion of *SMAD4* at the DP stage resulted in deficits of RORγT$^+$ Vα14*i* NKT cells that were similar to those observed in the absence of TGF-βRII, demonstrating that the effect of TGF-β signaling on NKT17 development was SMAD-dependent (Havenar-Daughton et al. 2012). NKT17 thymocytes also exhibited higher basal levels of TGRβRII and phospho-SMAD 2/3 than other Vα14*i* NKT thymoyctes, but they express relatively low levels of the IL-15Rβ receptor CD122 and are less responsive to IL-15, as measured by the phosphorylation of STAT5. These data distinguish the response to cytokines by NKT17 cells from that of the NKT1 subset. The latter are known to express high levels of CD122 and are dependent upon the IL-15 signaling pathway for their homeostasis. TGF-β signaling also functions to support the T-bet/IL-15/CD122 axis necessary for NKT1 cells, as well as to promote the survival of Stage 1 NKT thymocytes, but through pathways independent of SMAD4 (Doisne et al. 2009).

NKT17 development also is limited by the activity of the BTB-POZ family member Th-POK/c-krox/Zbtb7b (Th-POK). Th-POK directs much of the CD4 CD4 SP T cell gene expression program, including the expression of CD4 and the inhibition of CD8 co-receptor expression in differentiating MHC class II reactive thymocytes (He et al. 2005; Sun et al. 2005). Similarly, whereas WT Vα14*i* NKT cells are either CD4 SP or DN, therefore completely lacking expression of CD8, in mice without a functional -POK gene they are either CD8SP or DN (Engel et al. 2010; Wang et al. 2010; Enders et al. 2012). Th-POK also represses NKT17 cell number in both the thymus and the periphery in a gene dose-dependent manner, and conversely supports Stage 3/NKT1 development in the thymus (Engel et al. 2012; Enders et al. 2012). It is interesting to note that cells are mostly DN, while NKT2 cells are nearly all CD4SP. Because CD4 expression is dependent upon Th-POK, these data are consistent with a model in which high Th-POK activity prevents NKT17 differentiation.

3.4 NKT2 Cells and Innate-Like CD8SP Thymocytes

NKT2 thymocytes were discovered in part through analysis of the requirements for the development of a polyclonal population that displays a robust, unprimed IFNγ response to antigen (Lee et al. 2013; Atherly et al. 2006; Broussard et al. 2006). These innate-like CD8SP cells are rare in C57BL/6 mice, but are relatively numerous in the BALB/c strain, as well as in C57BL/6 mice with any of a number of genetic alterations, including null mutations in the Tec family non-receptor tyrosine kinase Itk, the Kruppel-like factor 2 (KLF2) and CRE binding protein (CBP) transcription factors, the E protein inhibitor Id3, and point mutations in the

SLP76 signal transduction protein (Alonzo et al. 2010; Atherly et al. 2006; Broussard et al. 2006; Fukuyama et al. 2009; Jordan et al. 2008; Verykokakis et al. 2010b; Weinreich et al. 2009, 2010). Further analyses revealed that strains that were permissive for the generation of innate-like CD8 SP had increased populations of NKT-like populations, and that IL-4 produced by these NKT cells was necessary to support innate-like CD8 development. Analysis of Vα14*i* NKT cells in BALB/c mice revealed the identity of the NKT2 subset, which is much more numerous in BALB/c as compared to C57BL/6 mice, as the source of IL-4 driving the outgrowth of innate CD8SP T cells (Lee et al. 2013). NKT2 cells have been shown to be under the control of Krüppel-like (KLF) transcription factor family members, as their relative abundance in the BALB/c background is dependent on the function of KLF13, while the deletion of *KLF2* leads to increased NKT2 numbers in C57BL/6 mice (Weinreich et al. 2009; Lai et al. 2011). However, the levels of KLF2 and 13 are similar in these two mice strains, indicating that other as yet undetermined factors must account for the difference in NKT2 cell frequency between BALB/c and C57BL/6 mice. It should be noted that some of the mutations that result in increased innate-like CD8 T cells do so at least in part through the expansion of other innate-like T lymphocytes, including $\gamma\delta$ T cells with an NKT phenotype. Also, innate-like CD8 T cells could be augmented by poly-clonal CD4 T lymphocytes that were generated in mice with enforced expression of MHC class II on DP T cells in the absence of epithelial MHC class II, which resulted in CD4SP cells that were selected in the context of SLAM-SAP signaling that adopted NKT-like characteristics (Verykokakis et al. 2010a; Li et al. 2005). Therefore, the ability to promote the differentiation of innate-like CD8 T cells does not absolutely require the expression of CD1d or the specificity of Vα14*i* NKT cells.

Cells in both the NKT2 and NKT17 subsets express high levels of GATA-3, which has been shown to be associated with MHC class II-reactive CD4 SP Th2 cells, as well as IRF-4, which is expressed by both the Th2 and Th17 conventional CD4 T lymphocyte subsets. Studies of the role of GATA-3 in Vα14*i* NKT cell development and function were published prior to the characterization of functional subsets. However, deletion of *GATA3* in DP thymocytes results in a markedly reduced number of Vα14*i* NKT cells with a Stage 2 phenotype, consistent with a role for GATA-3 in supporting the development of NKT2 cells, which are the major population in the Stage 2 phenotype gate (Kim et al. 2006). GATA-3 has been shown to positively regulate Th-POK expression, and it is necessary for CD4 expression in conventional T as well as Vα14*i* NKT cells (Wang et al. 2008, 2010). However, in contrast to GATA-3 null mice, Th-POK-deficient animals display a dramatic increase in the numbers of Stage 2 Vα14*i* NKT cells, as a consequence of the expansion of the NKT17 subset (Kim et al. 2006; Engel et al. 2012). Taken together, these data imply that GATA-3 acts independently of Th-POK to regulate NKT cell subset development. The effects of IRF4 deficiency on the NKT2 and NKT17 subsets also have not been examined by flow cytometric analyses. However, although IRF4 has been shown to be necessary for IL-17 and IL-22 production in by mainstream Th17 cells, peripheral Vα14*i* NKT cells do not display a defect in

the production of these cytokines in the absence of IRF4 (Raifer et al. 2012). It is not yet understood why NKT17 cells produce very low levels of Th2 cytokines, nor why NKT1 cells make appreciable amounts of Th2 cytokines upon stimulation, despite expressing relatively low levels of both GATA-3 and IRF4 (Lee et al. 2013). One possibility is that Th2 cytokine production by the NKT1 subset could be a consequence of PLZF expression, as suggested by the unpolarized cytokine response profiles observed in T cells from PLZF transgenic mice (Savage et al. 2008; Kovalovsky et al. 2010). Presumably the response profile of the NKT17 lineage is restricted, even in the presence of PLZF, by RORγT or other NKT17-specific factors.

The IL-17RB surface marker that is expressed by both the NKT2 and NKT17 subsets, but not by NKT1 cells, is a receptor for IL-25 (Terashima et al. 2008; Stock et al. 2009). Treatment of sorted NKT2 cells with IL-25 elicited the production of several cytokines, in particular IL-9 and IL-10 and IL-13 (Watarai et al. 2012). IL-25 has also been shown to induce expression of the basic leucine zipper (bZIP) transcription factor E4BP4, which in turn acted to drive expression of IL-10 and IL-13 in bulk Vα14*i* NKT cells as well as conventional and regulatory T lymphocytes subsets (Motomura et al. 2011). Consistent with E4BP4 controlling expression of these cytokines, NKT2-enriched populations from E4BP4-null mice had decreased production of IL-9, IL-10 and IL-13 in response to IL-25 (Watarai et al. 2012). Interestingly, NKT2 cells stimulated with IL-25 also produced IL-22, which is more typically associated with the Th17 and NKT17 subsets, and this IL-22 expression was also dependent upon E4BP4 (Watarai et al. 2012).

3.5 E Proteins and Thymic NKT Subset Specification

Three separate studies show that E proteins, in addition to their functions in inhibiting the initial selection of Vα14*i* NKT cells, also regulate Vα14*i* NKT thymocyte subset differentiation. One report found that Vα14*i* NKT thymocytes in mice lacking Id3 exhibited decreased numbers of Stage 3 cells (Verykokakis et al. 2013). Another group found a similar (though somewhat milder) effect of Id3 deficiency on Stage 3 NKT development, and furthermore reported that deletion of either the *Id2* or *Id3* genes resulted in a bias towards NKT2 and against NKT1 development (D'Cruz et al. 2014). A third study used a different approach to increase E protein activity, through transgenic expression of a protein that interfered with Id-mediated inhibition of E protein DNA binding by competing with Id proteins for dimerization with the E proteins, while stimulating rather than inhibiting their transcriptional activity. This report demonstrated that enhancing E protein function increased both NKT2 and NKT17 subset numbers at the expense of NKT1 cells (Hu et al. 2013).

4 Peripheral Vα14*i* NKT Cells

4.1 Localization and Function

The three Vα14*i* NKT thymic subsets discussed here have distinct peripheral localization profiles. NKT1 cells account for the vast majority of liver Vα14*i* NKT cells in all mouse strains that have been examined, and they are also found in large numbers in the spleen (Lee et al. 2013; Watarai et al. 2012). In contrast, NKT2 and NKT17 cells tend to be excluded from the liver and spleen to varying extents, depending on the mouse strain, but they comprise large fractions of the smaller Vα14*i* NKT populations in the lung, peripheral LN and skin (Lee et al. 2013; Watarai et al. 2012). The dominance of the NKT1 subset in the some of the sites where Vα14*i* NKT cells are most abundant in the periphery is correlated with their abundance in the thymus, particularly in C57BL/6 mice. However, studies designed to identify recent thymic emigrants have led to the paradoxical observation that the Vα14*i* NKT cells that exited the thymus were primarily NK1.1^{-}, despite the fact that NKT1 cells comprise only a small fraction of NK1.1^{low} Vα14*i* NKT thymocytes (Benlagha et al. 2002; Pellicci et al. 2002; Watarai et al. 2012). Acquisition of NK1.1 expression by Vα14*i* NKT cells after emigration from the thymus required the peripheral expression of CD1d, suggesting that the final maturation of most liver and spleen Vα14*i* NKT cells depended on TCR recognition (McNab et al. 2005; Wei et al. 2005). Recent data suggest that NKT2 and NKT17 cells do not change phenotype after sorting and adoptive transfer, likely ruling out the possibility that peripheral NKT1 cells are principally derived from NKT2 or NKT17 precursors recently emigrated from the thymus (Lee et al. 2013; Watarai et al. 2012). Taken together, these findings suggest that NK1.1^{low} NKT1 cells are either exported more efficiently from the thymus or have survival or proliferative advantages in the periphery relative to NKT2 and NKT17 cells. Studies demonstrating that a high fraction of peripheral NKT17 cells are recent thymic emigrants are consistent with the hypothesis that the NKT17 subset may be short-lived outside of the thymus (Milpied et al. 2011).

As expected, mutations that severely compromised Vα14*i* NKT development in the thymus typically led to dramatic reductions of peripheral Vα14*i* NKT cells. Interestingly, deletion of in the T cell lineage dramatically reduced Vα14*i* NKT cells in the liver and spleen, without having a major effect on overall NKT thymic cellularity (Kim et al. 2006). However, while a deficiency for GATA-3 did not affect Stage 3 Vα14*i* NKT thymocyte cell numbers, Stage 2 NKT cells were severely reduced. In light of our current understanding of thymic Vα14*i* NKT cell subsets, these data imply that the homeostasis of NK1.1^{low} NKT1 cells may be compromised in the absence of GATA-3, leading to the reduced peripheral NKT1 numbers. The effect of a deficiency in the GATA-3 target Th-POK on Vα14*i* NKT cell numbers in the spleen and liver was relatively mild, suggesting that the role for GATA-3 in supporting spleen and liver Vα14*i* NKT cellularity is largely independent of Th-POK (Wang et al. 2010; Engel et al. 2010; Enders et al. 2012).

The mechanisms underlying the peripheral localization of the Vα14*i* NKT cell subsets have not been fully worked out, although these subsets differ to some extent in their expression of chemokine receptors, with CCR6 expression characterizing the NKT17 subset. Interestingly, it has been shown that expression of CXCR6 is necessary more for the survival than for the homing of resident liver Vα14*i* NKT cells, which are overwhelmingly NKT1-like in the BALB/c as well as C57BL/6 strains (Geissmann et al. 2005; Germanov et al. 2008; Shimaoka et al. 2007; Lee et al. 2013). CXCR6 expression and NKT survival in the liver are defective in the absence of Id2 (Monticelli et al. 2009). However, the effect of Id2 deficiency on liver Vα14*i* NKT numbers was more severe than that observed in mice deficient for CXCR6 signaling, suggesting that Id2 was necessary to support CXCR6-independent survival pathways, perhaps involving Bcl-2 family members (Monticelli et al. 2009).

The hallmark feature of liver and spleen Vα14*i* NKT cells is their ability to mount a vigorous and rapid response to CD1d-presented agonists in the absence of priming, featuring the production of both Th1 and Th2-associated cytokines. Furthermore, both IL-4 and IFNγ RNA transcripts can be detected in Vα14*i* NKT cells even without exogenous antigen exposure (Stetson et al. 2003; Matsuda et al. 2003), although stimulation increases the mRNA concentration greatly. A number of transcription factors have been shown to support Vα14*i* NKT cell cytokine production in response to TCR agonists. In general, transcription factor mutations or targeted gene deletions that impacted the TCR signal transduction pathway required for Vα14*i* NKT cell positive selection, or that inhibited NKT1 maturation, tended to have corresponding effects on the cytokine response of the remaining Vα14*i* NKT cells to antigens such as αGalCer. However, the phenotype of Vα14*i* NKT cells with a deficiency for Hobit presents a somewhat more complex story. Hobit is necessary for the complete maturation of thymic NKT1 cells, as assessed by phenotype, and it also acted to promote NK1.1 expression in liver and spleen Vα14*i* NKT cells (van Gisbergen et al. 2012). However, Hobit-deficient NKT cells actually displayed an enhanced in vivo cytokine response to αGalCer, particularly with regard to IFNγ production (van Gisbergen et al. 2012). However, Hobit was necessary for granzyme B expression by Vα14*i* NKT cells, both in the thymus and in the periphery, particularly in response to antigen-independent stimulation by exposure of to poly I:C or viral infection (van Gisbergen et al. 2012). These roles for Hobit suggest a functional similarity with its homolog Blimp-1, which inhibits IFNγ and promotes granzyme B in conventional CD8SP T and NK cells (Kallies et al. 2009, 2011; Rutishauser et al. 2009; Shin et al. 2009; Smith et al. 2010).

Although Vα14*i* NKT thymocytes lacking Th-POK function exhibited a profound shift towards the NKT17 lineage, the dominant populations in liver and spleen in these mice exhibited an NKT1-like phenotype with respect to the expression of and other markers (Engel et al. 2012; Enders et al. 2012). However, Th-POK-deficient peripheral Vα14*i* NKT cells exhibited deficiencies in their response to αGalCer in vivo (Engel et al. 2010). Interestingly, the Th2 cytokine response in the absence of functional Th-POK is much more profoundly inhibited

than was IFNγ, and IL-17 production by NKT17 cells was unaffected (Engel et al. 2010, 2012). Furthermore, Th-POK-null Vα14*i* NKT cells exhibited severely reduced levels of basal IL-4 transcripts, but basal transcription at the IFNγ locus was unimpaired (Engel et al. 2010). These data suggest that Th-POK may have a particularly important role in promoting the transcription of Th2 cytokine genes. This hypothesis is supported by studies examining the rescue of MHC class II-dependent CD4 T helper function by stimulation of Th-POK-deficient conventional T cells, which demonstrated that Th1, but not Th2 responses could be elicited either by TCR cross-linking together with the appropriate polarizing conditions, or by challenge of Th-POK-deficient mice with pathogens (Carpenter et al. 2012). Th-POK has an antagonistic relationship with members of the Runx family of transcriptional inhibitors, and Runx proteins are known to inhibit Th2 cytokine production (Setoguchi et al. 2008; Egawa and Littman 2008; He et al. 2008; Djuretic et al. 2007; Naoe et al. 2007). Thus Th-POK may function to support Th2 cytokine production by inhibiting Runx activity in Vα14*i* NKT cells; this hypothesis is supported by the demonstration that a dominant-negative form of Runx can restore Th2 cytokine production in activated Th-POK-deficient mainstream T cells (Carpenter et al. 2012).

Peripheral Vα14*i* NKT cell activation is also regulated by AP-1 factors, as demonstrated by the effects of a T-lineage specific deletion of *Fra2/Fosl2* (Lawson et al. 2009). Splenic Vα14*i* NKT cells from *CD4-cre Fosl2*$^{f/f}$ mice that were stimulated with αGalCer in vivo exhibit enhanced proliferative expansion. Furthermore, aberrantly high levels of IL-4 and IL-2 marked the in vivo αGalCer response of Fra-2/Fosl2-deficient Vα14*i* NKT cells from both the liver and spleen, although IFNγ production was unaffected. It is interesting to note that Fra-2/Fosl2 has also been identified as a limiter of developmental plasticity in the differentiation of conventional T helper subsets, where it acts to restrict cytokine expression to the appropriate Th lineage (Ciofani et al. 2012). It is conceivable that Fra-2/Fosl2 is playing an analogous role in preventing excessive non-Th1 cytokine expression in the NKT1 cells, although the response of NKT1 cells is not as polarized as that of conventional Th subsets.

The NF-kB signaling pathway is also critical for peripheral Vα14*i* NKT function. Deficiency for even single NF-κB transcription factors caused marked decreases in the ability of mature Vα14*i* NKT cells to secrete cytokines in response to TCR engagement. Deletion of either *RelA*, *NF-κB1* or *Rel* (which encodes the c-Rel protein) caused decreased synthesis of most cytokines by Vα14*i* NKT cells when measured on a per cell basis by intracellular staining (Stanic et al. 2004; Stankovic et al. 2011). There was some variation in degree of the effect on particular cytokines, however, and interestingly, IL-17 production constituted an exception, because it was not dependent on any one NF-κB transcription factor. Furthermore, when thymic CD4$^-$, NK1.1$^-$ Vα14*i* NKT cells were measured, as a surrogate for the NKT17 subset, there was no decrease in mice deficient for either the *Nfκb1*, *Rela* or *Rel* genes (Stankovic et al. 2011).

4.2 Peripheral Differentiation: NKT Subsets Generated in the Periphery

In addition to the three functional subsets that originate in the thymus, it is now clear that peripheral Vα14*i* NKT cells share at least some of the post-thymic differentiation capacity of MHC class II-reactive CD4SP T cells. For example, challenge with αGalCer leads to the acquisition of markers of follicular helper cells in a significant fraction of splenic Vα14*i* NKT cells, and treatment with αGalCer-conjugated proteins or haptens results in the formation of Vα14*i* NKT-B germinal centers (King et al. 2011; Chang et al. 2011). As is the case for conventional T_{FH}, the generation of NKT_{FH} depends on IL-21 and the BTB-POZ transcription factor Bcl-6 (Chang et al. 2011; King et al. 2011). There have also been reports that *i*NKT cells from both mouse and humans can be induced to express FoxP3 upon exposure to TGF-β, and thereby assume a regulatory T phenotype; and that treatment of Vα14*i* NKT cells with TGF-β and IL1-β can promote the expression of RORγT and the acquisition of Th17-like function (Engelmann et al. 2011; Moreira-Teixeira et al. 2012; Monteiro et al. 2013). These reports imply that master regulatory transcription factors of specific conventional T subsets have the potential to similarly direct the differentiation of Vα14*i* NKT cells.

5 Other Invariant T Cell Subsets

There are several other T cell populations that express TCRs with a limited diversity, and exhibit at least some of the properties of Vα14*i* NKT cells, such as PLZF expression and rapid response kinetics. Most of these populations have not been examined to the extent that Vα14*i* NKT cells have, and so our knowledge of the transcription factors that control their development and function are more limited. One of these populations is the mucosal associated invariant T (MAIT) cell subset, which like *i*NKT cells are a conserved population that expresses an invariant TCR α chain and that recognizes non-peptidic antigens presented by a nonclassical class I molecule (Le Bourhis et al. 2013). These cells share a number of developmental features with iNKT cells, including the expression of PLZF (Walker et al. 2012). Also, it should be noted that the $\gamma\delta$ T cell pool contains populations that exhibit different cytokine response profiles, as well as both transcription factor and surface marker expression patterns, that are similar to the NKT1, 2 and 17 subsets (Bonneville et al. 2010). Each of these $\gamma\delta$ subsets is marked by the expression of different TCR V regions, suggesting that their developmental fate may be dictated by their antigen specificity or by encounter with antigen in the thymus (Jensen et al. 2008). Recent studies have also revealed that the three thymic Vα14*i* NKT subsets express distinct Vβ repertoires while having the same Vα (Lee et al. 2013). Although these variations in Vβ usage are subtler than the differences in the TCRs expressed by each of the $\gamma\delta$ subsets, they nevertheless imply that Vα14*i* NKT subset

differentiation may also be controlled by TCR structure and specificity. The epigenetic and transcriptional signatures of some invariant $\gamma\delta$ T populations have been recently characterized (Schmolka et al. 2013). Future studies are likely to explore the similarities in how $\gamma\delta$ T and Vα14*i* NKT subsets are transcriptionally regulated. It will also be interesting to explore the extent to which the transcription factors that govern $\gamma\delta$ T cell and Vα14*i* NKT cell functional differentiation are related to those that control the differentiation of the recently characterized populations of non-T, innate lymphoid cells (ILCs), which also are divided into functional subsets with similarities to the $\gamma\delta$ T and Vα14*i* NKT populations.

6 Summary and Conclusions

Knowledge of the transcriptional control of Vα14*i* NKT cell differentiation and function has grown very rapidly. Transcription factors exemplified by Tox and the Id proteins, with profound effects on Vα14*i* NKT cell differentiation, also influence the development of mainstream thymocytes. Other transcription factors with similarly profound effects on Vα14*i* NKT cell differentiation, such as members of the NF-κB family and T-bet, are not required in the thymus for conventional T lymphocytes, but are known instead to influence the effector responses of the mainstream cells. A third category includes those transcription factors, such as PLZF and Hobit, which are important for Vα14*i* NKT cell differentiation but that are not known to have significant effects on CD4 or CD8SP T lymphocyte differentiation or function. The complexity of the Vα14*i* NKT cell transcriptional program likely reflects two facts about these cells. First, their differentiation in the thymus includes rounds of expansion and the acquisition of effector functions, so in a sense it recapitulates aspects of both thymocytes positive selection and the induction of an immune response in the periphery. Second, global analyses of transcription have confirmed the hybrid character of Vα14*i* NKT cells, combining aspects of natural killer (NK) cells, part of the innate immune system, and genes characteristic of adaptive immune cells, particularly CD8 effector T cells (Cohen et al. 2013). Future work undoubtedly will shed more led light on the factors governing the differentiation of the recently discovered functional subsets Vα14*i* NKT cells in the thymus, which is curious in a population of cells with highly similar TCRs, and the extent to which the gene programs so identified apply to other innate-like lymphocyte populations in mice and humans.

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Transcriptional Control of Regulatory T cells

Michael Delacher, Lisa Schreiber, David M. Richards, Carla Farah, Markus Feuerer and Jochen Huehn

Abstract Regulatory T cells (Tregs) constitute unique T cell lineage that plays a key role for immunological tolerance. Tregs are characterized by the expression of the forkhead box transcription factor Foxp3, which acts as a lineage-specifying factor by determining the unique suppression profile of these immune cells. Here, we summarize the recent progress in understanding how *Foxp3* expression itself is epigenetically and transcriptionally controlled, how the Treg-specific signature is achieved and how unique properties of Treg subsets are defined by other transcription factors. Finally, we will discuss recent studies focusing on the molecular targeting of Tregs to utilize the specific properties of this unique cell type in therapeutic settings.

Contents

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1 Introduction: Development and Functional Properties of Foxp3$^+$ Tregs

To protect our body from harmful immune responses toward self-antigens or innocuous environmental antigens such as food or commensal microbiota, we rely on both centrally and peripherally mediated mechanisms to establish and maintain immune tolerance. Central tolerance is achieved by negative selection of self-reactive lymphocytes during their development in thymus (T cells) or bone marrow (B cells), leading to the elimination of most self-reactive clones. A significant part of peripheral tolerance is mediated by dominant suppression (Sakaguchi et al. 2008), and first impressions of such an immune regulatory cell type were presented by Powrie and co-workers already in 1993. They utilized the surface marker CD45RB to stratify CD4$^+$ T cells into a regulatory (CD45RB^{low}) and an inflammatory (CD45RB^{high}) cell type. When CD45RB^{high} CD4$^+$ T cells were transferred into immunodeficient mice, severe autoinflammatory wasting disease occurred, which could be prevented by co-transfer of CD45RB^{low} CD4$^+$ T cells, indicative of a regulatory cell type being present in this population (Powrie et al. 1993). In a landmark paper in 1995, Sakaguchi and co-workers then identified CD25-expressing CD4$^+$ T cells as the primary mediators of self-tolerance (Sakaguchi et al. 1995), and designated this cell type regulatory T cells (Tregs). They described that Tregs can rescue mice from organ-specific and systemic autoimmune disease caused by either postnatal thymectomy or by transfer of CD25-depleted CD4$^+$ T cells into *nu/nu* recipient mice (Sakaguchi et al. 1995; Asano et al. 1996). Furthermore, Tregs were also shown to inhibit transplant rejection and promote tumor immune escape (Shevach 2000). As CD25 is also expressed on activated T cells, a

search for Treg-specific markers was initiated. In 2001, a human fatal autoimmune disease, IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), and its murine homologue (scurfy mouse) were linked to mutations in the forkhead box p3 (*Foxp3*) gene (Brunkow et al. 2001; Wildin et al. 2001). The disease profile was reminiscent of autoimmune disease caused by transfer of $CD45RB^{high}$ T cells or CD25-depleted T cells into immunodeficient mice. Two years later, Foxp3 was described as the pivotal transcription factor (TF) defining the Treg lineage in mice (Hori et al. 2003; Fontenot et al. 2003; Khattri et al. 2003). It was shown that retroviral over-expression of Foxp3 induces a regulatory phenotype within conventional T cells (Tconv) (Hori et al. 2003), that the conditional excision of *Foxp3* results in autoimmune disease (Fontenot et al. 2003) and that *Foxp3* expression is critically required for the immunosuppressive phenotype of Tregs (Gavin et al. 2007; Lin et al. 2007). Together, these data demonstrated that Foxp3 acts as the key Treg lineage determination factor.

The vast majority of $Foxp3^+$ Tregs are generated during thymic development (called thymus-derived, tTregs) and are believed to be selected for self-antigen recognition. It was shown that tTreg development relies on factors such as a high-affinity T cell receptor (TCR) binding to antigens presented on thymic antigen-presenting cells (APCs), low clonal frequency of antigen-specific Treg precursors, and a specific cytokine environment in combination with co-stimulation by thymic APCs (Hsieh et al. 2012). A two-step model was proposed, where TCR- and APC-dependent signals trigger thymocytes to develop into a Treg precursor state, followed by a second "maturation" step independent of TCR stimulation, but dependent on the cytokine environment, namely interleukin-2 (IL-2) and IL-15, to induce stable *Foxp3* expression and conserve the immunosuppressive phenotype of Tregs (Lio and Hsieh 2008; Burchill et al. 2008).

In the periphery, the tTreg repertoire needs to be complemented by Tregs with specificities directed against nonpathogenic foreign antigens, including commensal microbiota, food, and fetal antigens. This expansion of the $Foxp3^+$ Treg pool is achieved by peripheral conversion of $Foxp3^-$ Tconv into $Foxp3^+$ Tregs (called peripherally derived, pTregs) (Curotto de Lafaille and Lafaille 2009; Haribhai et al. 2011; Lathrop et al. 2011; Cording et al. 2013) and these pTregs have been demonstrated to play a central role in the acquisition of mucosal as well as feto-maternal tolerance (Josefowicz et al. 2012b; Samstein et al. 2012b).

In addition to tTregs and pTregs, in vitro induced Tregs (iTregs) comprise the third commonly investigated group of Tregs (Abbas et al. 2013). To generate iTregs, Tconv require stimulation of the TCR in conjunction with cytokine stimulation through IL-2 and transforming growth factor β (TGF-β) (Chen et al. 2003). All three Treg groups, tTregs, pTregs, and iTregs depend on proper *Foxp3* expression for the acquisition of an immunosuppressive phenotype. Thus, in this review we summarize recent findings about the regulation of *Foxp3* gene expression and downstream effects of Foxp3.

2 Molecular Basis for Determination of Treg Lineage Commitment: Transcriptional Regulation of *Foxp3* Gene Expression

2.1 Signaling Pathways Involved in Foxp3 Expression

T cells have the exclusive ability to express *Foxp3*, thereby differentiating into immunosuppressive Tregs. Recently, several signaling pathways have been identified to critically control *Foxp3* gene expression (Fig. 1), among which TCR-downstream events have been intensively studied (Huehn et al. 2009; Hsieh et al. 2012). The examination of several TCR/cognate antigen double-transgenic mice has shown that TCR specificity to self-antigens is a crucial requirement for the development of tTregs (Jordan et al. 2001; Apostolou et al. 2002; Hsieh et al. 2004; Knoechel et al. 2005), with the overall strength of TCR signaling as a major determinant. It is thought that for tTreg induction, the level of self-reactivity lies above the level that allows positive selection but below the one inducing negative selection (Cozzo Picca et al. 2011). Furthermore, CD28 co-stimulation is known to amplify TCR signal transduction and is therefore difficult to dissect from TCR signals during tTreg differentiation. CD28 co-stimulation was shown to be essential for tTreg development, and the contribution of unique, yet unidentified signals that go beyond TCR signal amplification have been proposed (Tai et al. 2005; Hinterberger et al. 2011). Eventually, TCR/CD28 engagement triggers an array of signaling cascades and TFs that are critically involved in both Treg development and the direct control of *Foxp3* transcription (Fig. 1). Among these, the NF-κB (nuclear factor/immunoglobulin κ-chain enhancer/B lymphocyte) pathway seems to play a central role (Long et al. 2009b; Ruan et al. 2009; Zheng et al. 2010). Dramatically impaired induction of *Foxp3* expression can be observed in the thymus of mice lacking key NF-κB-signaling molecules, including protein kinase C θ (PKCθ) (Gupta et al. 2008) and TGF-β-activated kinase 1 (TAK1) (Wan et al. 2006; Sato et al. 2006) as well as compounds of the Carma-1/Bcl-10/Malt-1 (CBM) complex (Barnes et al. 2009; Medoff et al. 2009; Molinero et al. 2009; Schmidt-Supprian et al. 2004) and IκB kinase β (IKKβ) (Schmidt-Supprian et al. 2003). Importantly, IKKα, which is a key molecule in nonclassical NF-κB signaling, was not able to compensate for the loss of IKKβ, indicating that classical, but not nonclassical NF-κB signaling is important for proper *Foxp3* expression. On the other hand, enforcing the permanent activation of classical NF-κB by the over-expression of constitutively active IKKβ was sufficient to induce *Foxp3* expression even in T cells that were unable to activate NF-κB and otherwise would not express *Foxp3* (Long et al. 2009b).

It came as a surprise that TCR engagement can also negatively influence *Foxp3* expression, via a different signaling branch that is usually associated with cellular metabolism processes. At the heart of this pathway lies mammalian Target of Rapamycin (mTOR) (Haxhinasto et al. 2008; Sauer et al. 2008; Delgoffe et al. 2009),

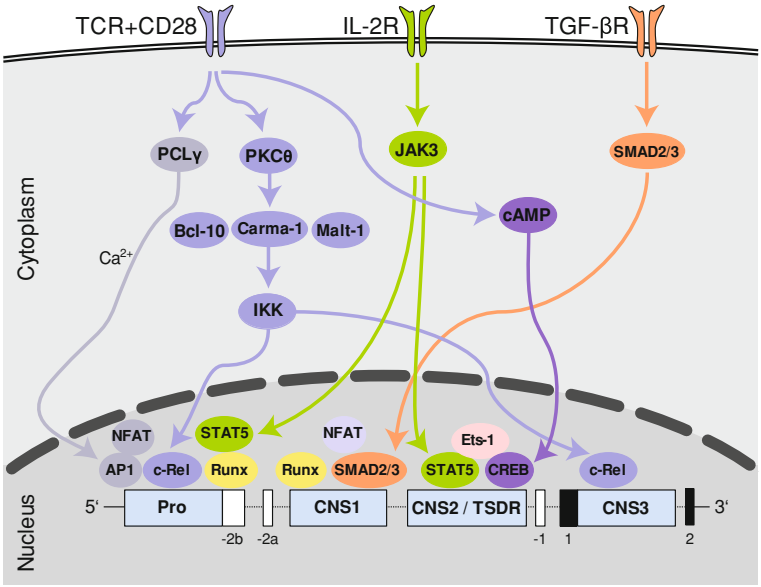

Fig. 1 Signaling pathways and genetic elements contributing to *Foxp3* gene expression

a kinase that forms two distinct signaling complexes (mTORC1 and mTORC2), which are regulated by the upstream kinases phosphoinositide 3-kinase (PI3K), protein kinase B (PKB, usually referred to as Akt), and serum/glucocorticoid regulated kinase 1 (SGK1) (Hedrick et al. 2012). Apart from TCR/CD28 signals (Okkenhaug and Vanhaesebroeck 2003), the PI3K-Akt-mTOR signaling cascade in Tregs can be triggered by common gamma chain (γ_C)-cytokine receptors such as the IL-2R (Stahl et al. 2002), receptors ligating the complement fragments C3a and C5a (C3aR and C5aR) (Strainic et al. 2013), lipid binding sphingosine-1-phosphate receptor 1 (S1PR1) (Liu et al. 2009) and the T cell inhibitory receptor programmed death 1 (PD-1) (Francisco et al. 2009). Forced over-expression of constitutively active Akt leads to impaired *Foxp3* induction, whereas the addition of rapamycin, a prominent inhibitor of mTOR activity, restored Foxp3 protein levels (Haxhinasto et al. 2008). Likewise, pharmacological inhibitors of PI3K, Akt and mTOR lead to the induction of *Foxp3* expression in Tconv (Sauer et al. 2008). The selective disruption of only mTORC1 or mTORC2 is not sufficient but the elimination of both is required for *Foxp3* induction to take place (Delgoffe et al. 2011). In line with this, strong and long-lasting TCR/co-stimulation signaling counteracts efficient *Foxp3* induction (Sauer et al. 2008), and the activation level of Akt is lower in Tregs than in Tconv upon TCR or IL-2 stimulation (Crellin et al. 2007). Finally, it is the Foxo family of TFs that is central to the integration of Akt-mTOR signals on the level of gene expression (Hedrick et al. 2012). Akt and SGK1 phosphorylate Foxo proteins in a mTOR-dependent manner, causing their cytoplasmic retention and degradation.

Consequently, disruption of Akt-mTOR signaling activates Foxo TFs, which are essentially involved in the differentiation of Tregs and sufficient for *Foxp3* expression (Merkenschlager and von Boehmer 2010).

Apart from the TCR and CD28, the development of Tregs crucially depends on cytokine signaling (Fig. 1). According to the above-mentioned two-step model, initial TCR and co-stimulatory signals give rise to Treg precursors, which have up-regulated the high affinity IL-2R α-chain CD25 that renders them highly responsive to γ_C-dependent cytokines. In a second TCR-independent step, IL-2 and to a weaker extent IL-15 and IL-7, allow efficient signaling via the signal transducer of activated T cells-5 (STAT-5) to induce *Foxp3* expression (Lio and Hsieh 2008; Burchill et al. 2008). Although efficient *Foxp3* induction can be observed in T cells lacking IL-2 or CD25 (D'Cruz and Klein 2005), mice that are deficient for the γ_C cytokine receptor subunit (which is critical for the assembly of the IL-2, IL-7 and IL-15 receptor complexes) or triple deficient mice for IL-2/7/15 are completely devoid of Tregs (Burchill et al. 2007; Vang et al. 2008). Thus, the loss of IL-2/CD25 can be compensated by the γ_C-dependent cytokines IL-15 and IL-7, and γ_C-mediated signaling is indispensable for *Foxp3* expression (Vang et al. 2008; Bayer et al. 2008). Furthermore, *Foxp3* expression can be induced in Tconv in a TCR- and TGF-β-dependent manner (Huehn et al. 2009), and the corresponding downstream TFs nuclear factor of activated T cells (NFAT) and SMAD (coined from SMAD homologs Sma [small mutants] and Mad [mothers against decapentaplegic]) seem to be involved in this process by binding to the *Foxp3* gene locus (Tone et al. 2008; Schlenner et al. 2012). Although there is substantial data showing that TGF-β is important for the peripheral conversion of Tconv into pTregs and also for the generation of iTregs in vitro (Kretschmer et al. 2005; Chen et al. 2003; Fantini et al. 2004), its role in tTreg development remains controversial (Marie et al. 2005; Liu et al. 2008). The current model suggests that TGF-β contributes to tTreg development by protecting them from negative selection but is dispensable for induction of *Foxp3* expression (Ouyang et al. 2010a).

2.2 The Foxp3 Gene Locus Includes Four Conserved Noncoding Regions with Regulatory Function

The *Foxp3* gene was mapped to the X-chromosome locus Xp11.23 and, in females, is subject to random X-chromosome inactivation (Brunkow et al. 2001; Wildin et al. 2001). The human as well as the mouse *Foxp3* gene contains 14 exons stretching over a length of roughly 15.5 kilo base pairs (bp). The first three exons are not translated into protein (exons -2b, -2a, -1) whereas exons 1–11 encode the Foxp3 protein (Brunkow et al. 2001; Wildin et al. 2001).

Comparing *Foxp3* gene sequences of various species revealed high sequence homology not only for the exons (Brunkow et al. 2001; Wildin et al. 2001), but

also for four evolutionarily highly conserved noncoding sequences (CNS) (Fig. 1). The most 5′ CNS lies 6.5 kb upstream of exon 1 and has been identified as a classical promoter. This region is defined by a TATA box, a GC box, and a CAAT box as well as multiple NFAT and activator protein 1 (AP-1) TF binding sites within approximately 500 bp upstream of the transcriptional start site (TSS) (Brunkow et al. 2001; Wildin et al. 2001; Mantel et al. 2006; Tone et al. 2008). The promoter is transcriptionally responsive to TCR stimulation, although its activity is typically very weak as measured by in vitro reporter assays (Tone et al. 2008; Kim and Leonard 2007; Polansky et al. 2010). In order to achieve efficient expression of *Foxp3* it was conceivable that additional regulatory elements enhance *Foxp3* promoter activity. Indeed, the other three conserved elements numbered CNS1-3 integrate multiple predicted TF binding sites (Fig. 1) and in combination with a promoter, CNS1 and CNS2 possess transcriptional enhancer activity in in vitro reporter assays. Further comprehensive studies revealed that each of the three CNS elements appears to fulfill a distinct function in order to enable efficient *Foxp3* expression during Treg differentiation.

The most proximal conserved element, **CNS1**, is located within the first intron, approximately 2 kb downstream of the promoter (Tone et al. 2008). CNS1 is a TGF-β-sensitive enhancer, which is critical for the TGF-β-mediated generation of both iTregs (Tone et al. 2008; Zheng et al. 2010; Schlenner et al. 2012) and pTregs (Josefowicz et al. 2012b; Weiss et al. 2012). This is best exemplified by the development of severe allergic inflammation at mucosal sites in CNS1-deficient mice, which is the consequence of the lack of pTregs (Josefowicz et al. 2012b). In contrast, CNS1 is dispensable for the generation of tTregs (Zheng et al. 2010; Josefowicz et al. 2012b).

CNS2 (also named TSDR, see Sect. 2.3) also lies within the first intron, roughly 4 kb downstream of the TSS. It is around 1200 bp in length (although boundaries of all CNS elements are arbitrary and can differ in the various studies) and is enriched in CpG motifs (a cytosine followed by a guanine within one DNA strand) and predicted TF binding sites (Floess et al. 2007). CNS2 is dispensable for the induction but plays a crucial role in the stabilization of *Foxp3* gene transcription and translation, which is necessary for the heritable maintenance of Foxp3 protein expression in tTregs and pTregs (Zheng et al. 2010). For example, CNS2-deficient Tregs lose *Foxp3* expression upon in vitro stimulation or adoptive transfer into T cell-deficient recipient mice, and Treg numbers were reduced in the periphery of CNS2-deficient mice older than six months.

The last CNS within the *Foxp3* locus, designated **CNS3**, is located immediately downstream of the first coding exon, approximately 7 kb downstream of the TSS, and was shown to be essential for the differentiation of both tTregs and pTregs (Zheng et al. 2010). Although CNS3 does not show enhancer activity in vitro (Zheng et al. 2010; Schuster et al. 2012), it was shown to play a critical role in the initiation of *Foxp3* expression, but to be dispensable once *Foxp3* is expressed and is therefore also referred to as a pioneer element (Zheng et al. 2010).

2.3 Epigenetic Regulation of the Foxp3 Gene Locus

Epigenetic processes are well known to play an important role in gene regulation during development as they can fix pre-established genetic signatures and provide an inheritable memory of transcriptional activities without changing the DNA sequence. DNA methylation and histone modifications are two major epigenetic mechanisms that participate in establishing and maintaining chromatin structures. Within the *Foxp3* locus, the four conserved genetic elements are the primary targets of epigenetic regulation. A distinct DNA methylation pattern combined with the formation of characteristic histone modifications establishes an open chromatin structure, thereby imprinting *Foxp3* gene expression in Tregs.

CpG motifs within the *Foxp3* promoter are almost fully demethylated in *ex vivo* isolated Tregs in humans as well as in mice, while Tconv have a much more pronounced methylation at the promoter which is even increased upon TCR stimulation in vitro (Kim and Leonard 2007; Janson et al. 2008; Zheng et al. 2010). In TGF-β-induced iTregs, *Foxp3* promoter methylation declines nearly to the level of *ex vivo* isolated Tregs (Kim and Leonard 2007; Zheng et al. 2010). Mechanistically, the SUMO E3 ligase protein inhibitor of the activated STAT-1 (PIAS1) maintains promoter methylation and concomitantly a repressive chromatin state by binding to the methylated *Foxp3* promoter and recruiting DNA methyltransferases and heterochromatin protein 1, thus restricting *Foxp3* expression and Treg differentiation (Liu et al. 2010). As soon as PIAS1 is eliminated from the *Foxp3* promoter, the chromatin becomes accessible to the transcription machinery. In agreement with enhanced demethylation and a more accessible chromatin structure (Kim and Leonard 2007; Liu et al. 2010), permissive histone modifications such as histone 3 (H3) and H4 acetylation as well as di- and trimethylated H3-lysine4 (H3K4) accumulate at the promoter in Tregs but not in Tconv (Tone et al. 2008; Sekiya et al. 2013; Rudra et al. 2009; Zheng et al. 2010). In contrast, trimethylation of H3K27 was not observed in Tregs but in Tconv (Xiong et al. 2012). It was proposed that a Polycomb response element/Krueppel-like factor (KLF) binding site within the *Foxp3* promoter is associated with Polycomb repressor complex 2 comprising histone methyltransferases, which keep the *Foxp3* promoter silenced in Tconv (Xiong et al. 2012). Under *Foxp3*-inducing conditions, KLF-10 and the associated histone acetyltransferase PCAF (p300/CBP-associated factor) replace the Polycomb repressor complex, which is accompanied by the opening of chromatin, imparting full *Foxp3* inducibility.

The TGF-β-sensitive enhancer CNS1 is devoid of any CpG motifs and therefore solely regulated by histone modifications, and permissive histone modifications such as H4/H3 acetylation and H3K4 di- and trimethylation are enriched in both tTregs and iTregs compared to Tconv (Tone et al. 2008; Xu et al. 2010; Zheng et al. 2010; Sekiya et al. 2011).

Striking differences in DNA methylation are found at *Foxp3* CNS2. Analyses of the methylation status of the 14 CpG motifs within CNS2 have revealed that this

region is completely demethylated only in *ex vivo* isolated Tregs. In contrast, Foxp3$^-$ T cells and all other cell types analyzed so far display a fully methylated CNS2 and therefore this element has been designated the Treg-specific demethylated region (TSDR) (Floess et al. 2007; Kim and Leonard 2007; Baron et al. 2007; Nagar et al. 2008). Notably, the differential methylation status of the TSDR is conserved between mice and humans and the demethylated TSDR presents an epigenetic marker for stable *Foxp3* expression and true Treg lineage affiliation (Huehn et al. 2009; Miyao et al. 2012). For example, iTregs retain a fully methylated TSDR that correlates with a progressive loss of *Foxp3* expression (Polansky et al. 2008; Kim and Leonard 2007). Moreover, forced demethylation of the TSDR in Tconv by the inactivation of methyltransferase enzymes results in sustained, heritable *Foxp3* expression (Polansky et al. 2008; Nagar et al. 2008; Josefowicz et al. 2009). Accordingly, transcriptional enhancer activity of the TSDR is controlled by its methylation status, allowing potent activity only in its demethylated state (Polansky et al. 2010; Kim and Leonard 2007).

TSDR demethylation can be induced during tTreg development in a step-wise progress that is finalized upon Treg egress to the periphery (Toker et al. 2013). However, pTregs also display a demethylated TSDR and stable *Foxp3* expression (Polansky et al. 2008; Ohkura et al. 2012). Mechanistically, compelling evidence exists that in developing Tregs, TSDR demethylation is carried out through an active, mitosis-independent mechanism, necessitating more than the removal of DNA methyltransferases from the TSDR (Toker et al. 2013). It has been proposed that active demethylation of the TSDR is facilitated by enzymatic hydroxylation of methylated cytosines through members of the Ten eleven translocation (Tet) family (Toker et al. 2013), a process that has recently been described to represent the initiating step of active DNA demethylation in mammalian cells (Branco et al. 2012). Nevertheless, the precise molecular pathways and mechanisms that conduct specific demethylation at the TSDR remain to be fully elucidated. PIAS1, which hinders *Foxp3* promoter demethylation by the recruitment of DNA methyltransferases, is not involved in the regulation of TSDR methylation (Liu et al. 2010; Toker and Huehn 2011). Along with its demethylated DNA, the TSDR contains increased levels of H3K4 methylation as well as H3/H4 acetylation (Floess et al. 2007; Zheng et al. 2010; Sekiya et al. 2011). Taken together, DNA demethylation and permissive histone modification generate an open chromatin status at the TSDR that allows binding of TFs and promotes stabilization of *Foxp3* expression.

Finally, the third conserved regulatory element, CNS3, is subject to epigenetic modifications and is enriched in H3K4 mono- and dimethylation (but not trimethylation) in Tregs. Interestingly, these permissive histone modifications are already markedly increased in Foxp3$^-$CD4SP and even CD4$^-$CD8$^-$ thymocytes, cell populations which include Treg precursors (Zheng et al. 2010). Therefore, it is thought that CNS3 is the first element within the *Foxp3* gene locus to become transcriptionally active, facilitating the opening of the *Foxp3* gene locus already in Treg precursors (Zheng et al. 2010).

2.4 Multiple TFs Contribute to the Regulation of Foxp3 Expression

Extensive studies in the recent years have provided a comprehensive picture of the molecular players that are involved in the transcriptional regulation of *Foxp3*. An array of TFs have been identified that are activated during Treg differentiation and that associate with their recognition sites located within the regulatory genetic elements of the *Foxp3* gene locus in order to control *Foxp3* transcription.

2.4.1 Initiating *Foxp3* Expression

For the initiation of *Foxp3* expression, TCR and co-stimulatory signals are mandatory, and a prominent downstream signaling event results in Ca^{2+} mobilization and nuclear translocation of NFAT (Fig. 1). Several NFAT/AP1 binding sites have been identified within the *Foxp3* promoter and were proven to be critical for its transcriptional activity (Mantel et al. 2006; Ruan et al. 2009). It was further demonstrated that *Foxp3* expression could not be induced in iTreg cultures in the presence of Cyclosporin A, an inhibitor of Ca^{2+}-mediated NFAT activation (Mantel et al. 2006; Tone et al. 2008). Moreover, mice with a T cell-specific deficiency of the two Ca^{2+} sensors STIM1/2, resulting in the complete loss of Ca^{2+} influx and cytosolic retention of NFAT, are severely impaired in *Foxp3* expression and hence Treg development (Oh-Hora et al. 2013). However, the role of individual, redundantly acting NFAT members is difficult to address. For example, deficiency of one individual NFAT member or combined deficiency of NFAT1/4 or NFAT1/2 does not show drastic alterations of *Foxp3* expression in thymocytes (Bopp et al. 2005; Vaeth et al. 2012; Oh-Hora et al. 2013). In contrast, several studies indicate that NFAT is essential for proper *Foxp3* induction in iTregs (Ruan et al. 2009; Vaeth et al. 2012). In line with this, NFAT was shown to cooperate with TGF-β-activated SMAD proteins to bind to the highly conserved SMAD and NFAT binding site within the TGF-β-sensitive enhancer CNS1 (Tone et al. 2008; Schlenner et al. 2012) (Fig. 1). Importantly, NFAT-deficient Tregs have unaltered levels of Foxp3 and are suppressive (Bopp et al. 2005; Mantel et al. 2006; Vaeth et al. 2012), indicating that the predominant role of NFAT is during the establishment of *Foxp3* expression but may be dispensable in mature Tregs. Hence, NFAT and SMAD proteins play a central role in the TGF-β-mediated induction of *Foxp3*, which is mainly mediated by binding to the CNS1 enhancer. However, TGF-β-mediated signals can also directly target the *Foxp3* promoter via TGF-β-inducible early gene 1 (TIEG1, also known as KLF-10) (Venuprasad et al. 2008; Cao et al. 2009).

NF-κB activation has been recognized to link TCR ligation with the induction of *Foxp3* expression, however, the precise molecular mechanisms are only incompletely understood. The mammalian NF-κB family is composed of p65 (also known as RelA; the Rel homology domain is characteristic for all NF-κB

proteins), RelB and c-Rel, p50 and p52. Of all NF-κB proteins the absence of c-Rel has the strongest impact on Treg differentiation resulting in heavily compromised *Foxp3* expression in both tTreg and pTregs (Isomura et al. 2009; Ruan et al. 2009; Visekruna et al. 2010; Vang et al. 2010; Deenick et al. 2010). Importantly, the effect of c-Rel was Treg-intrinsic and could not be attributed to an anti-apoptotic function (Isomura et al. 2009; Ruan et al. 2009). Apart from c-Rel, deficiency of RelA or the atypical IκB protein $I\kappa B_{NS}$ resulted in compromised *Foxp3* induction (Isomura et al. 2009; Schuster et al. 2012). Like NFAT, both c-Rel and $I\kappa B_{NS}$ are not required for sustained expression of *Foxp3* and the suppressive capacity of Tregs (Isomura et al. 2009; Deenick et al. 2010; Schuster et al. 2012), indicating that they play a key role merely during Treg development but are dispensable in mature Tregs. Therefore, c-Rel has been proposed to act as a pioneer TF that initiates *Foxp3* expression in thymic Treg precursors. Both c-Rel and $I\kappa B_{NS}$ were shown to bind to the pioneer element CNS3 (Fig. 1), where chromatin starts to open in $CD4^{-}CD8^{-}$ thymocytes and earlier than any other element in the *Foxp3* locus (Zheng et al. 2010; Schuster et al. 2012). In addition, CNS3- and c-Rel-deficient mice have similar phenotypes, both displaying severe Treg developmental defects, but the remaining Tregs present normal *Foxp3* expression levels (Isomura et al. 2009; Zheng et al. 2010). These data further support the idea that, in analogy to the *Il-2* locus (Rao et al. 2003), c-Rel facilitates the opening of the *Foxp3* locus (Zheng et al. 2010) during tTreg development and $I\kappa B_{NS}$ may contribute to this process. In an experimental setup utilizing iTregs to follow *Foxp3* induction, it was shown that the *Foxp3* promoter is initially activated by a complex of c-Rel/p65 and NFAT (Ruan et al. 2009; Long et al. 2009a). At later time points, other TFs that have initially been recruited to the enhancer elements (such as SMAD and CREB [cAMP response element binding protein] to CNS1 and CNS2, respectively) relocate to the promoter (Ruan et al. 2009). It was proposed that the successive recruitment of TFs forms a Treg-specific multiprotein complex at the *Foxp3* promoter, designated the Foxp3-specific enhanceosome, in order to promote *Foxp3* expression selectively in Tregs (Ruan et al. 2009). While in iTregs, NF-κB binding to the TSDR cannot be detected, another study demonstrates that c-Rel binds to the TSDR in primary $CD4^{+}$ T cells (Long et al. 2009a). A functional role for NF-κB in the control of the TSDR has not been proven so far. In fact, we found strong indication that NF-κB signaling is dispensable for TSDR transcriptional activity, and the TSDR was completely demethylated in c-Rel deficient Tregs (Schreiber et al. 2014).

Recently, the TF family of nuclear orphan receptors Nr4a1/2/3 has been identified to be critically involved in the regulation of *Foxp3* (Sekiya et al. 2013). The triple knock-out of Nr4a1/2/3 results in complete loss of *Foxp3* expression, causing systemic lethal autoimmunity reminiscent of the scurfy phenotype. Mechanistically, it has been proposed that Nr4a proteins translate TCR signaling strength into *Foxp3* transcriptional control as moderate activation of Nr4a has the ability to bypass essential TCR signals thereby driving *Foxp3* expression, but strong activation of Nr4a instead induces cell death. All Nr4a members initiate *Foxp3* expression upon ectopic expression probably by direct binding to and

stimulation of the *Foxp3* gene locus (promoter and partially CNS1/2) (Sekiya et al. 2011, 2013). In addition, at least Nr4a2 has the capacity to induce permissive histone modifications at the *Foxp3* promoter (Sekiya et al. 2011). Although TSDR methylation is not altered in Nr4a2 deficient cells, *Foxp3* expression is lost upon adoptive transfer into T cell-deficient mice, and cooperative action of Nr4a with Runx proteins (see below) promoting TSDR activity has been suggested (Sekiya et al. 2011).

A somewhat unexpected finding was that signals emanating from the TCR could counteract *Foxp3* expression (Sauer et al. 2008), which can in part be attributed to cytoplasmic retention of TCR/Akt-phosphorylated Foxo TFs (see above). Apparently, initial TCR signals, including the activation of NF-κB, are essential for the induction of *Foxp3* expression but the signaling must cease at a premature state enabling Foxo activation and nuclear translocation (Sauer et al. 2008; Haxhinasto et al. 2008; Ouyang et al. 2010b; Harada et al. 2010; Kerdiles et al. 2010). Two of three Akt-regulated mammalian Foxo proteins, Foxo1 and Foxo3a, are involved in the direct control of *Foxp3* gene expression by binding to both the *Foxp3* promoter and the TSDR in Tregs, but not to CNS1 (Ouyang et al. 2010b; Harada et al. 2010; Kerdiles et al. 2010). Consequently, mice with a T cell-specific deficiency of Foxo1/3a show a considerable reduction of thymocytes capable of expressing *Foxp3* and the remaining Foxp3$^+$ thymocytes express considerably reduced levels of Foxp3 (Ouyang et al. 2010b). Likewise, TGF-β-mediated *Foxp3* expression is impaired in Foxo-deficient T cells (Kerdiles et al. 2010; Harada et al. 2010).

2.4.2 Maintaining *Foxp3* Expression

The predominant function of the above-mentioned TFs is their capacity to induce *Foxp3* expression. Nevertheless, continuous *Foxp3* expression is fundamental to Treg physiology. The Runt-related TFs (Runx) and their essential interaction partner core-binding factor subunit β (Cbfβ), which increases DNA binding affinity, are critical mediators of *Foxp3* expression induction and maintenance (Klunker et al. 2009; Bruno et al. 2009; Kitoh et al. 2009; Rudra et al. 2009). Of the three Runx proteins, Runx1 and Runx3 as well as Cbfβ are indispensable for normal *Foxp3* expression (Fig. 1). *Foxp3* induction is regulated directly through Runx binding to the *Foxp3* promoter (Klunker et al. 2009; Bruno et al. 2009; Kitoh et al. 2009), but Runx binding to the pioneer element CNS3 has also been described (Zheng et al. 2010). Furthermore, Runx is up-regulated by TCR/TGF-β stimulation and required for TGF-β-mediated *Foxp3* induction (Klunker et al. 2009), consistent with a study where Runx was detected to bind to the TGF-β-responsive element CNS1 (Kitoh et al. 2009). Finally, Runx proteins also interact with the TSDR (Bruno et al. 2009; Kitoh et al. 2009), which may explain why Runx TFs are crucial not only for the initiation but for the maintenance of *Foxp3* gene expression (Bruno et al. 2009; Rudra et al. 2009). For example, Cbfβ-deficient Tregs and Tregs in which Runx proteins compete with a dominant-negative Runt

DNA binding domain for Runx recognition sites progressively lose *Foxp3* expression over time (Bruno et al. 2009; Rudra et al. 2009). Despite Runx association with the TSDR, mutations of Runx binding sites have no discernible effect on the transcriptional enhancer activity of the TSDR, a finding that may point to a chromatin modulatory function of Runx proteins rather than direct transcriptional activity (Rudra et al. 2009; Kitoh et al. 2009). Indeed, ablation of Cbfβ in Tregs reveals a reduction in permissive chromatin modifications (H3K4 trimethylation) but an increase in repressive modifications (H3K9 trimethylation) at the *Foxp3* gene locus compared to wild-type (WT) Tregs (Rudra et al. 2009). In addition, the cooperative interaction of Runx with Nr4a and Runx-mediated recruitment of Foxp3 protein itself to the TSDR has been described to be important for TSDR activity. Of note, Runx/Foxp3 binds selectively to the demethylated but not the methylated TSDR (Zheng et al. 2010; Sekiya et al. 2011). In line with this, transfer of "wannabe" Tregs, which have the *Foxp3* gene locus replaced with a green-fluorescence protein (GFP)-encoding reporter sequence, into lymphopenic mice leads to loss of *GFP* expression, suggesting that Foxp3 protein is required for sustained *Foxp3* gene expression but not for its initiation (Gavin et al. 2007; Lin et al. 2007). Consequently, the cooperative function of Runx/Foxp3 and Nr4a seems to be part of a positive feedback loop maintaining the continuous expression of *Foxp3* that is however restricted to fully differentiated Tregs containing a demethylated TSDR (Zheng et al. 2010; Polansky et al. 2010; Bruno et al. 2009).

CREB, the first protein found to selectively bind to the demethylated TSDR (Fig. 1), has been implicated to have a functional role in the regulation of the TSDR and therefore represents an interesting candidate to impart stable *Foxp3* expression, potentially by retaining the TSDR in its demethylated state (Kim and Leonard 2007). A similar function has also been proposed for ETS-1 (Erythroblastosis virus E26 oncogene homolog-1) (Mouly et al. 2010; Polansky et al. 2010). *Foxp3* transcript and protein levels are reduced in ETS-1-deficient Tregs and, interestingly, the TSDR was significantly more methylated in knock-out than in WT Tregs (Mouly et al. 2010). Indeed, the TSDR contains two ETS-1 binding sites, which are crucial for enhancer activity and bound by ETS-1 in Tregs (Polansky et al. 2010). Finally, the master TF of T_H2 effector cells, GATA-binding protein 3 (GATA3), has initially been reported to inhibit *Foxp3* transcription as it binds to and represses *Foxp3* promoter activity in effector T cells (Mantel et al. 2007). However, more recent literature points to a cardinal role for GATA3 in Treg physiology (Wohlfert et al. 2011; Wang et al. 2011), and a Treg-specific deletion results in significantly reduced levels of *Foxp3* transcript and protein per cell despite normal Treg frequencies. Consistent with lower *Foxp3* expression levels, GATA3 associates with the TSDR in Tregs but not with the other *Foxp3* regulatory elements (Wohlfert et al. 2011; Wang et al. 2011).

IL-2 signals are indispensable for Treg development and survival. The importance of IL-2 for *Foxp3* expression could be linked to the requirement of STAT5 as its ablation dramatically reduced thymic *Foxp3* expression, similar to IL-2/7/15 triple- or γ_C-deficiency in mice (Burchill et al. 2007; Vang et al. 2008). Most likely, STAT5 directly regulates *Foxp3* transcription as it binds to the *Foxp3*

promoter and the TSDR in cells stimulated with IL-2 (Burchill et al. 2007; Yao et al. 2007; Mouly et al. 2010; Liu et al. 2010) (Fig. 1). A potential contribution of IL-2/STAT5 signaling to DNA demethylation has been proposed. In $CD25^{high}$ Treg precursors, IL-2 signaling alone was enough to potently induce *Foxp3* expression (Lio and Hsieh 2008), which was accompanied by demethylation of the TSDR (Toker et al. 2013). In addition, in vivo administration of an IL-2/anti-IL-2 complex has been shown to increase TSDR demethylation and stability of iTregs after adoptive transfer (Chen et al. 2011b). On the other hand, IL-2 is not capable to induce TSDR demethylation in Tconv in vitro, and only enforced opening of the promoter allowed STAT5 to bind even in Tconv (Liu et al. 2010). Therefore, the role of STAT5 in the TSDR-mediated stabilization of *Foxp3* expression awaits further investigations.

Despite the identification of such a broad range of TFs that control *Foxp3* expression, many TFs are not uniquely expressed in Tregs and it is unlikely that simply the presence or absence of such factors can determine cell lineage specificity and function. It appears that the function of each factor is context-dependent and determined by more parameters, including timing, dosage, posttranslational modifications, and co-factors. Therefore, future studies shall shed further light on the intricate network of TFs controlling *Foxp3* expression.

3 Foxp3-Independent Epigenetic Imprinting and Treg Lineage Stability

Foxp3 is widely recognized as the most prominent factor with a pivotal role in Treg function. However, mounting evidence suggests that Treg lineage commitment is not solely determined by *Foxp3* expression. First, Foxp3 alone is not sufficient to implement the full Treg-specific phenotype in Tconv (Fig. 2). For example, *Foxp3* expression at low levels can be observed in activated Tconv of mice and human origin without conferring a Treg phenotype (Allan et al. 2007; Miyara et al. 2009; Miyao et al. 2012). Furthermore, transcriptome analyses in Tregs and Foxp3-transduced cells have shown that Foxp3 accounts only for a fraction of genes characteristically expressed in Tregs (Sugimoto et al. 2006; Hill et al. 2007). The presence of Foxp3 in these cells, as well as in iTregs, is not enough to establish the full Treg-specific gene expression profile and hence generate genuine Tregs (Sugimoto et al. 2006; Hill et al. 2007; Tran et al. 2007). Second, a partial Treg-specific expression profile, as well as TSDR demethylation and characteristic histone modifications at the *Foxp3* gene locus, can be established even in the absence of *Foxp3* (Gavin et al. 2007; Lin et al. 2007; Zheng et al. 2010). Apparently, other, Foxp3-independent mechanisms pave the way for Treg differentiation even before *Foxp3* expression takes place, implying that Foxp3 may be dispensable for Treg lineage commitment but not for consolidation and stability of Treg lineage identity (Fig. 2).

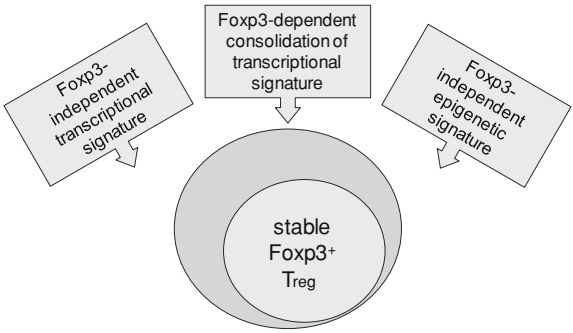

Fig. 2 Signatures involved in the definition of stable Foxp3$^+$ Tregs

In fact, genome-wide epigenetic changes take place in the course of Treg development and DNA demethylation can be observed at Treg signature gene loci apart from *Foxp3*, including *Ctla-4*, *Ikzf4* (*EOS*) and *Tnfrsf18* (*GITR*) (Ohkura et al. 2012). Importantly, this so-called Treg-specific epigenetic signature develops independently of Foxp3 and, interestingly, Treg-specific gene regulation was far more dependent on the epigenetic imprinting of signature genes than on *Foxp3* expression (Ohkura et al. 2012) (Fig. 2). Only few Treg signature genes seem to be regulated by both Foxp3 and epigenetic regulation, among them *CD25*, *Ctla-4,* and *Foxp3* itself (Ohkura et al. 2012). Recently, it was shown that thymic Treg precursors, defined by their high expression of CD25, already present a partial Treg-specific gene expression pattern and, upon IL-2 stimulation, up-regulate *Foxp3* and initiate TSDR demethylation, indicating that these cells are already primed to become stable Foxp3-expressing Tregs. In line with this, there is some indication that Foxp3$^-$ Tconv already provide a pre-existing chromatin landscape that is favorable for Treg development. Samstein et al. performed genome-wide DNase I hypersensitivity assays in Tregs and Tconv and found that only 2 % of the enhancers became accessible specifically in Tregs, among them the TSDR and Treg-characteristic genes such as *Ctla-4* and *Helios*. However, most Foxp3-binding enhancers (98 %) pre-exist in an accessible chromatin formation in Tconv, which is preserved by the structurally related protein Foxo1 in the absence of Foxp3, and subsequently replaced by Foxp3 as soon as it is expressed (Samstein et al. 2012a). This finding may imply a more superordinate function for Foxo1 in Treg differentiation than solely the regulation of *Foxp3*. In addition, Foxo1 regulates a large fraction of Treg-characteristic genes, mostly independently of Foxp3 (Ouyang et al. 2010b).

Despite Treg identity being well defined on the molecular (epigenetic profile, *Foxp3* expression, Treg-specific expression signature) and functional (suppressive activity) level, whether Treg lineage commitment is irreversible or whether fully differentiated Tregs may retain the capacity to reprogram into other T cell types remains a controversial issue. Loss of *Foxp3* expression has been observed in a subpopulation of Foxp3$^+$ T cells that was able to produce pro-inflammatory cytokines (Yang et al. 2008; Zhou et al. 2009). On the other hand, stable *Foxp3*

expression even in an inflammatory milieu has been demonstrated (Rubtsov et al. 2010). The study by Miyao et al. suggests that these controversial findings may simply be attributed to a small fraction of Foxp3$^+$ T cells lacking hypomethylation of the TSDR and which therefore may not be fully committed to the Treg lineage (Miyao et al. 2012). Treg-fate studies demonstrated that TSDR demethylation—as part of the Treg-specific epigenetic signature—serves as an epigenetic memory that ensures Treg lineage stability, as Foxp3$^-$ cells presenting a demethylated TSDR can reacquire *Foxp3* expression and become functional Tregs. In contrast, T cells expressing Foxp3 but lacking TSDR hypomethylation easily lose *Foxp3* expression and produce pro-inflammatory effector molecules (Miyao et al. 2012; Ohkura et al. 2012).

In conclusion, it is now clear that Foxp3 is essential to amplify pre-established activities and solidify the Treg-specific phenotype but is not sufficient to confer stable Treg lineage identity. It appears that the Treg-specific epigenetic signature and transcriptional program is initiated at a higher level of regulation upstream of *Foxp3*. The precise mechanisms of epigenetic imprinting in differentiating Tregs remain to be elucidated. As of now, the Treg-specific demethylation pattern combined with *Foxp3* expression provides reliable criteria that indicate long-term Treg lineage stability and function.

4 Downstream Targets of Foxp3

4.1 Structural and Functional Domains of Forkhead Box Proteins and Foxp3

The forkhead box (Fox) family of TFs comprises more than 100 proteins. All Fox family members share a highly conserved winged helix DNA binding domain consisting of 100 amino acids (Jonsson and Peng 2005). More than 18 subclasses, FoxA through FoxQ, have been identified and classified along a unified nomenclature (Tuteja and Kaestner 2007). The three-dimensional structure of their shared forkhead domain has been determined via crystallography to be a butterfly structure containing a helix-turn-helix core of three or four α helices flanked by two loops (Clark et al. 1993). Forkhead box proteins generally bind DNA as monomers, and the DNA-binding structure is highly conserved between species to recognize DNA binding motifs of 15–17 bp in length (Carlsson and Mahlapuu 2002). Upon DNA-binding, winged helix proteins such as forkhead box proteins can open chromatin structures and promote gene activation through the assembly of enhancer complexes (Cirillo and Zaret 1999), whereas other forkhead proteins also repress gene transcription. Repression and/or activation are mediated through distinct domains, which show high structural variation between different forkhead box proteins (Carlsson and Mahlapuu 2002).

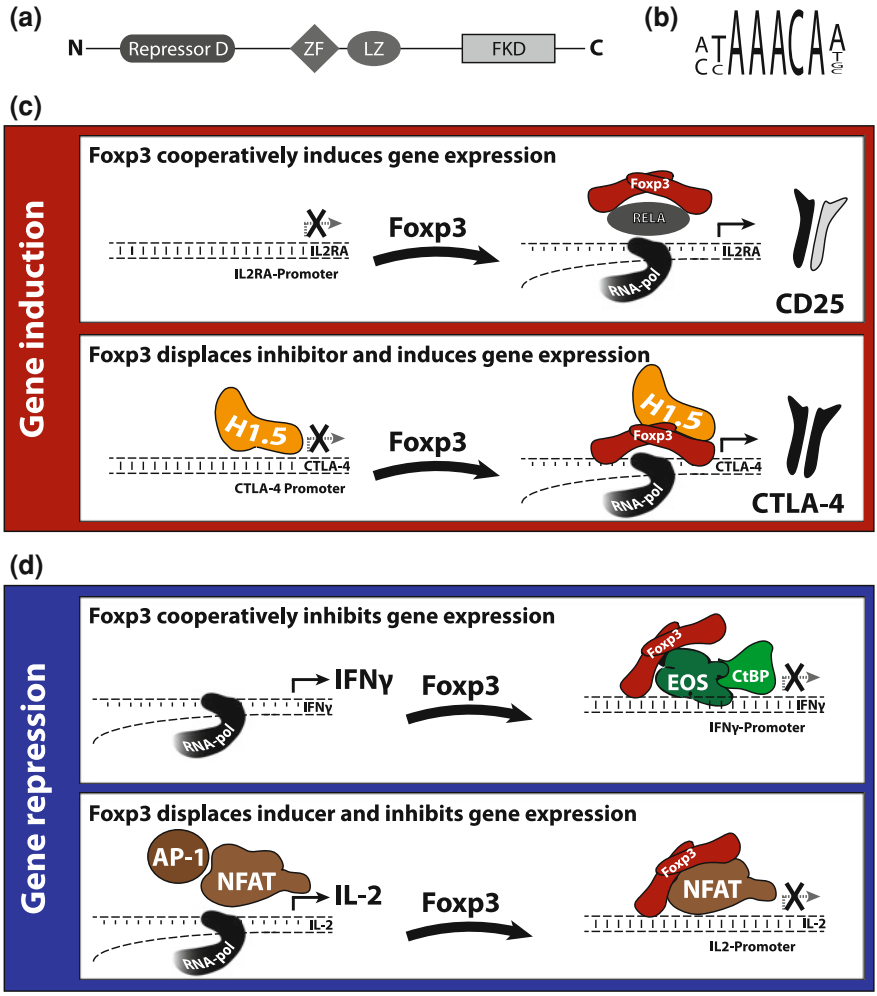

Fig. 3 Foxp3 protein and its mode of action. **a** Structural and functional domains of the Foxp3 protein. ZF, zinc finger; LZ, leucine zipper; FKD, forkhead domain. **b** Consensus DNA target sequence of Foxp3. **c** Mechanisms of Foxp3-guided gene induction. **d** Mechanisms of Foxp3-guided gene repression

The lineage-defining TF in Tregs, Foxp3, is a member of the forkhead box family of TFs and contains a C-terminal forkhead (FKH) domain followed by a central tandem zinc-finger-leucine-zipper oligomerization domain. A repressor domain forms the N-terminal end of the protein (Zhou et al. 2008c) (Fig. 3a). Structurally, the central zinc-finger-leucine-zipper domain is important for Foxp3 homodimerization, and mutations in this domain results in impaired transcriptional repressor activity and loss of Treg suppressor activity in vitro and in vivo (Chae et al. 2006; Song et al. 2012). The C-terminal FKH domain is relevant for DNA

binding as well as nuclear localization (Lopes et al. 2006). To enter the nucleus, the Foxp3 protein is thought to bind other nuclear factors via three distinct nuclear import domains which assist in nuclear shuttling (Hancock and Ozkaynak 2009). Finally, the N-terminal repressor domain consists of two subunits, one involved in general repression of transcription by Foxp3 and the other mediating specifically NFAT-NF-κB interaction-mediated repression (Ziegler 2006).

4.2 Molecular Targets of Foxp3

In order to induce or repress gene transcription and translation, Foxp3 has to bind DNA and a common binding motif for Foxp3 proteins has been identified (Koh et al. 2009). The importance of the leucine-zipper domain for Foxp3 oligomerization and DNA binding was determined as mutation of the leucine zipper inhibits DNA probe-binding to Foxp3. These data were in agreement with other reports, where crystallographic studies showed Foxp3 binding to DNA as a domain-swapped dimer bridging two DNA molecules (Bandukwala et al. 2011). Additionally, it was shown that leucine zipper mutations identified in IPEX patients inhibit Foxp3 homodimerization (Lopes et al. 2006). Finally, different oligonucleotide consensus sequences were tested and the common DNA binding motif 3′-**GTAAACA**-5′ was found to be crucial for binding a Foxp3 construct (Fig. 3b). The authors observed increased binding through doubling of the target sequences with 10 nucleotide spacing 3′-**GTAAACAnnnGTAAACA**-5′, indicating that dimerization of Foxp3 translates into a preference for two binding sites, one for each monomer. This binding motif has been confirmed by another study, which utilized primary human Tregs isolated from cord blood (Sadlon et al. 2010). In this study, the authors identified a common human Foxp3 binding motif in 1,000 Foxp3-bound genomic regions. 922 of 1,000 sequences contained one or more FKH binding motifs, with the consensus sequence of **G**(/A)**T**(/A)**AAAC**(/A)**A**A closely related to the findings described above. But despite this identification of a general Foxp3 DNA target consensus sequence, promiscuous Foxp3 binding to non-consensus sequences, such as the *Il-2* promoter, has been reported (Wu et al. 2006). These findings suggest an additional role of Foxp3-associated proteins in mediating target DNA binding.

As mentioned above, the Foxp3 target DNA consensus sequence alone is not sufficient to predict Foxp3-DNA binding sites. Therefore, two studies in 2007 used chromatin-immunoprecipitation (ChIP) to investigate putative Foxp3 binding sites. In one study, Marson and colleagues transduced a Foxp3^{-}CD4^{+} T cell hybridoma with FLAG-tagged Foxp3 (Marson et al. 2007). ChIP studies of stimulated Foxp3-transduced T cell hybridoma revealed more than 1,100 promoter regions to be bound by FLAG-Foxp3. Gene annotation revealed that most targets of Foxp3 are associated with the TCR signaling cascade, from the levels of cell surface receptors (e.g., CD28, PD-1, CD3) through signaling components (e.g., ZAP70, MAPK signaling pathway, BCL-10) to transcriptional regulators (NF-κB, NFAT).

To decipher whether genes were positively or negatively regulated upon Foxp3 binding, the authors performed gene expression profiling, and found most of the genes down-regulated upon binding of Foxp3 to the respective promoters. Down-regulation was especially observed for genes involved in TCR signaling, T cell activation and cytokine production. Another study used nuclear extracts from freshly isolated murine Tregs and compared them to $CD4^+$ T cells from $Foxp3^{-/-}$ mice (Zheng et al. 2007). On a genome-wide basis, they detected more than 1,200 regions that significantly bind Foxp3, with 34 % of binding sites 50 kb upstream or in the 5′ untranslated region (5′UTR) of known or predicted genes. Of the remaining binding sites, 29 % were detected in intronic regions, 24 % in intergenic regions, and less than 1 % were detected in 3′-UTR or coding exons. The authors emphasized the substantial enrichment of Foxp3 binding sites within 10 kb upstream of TSSs, with decreasing Foxp3-binding frequency when increasing the distance from the TSS (Zheng et al. 2007). Gene ontology analysis of target genes revealed that Foxp3 is involved in regulation of TCR signaling pathway genes, cell communication genes, and genes involved in transcriptional regulation, confirming some of the data from Marson and colleagues. Finally, these data cooperate to define an important role of Foxp3 to adapt Tregs to chronic TCR stimulation without differentiation into pro-inflammatory effector cells, therefore conserving their unique suppressive function (Sundrud and Rao 2007). In addition, this study investigated the role of Foxp3 during thymic Treg development, where Foxp3 mostly up-regulates genes in developing tTregs, whereas in the periphery, Foxp3 acts predominantly as repressor of gene activation.

A third study investigated Foxp3-binding genes in in vitro expanded primary human cord blood Tregs (Sadlon et al. 2010). They localized the preferred Foxp3 binding site starting 2 kb upstream of the TSS and extending 3 kb downstream. Pathway analysis revealed Foxp3 target genes to be involved in TCR signaling, immuno-regulatory cytokines and growth factors, confirming what had already been described in the murine system. When comparing mouse and human data, more than 50 % of Foxp3-binding sites are thought to be species-specific, whereas 45 % of binding sites identified in the human study were also identified in either one of the above-mentioned mouse Foxp3 binding studies (Sadlon et al. 2010).

Two independent studies used the aforementioned “wannabe” Treg approach to indirectly analyze Foxp3-regulated target genes (Gavin et al. 2007; Lin et al. 2007). Both studies revealed an up-regulation of Treg-characteristic surface markers such as CD25 and CTLA-4, but expression levels were lower compared to Tregs with intact Foxp3. In conclusion, several key Treg characteristics were induced in “wannabe” Tregs despite the lack of functional Foxp3 protein. However, these cells failed to suppress T cell proliferation in vitro or effector T cell expansion in co-adoptive transfer studies in vivo. Both studies confirm the role of Foxp3 for suppressive function and proliferative potential of Tregs, thus Foxp3 not only induces, but also stabilizes the expression of several Treg lineage genes to prevent the differentiation of Treg precursors into inflammatory T cell lineages (Gavin et al. 2007; Lin et al. 2007). A direct comparison of Foxp3-bound genes, which have been identified via ChIP, with differentially expressed genes in

Foxp3^{+} versus GFP^{+} "wannabe" Tregs revealed 20-30 % of Foxp3-dependent genes to be directly regulated by Foxp3 (Josefowicz et al. 2012a). These data are in agreement with a publication from Hill and colleagues, where they showed that Foxp3 controls only a minority of the identified Treg-characteristic signature genes (Hill et al. 2007).

Finally, two studies reported on Foxp3-based regulation of noncoding RNA through binding to intergenically encoded miRNA regions (Sadlon et al. 2010; Zheng et al. 2007). These findings are consistent with reports about the conditional deletion of DICER or DROSHA, key enzymes for the posttranslational processing of pre-microRNA, leading to an autoimmune disease reminiscent of Foxp3$^{-/-}$ animals (Chong et al. 2008; Liston et al. 2008; Zhou et al. 2008b). This highlights the diversity of the Foxp3 protein in regulating gene expression not only on the DNA, but also on the RNA level.

5 The Foxp3 Complex

5.1 Mass Spectrometry-Based Decryption of the Foxp3 Complex

Additional mechanisms likely exist that could explain the binding of Foxp3 to nonconsensus DNA binding sites such as the *Il-2* gene promoter. Rudra and co-workers investigated the Foxp3 protein complex in a mass-spectrometric approach (Rudra et al. 2012). This analysis revealed a total of 361 proteins binding to the Foxp3 protein. They were able to show that Foxp3 forms large macromolecular complexes, with most of the Foxp3-binding complexes between 400 and 2,000 kilodaltons in molecular mass. Functional annotation of the Foxp3-interacting proteins revealed many partners (75) to be regulators of transcription like Foxp1, Foxp4, Runx1, Gata3, STAT3, and NFATc2. Furthermore, a substantial number of proteins were involved in RNA-processing (52) and regulation of RNA metabolic processing (44), indicating a potential unidentified role for Foxp3 in controlling gene expression on the RNA processing level. Foxp3 interaction partners interfering with chromosome organization (40), chromatin organization (32) and chromatin modification (28) may indicate a role of Foxp3 in controlling gene expression via chromatin alterations. Finally, the authors investigated whether the regulatory DNA elements controlling the expression of Foxp3-interacting proteins were bound by Foxp3 protein itself. They found that more than 50 % of regulatory regions controlling Foxp3 cofactors were targets of Foxp3. Interestingly, some Foxp3 cofactors also bound the *Foxp3* gene at promoter and enhancer sites, pointing towards a reciprocal regulation of gene expression between Foxp3 and its partner proteins (Rudra et al. 2012).

5.2 Experimental Identification of Foxp3-Interacting Partners

In addition to binding partners detected in the above-mentioned mass-spectrometric-based study, several additional groups have identified Foxp3-binding proteins in human and murine Tregs based on other techniques (Table 1). In general, Foxp3 can manipulate downstream target gene activity via several different mechanisms and we describe four possibilities that Foxp3 utilizes to either induce or repress target gene expression (Fig. 3c, d).

Foxp3 cooperatively induces gene expression. In order to induce Treg-specific target gene expression, Foxp3 can form a cooperative complex to directly bind the respective promoter region and boost assembly of the transcriptional machinery. This mechanism has been proposed for the complex of Foxp3 and p65/RelA and the synergistic action of Foxp3 with interferon regulatory factor 4 (IRF4). Prior to forming the Foxp3-RelA complex, stimulation of CD28 in developing Tregs recruits RelA to the *Il-2ra* gene locus. Then, close interaction of Foxp3 with RelA synergistically promotes expression of the *Il-2ra* gene (Camperio et al. 2012) (Fig. 3c). In a comparable way, Foxp3 and IRF4 bind the inducible T cell co-stimulator (*Icos*) gene promoter and selectively induce gene expression (Zheng et al. 2009).

Foxp3 displaces inhibitor and induces gene expression. In a variation of this process, Foxp3 can actively displace gene-inhibitory elements and then induce gene expression, as shown for the PLU-1/MOF system and the complex of Foxp3 and the linker histone H1.5 (H1.5). In the PLU-1/MOF system, PLU-1 is actively displaced and the cooperative binding of MOF with Foxp3 induces target gene expression (Katoh et al. 2011). Similarly, Foxp3-binding displaces H1.5 and thereby reverses epigenetic gene silencing of the *Ctla-4* gene locus and promotes *Ctla-4*-expression in Foxp3$^+$ Tregs (Mackey-Cushman et al. 2011) (Fig. 3c).

Foxp3 cooperatively inhibits gene expression. The cooperative binding between Foxp3 and its partner can also cause significant gene repression, as shown for STAT3, Tat-interactive protein 60 (TIP60), NF-κB, and EOS. The complex of STAT3 and Foxp3 has been shown to be important for down-modulation of *Il-6* and *Tgf-β* cytokine expression, whereas other genes are up-regulated (Chaudhry et al. 2009). Foxp3 can also utilize epigenetic mechanisms to silence target gene expression. The complex of Foxp3 and TIP60, a histone acetyltransferase, in concert with HDAC7/9 epigenetically silences the *Il-2* gene locus (Li et al. 2007). Another example for epigenetic silencing of pro-inflammatory target gene loci is the EOS-Foxp3 complex (Pan et al. 2009) (Fig. 3d). This complex binds the promoter regions of both *Il-2* and *Ifn-γ*, recruits the C-terminal binding protein (CtBP) and epigenetically silences this region. Finally, the down-modulation of NF-κB-target genes *A20* and *cIAP$_2$* is mediated via a complex of Foxp3 and NF-κB (Bettelli et al. 2005).

Foxp3 displaces inducer and inhibits gene expression. In addition, Foxp3 can actively displace pro-inflammatory gene inducers and silence gene expression through the formation of an inhibitory complex, shown for NFAT, acute myeloid

Table 1 Direct *Foxp3*-binding partners and target gene regulation

TF	Short functional description	Phenotype	Species	Literature
NFAT1	In Tregs, Foxp3 displaces AP-1 from target genes to form the *NFAT:DNA:Foxp3* complex repressing activation-induced genes and promoting anergy and an immunosuppressive phenotype	IL-2↓, CTLA-4↑, CD25↑, CD103↑, GITR↑	Mouse ms/hu	Wu et al. (2006) Bandukwala et al. (2011)
HIF1α	HIF1α can bind and mark Foxp3 for proteasomal degradation, thereby promoting a T_H17 phenotype over the Treg phenotype	Glycolysis↓ (Glut1↓, HK2↓, GPI↓, TPI↓, LDHα↓)	Mouse Mouse	Dang et al. (2011) Shi et al. (2011)
EOS	EOS forms a complex with Foxp3, recruiting CtBP1 to modify target gene histone methylation and acetylation. The EOS-Foxp3-CtBP1 complex represses the *Il-2* and *Ifn-γ* promoter in Tregs	IL-2↓, IFN-γ↓	Mouse	Pan et al. (2009)
AML1	AML1 initiates *Il-2* and *Ifn-γ* gene expression in Tconv. Close binding of Foxp3 with AML1 prevents inflammatory gene expression, creating the immunosuppressive phenotype	IL-2↓, IFN-γ↓, CTLA-4↑, CD25↑, GITR↑	Mouse Mouse Human	Rudra et al. (2009) Bruno et al. (2009) Ono et al. (2007)
NFκB	Foxp3 binds NFκB and directly blocks its transcriptional activity, leading to reduced expression of NFκB-dependent genes	A20↓, cIAP2↓	Mouse	Bettelli et al. (2005)
RelA	CD28-induced RelA and Foxp3 synergize in promoting gene expression at the *Il-2ra* gene locus	CD25↑	Human	Camperio et al. (2012)
RORγt	Foxp3 can inhibit RORγt-mediated induction of T_H17 cell differentiation, thus promoting the Treg phenotype	IL-17↓, IL-23r↓, IL-22↓	Mouse	Zhou et al. (2008a)
TIP60 HDAC7	Foxp3 forms a complex with HAT TIP60 and HDAC7 to mediate self-acetylation of Foxp3 and downstream repression of gene expression, e.g., *Il-2*	Foxp3↑ IL-2↓	Human	Li et al. (2007)
H1.5	Foxp3 and H1.5 cooperatively repress the induction of *Il-2* gene expression by enhancing H1.5 binding to the promoter region of the gene and thereby promoting epigenetic silencing. *Ctla-4* expression is induced through Foxp3-based inhibition of H1.5-binding to the *Ctla-4* promoter	IL-2↓ CTLA-4↑	Human	Mackey-Cushman et al. (2011)

(continued)

Table 1 (continued)

TF	Short functional description	Phenotype	Species	Literature
STAT3	STAT3 cooperates with Foxp3 to define Tregs capable of suppressing T_H17 responses. STAT3$^{-/-}$ Tregs can still suppress T_H1 and T_H2 responses, but not T_H17 responses	IL-10, CCR6, IL-1R IL6-R, IL-6, TGF-β	Mouse	Chaudhry et al. (2009)
MOF	Foxp3 can bind target DNA sequences and displace PLU-1, a histone H3K4 demethylase, followed by recruitment of MOF, which induces target gene transcription through epigenetic mechanisms	No information provided	Human	Katoh et al. (2011)
IRF4	Conditional deletion of IRF4 in IRF4 $^{fl/fl}$Foxp3^{Cre} mice causes severe autoimmune disease. IRF4 cooperates with Foxp3 to induce $\sim$20 % of Treg-specific genes	ICOS↑	Mouse	Zheng et al. (2009)
TCF1	Active canonical Wnt signaling disrupts Foxp3 suppressive activity on several pro-inflammatory target genes. Deletion of Wnt-pathway member TCF1 enhances Treg suppressive capacity	IL-2↑	Human Mouse	Van Loosdregt et al. (2013a)
USP7	USP7 co-localizes with Foxp3 and prevents its polyubiquitinylation, resulting in decreased Foxp3 degradation. USP7 knockdown in Treg cells decreases Treg-cell mediated suppressive capacity	IL-2↓	Mouse	Van Loosdregt et al. (2013b)
Stub1	In response to danger signals, the E3 ligase Stub1 assisted by heat-shock protein Hsp70 ubiquitinylates Foxp3 and thereby marks it for degradation	IFN-γ↑, IL-2↑, CD25↓, CTLA-4↓, GITR↓, IL-10↓	Human Mouse	Chen et al. (2013)

leukemia 1 protein (AML-1), T-cell factor 1 (TCF-1) and RORγt. NFAT can form complexes with AP1 to act as potent TFs in Foxp3$^-$ cells and for instance, inducing *Il-2* or *Il-4* gene expression. When Foxp3 is expressed, it displaces AP-1 and forms an inhibitory complex with NFAT, thereby inhibiting *Il-2* gene expression (Wu et al. 2006). In a study investigating the high-resolution crystallographic structure of a complex of Foxp3 and NFAT1, molecular mechanisms of the intimate binding between both partners have been revealed and confirm the NFAT-Foxp3 interaction (Bandukwala et al. 2011). Furthermore, it was shown that Foxp3 does not simply compete for the *Il-2* promoter region or displace NFAT, but forms a cooperative NFAT:FOXP3:DNA complex which replaces the NFAT:AP1 complex at the *Il-2* promoter (Wu et al. 2006). Additional experiments indicated that NFAT cooperates with Foxp3 not only to inhibit the *Il-2* gene expression, but also to promote the expression of *Il-2ra* and *Ctla-4* through promoter binding (Fig. 3d). AML-1 also forms a complex with NFAT to induce *Il-2* and *Ifng* gene expression, which can be inhibited by the Foxp3-NFAT complex (Ono et al. 2007; Rudra et al. 2009; Bruno et al. 2009). Additionally, a recent global transcription factor interaction analysis identified a close interaction between Foxp3 and TCF-1. The study shows that Foxp3 forms a complex with both the Wnt-signaling components TCF-1 and β-catenin at several Foxp3-target genes, for example at the *Il-2* promoter. They then induce gene expression, resulting in increased IL-2 mRNA and protein levels. When pre-treating Treg cells with Wnt signaling agonist or Wnt3a protein, the in vitro and in vivo suppressive capacity of Treg cells is attenuated. In contrast to this, knockout of TCF-1 renders Treg cells more suppressive, indicating TCF-1's role in promoting an inflammatory phenotype over the suppressive phenotype (van Loosdregt et al. 2013b). Finally, Foxp3 can displace RORγt, thereby inhibiting the expression of the T_H17-like cytokines IL-17 and IL-22 (Zhou et al. 2008a).

The hypoxia-inducible factor 1 (HIF1), another TF involved in Treg differentiation, promotes a Treg phenotype over the pro-inflammatory T_H17 cell phenotype through regulating cellular metabolism. Upon activation, T_H17 cells generate energy in a HIF1-dependent manner through expression of glycolytic enzymes. Under normoxic and hypoxic conditions, HIF1 can bind Foxp3 and target it for proteasomal degradation and thereby inhibit Treg induction (Dang et al. 2011). HIF1$\alpha^{-/-}$ mice display diminished T_H17 development in contrast to enhanced Treg generation, shifting the balance between both cell types towards immunosuppression. In a similar way, Stub1 responds to environmental stress or danger signals during inflammation by recruiting heat-shock protein Hsp70 and then ubiquitinylating Foxp3 for proteasomal degradation (Chen et al. 2013).

In contrast to the Foxp3-degrading role of HIF1 and Stub1, a recent study identified the Foxp3-protective function of the deubiquitinase USP7. It was shown to be up-regulated in Treg cells, and to interact with and de-ubiquitinate Foxp3 protein in the nucleus. The shRNA mediated knockdown of USP7 in murine Treg cells caused a marked decrease in *Foxp3* expression and thereby a failure of Treg-mediated suppression in an animal model of experimental colitis (van Loosdregt et al. 2013a).

5.3 Modeling the Transcriptional Signature of Tregs

A computer-based approached was used to model the specific contributions of TF interacting with Foxp3 (Fu et al. 2012). The authors analyzed gene expression profiles comparing mostly Tregs versus Tconv from different anatomical locations, such as lymph nodes, spleen, central nervous system, visceral adipose tissue, and thymus. Furthermore, different mouse strains and genotypes along with different Treg culture conditions were used to increase the overall sample size. The algorithm identified known Treg lineage-specific TFs such as Foxp3 as well as novel putative Treg-depending TFs such as lymphoid enhancer-binding factor (LEF1) and GATA-1. Using additional mathematical modeling, they characterized 10 master TFs that likely govern the genetic up- or down-regulation of more than 50 % of Treg target genes: Foxp3, PLAGL1, EOS, LEF1, GATA-1, XBP1, AHR, SATB1, HDAC9, HOPX. To validate this computer-generated dataset, they tested knockout mice deficient in four factors (EOS, GATA-1, XBP1, and Helios) and generated gene expression profiles. In loss-of-function experiments, they were not able to confirm that knockout mice of any of these specific genes cause a substantial change in the Treg signature. In contrast to this, retroviral transduction of activated $CD4^+$ T cells with EOS, IRF4, GATA-1, LEF1, and SATB1 acted synergistically with Foxp3 to shift the Treg signature, indeed validating the *in silico* predicted importance of these regulators (Fu et al. 2012). These data showed that Foxp3 and its cofactors locked the lineage-specific Treg signature.

The TFs EOS and IRF4 have been reported to bind Foxp3 (Pan et al. 2009; Zheng et al. 2009), as well as GATA-1, LEF1 and SATB1. The authors experimentally determined that the Foxp3-binding cofactor GATA-1 does not enable Foxp3 to bind to otherwise locked gene loci, but significantly enhances the DNA-promoter occupancy of Foxp3, serving as an enhancer (Fu et al. 2012). Additionally, Samstein and colleagues have recently reported that Foxp3 takes advantages of a pre-existing enhancer landscape where Foxp3 in an "opportunistic" manner utilizes a preformed transcription scenery and impinges a new interpretation (Samstein et al. 2012a). In this respect, they described that the TF Foxo1 serves as a pioneer at many Foxp3-binding loci in Treg precursors (Samstein et al. 2012a).

6 Treg Subsets Based on Transcription Factor Expression

6.1 Treg Heterogeneity and the Concept of Distinct Treg Subsets

Although Foxp3 is the most important transcriptional regulator of Treg function, recent evidence suggests that other TFs are required for the development of certain functionally distinct subsets of Tregs. The Treg subset concept arose from

accumulated evidence describing the heterogeneity of Tregs. They do not utilize a universal suppressor mechanism but have a very diverse and dynamic repertoire that changes depending on the inflammatory context as well as the location of the response. This is not surprising since Tregs are required to control a very complex and diverse array of immune effector responses. This ranges from preventing the initiation of immune responses in the secondary lymphoid tissue, for example against self-antigens, to the dampening of ongoing immune responses, or restoration of immune homeostasis, in peripheral, nonlymphoid tissues.

It has long been known that $CD4^+$ effector T cells can develop into distinct functional subsets depending on the cytokine signals that are present during their initial activation. For example, IFN-γ and IL-12 promote the development of T helper 1 (T_H1) effector cells, IL-4 promotes T_H2 development and TGF-β together with IL-6 result in T_H17 development. These cytokine signals produce stable T_H subsets through induction of differential expression of master TFs, such as T-box TF TBX21 (T-bet), GATA3, and RORγt. Similarly, it has been suggested that Tregs also develop into specific subsets depending on environmental signals present during their activation and, furthermore, these differentiation signals and resulting genetic signatures mimic that of the target cells or tissues (Fig. 4) (Feuerer et al. 2009b, 2010).

6.2 TFs Involved in Treg Subset Development

On a population level, Tregs exhibit tremendous diversity and plasticity regarding the mechanisms and extent of immune regulation. Interestingly, recent evidence has demonstrated that Treg-specific deletion of certain TFs disrupted the suppression of only certain types of immune responses (Fig. 4). For example, Treg-specific loss of IRF4 or GATA3 resulted in unregulated T_H2 responses (Zheng et al. 2009; Wang et al. 2011; Wohlfert et al. 2011). Similarly, deletion of STAT3, B-cell lymphoma 6 (Bcl6) or T-bet results in the loss of control of T_H17, follicular helper cell (T_{FH}) and T_H1 responses, respectively (Chaudhry et al. 2009; Chung et al. 2011; Koch et al. 2009).

Matched symmetry between T_H subsets and Treg subsets has some appeal, especially in light of the explosion in the number of T_H subsets. However, since Tconv are not the only targets of Treg-mediated control, identification of additional subsets was inevitable. Recently, the first tissue-specific Treg subset was identified that plays a direct role in controlling inflammation and maintain tissue homeostasis (Feuerer et al. 2009a). Tregs isolated from adipose tissue exhibited a distinct gene signature compared to Tregs isolated from secondary lymphoid tissues. In fact, this Treg subset expressed genes commonly associated with adipose tissue. This included the TF peroxisome proliferator-activated receptor γ (PPAR-γ), which serves as the major factor involved in differentiation and accumulation of adipose tissue Tregs (Cipolletta et al. 2012).

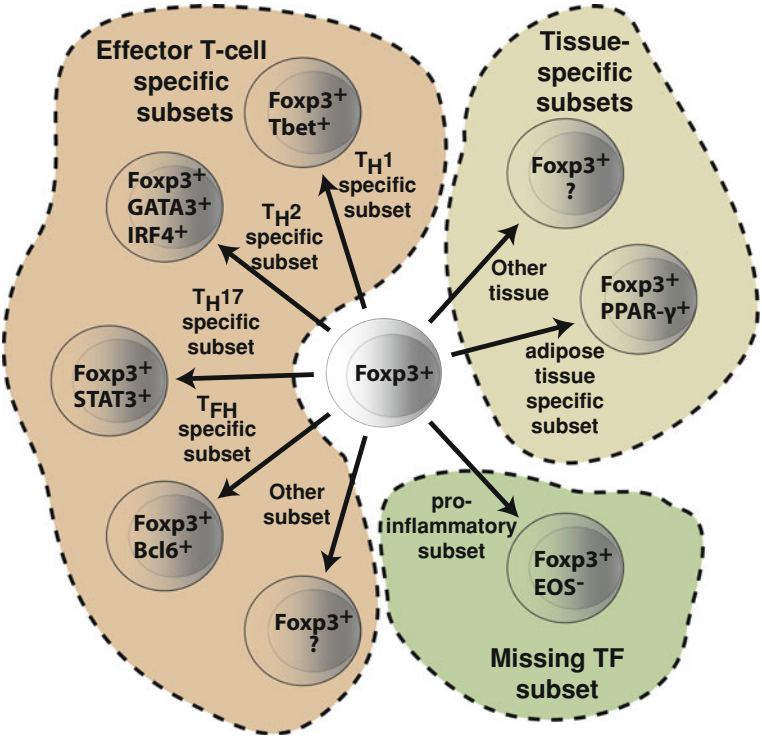

Fig. 4 Functional Treg subsets determined by transcription factor expression

A specialized population of Treg cells mediating the repair of skeletal muscle in injured mice has recently been described. Those muscle Treg cells over-express amphiregulin, a growth factor that enhances muscle regeneration. A key muscle-specific transcription factor has yet to be identified, but gene expression comparisons revealed a number of candidate TF (Burzyn et al. 2013).

6.3 TF-Dependent Treg Subset Regulatory Mechanisms

It appears that Treg plasticity and diversity is regulated by the unique interactions between the Treg master regulator Foxp3 and expression of additional TFs. The observation that target cells (or tissues) and their complimentary Tregs require expression of the same TFs raises questions about the mechanisms of target-specific suppression as well as the stability of these subsets.

Two distinct subset-specific mechanisms of TF-dependent immune suppression have been demonstrated. The first, which influences Treg migration patterns rather than intrinsic suppressive properties, is exemplified by T-bet. Effector T cells express T-bet in response to IFN- γ, IL-12, and STAT1-dependent signals produced

in the environment during activation. T-bet expression promotes IFN-γ secretion and expression of the chemokine receptor CXCR3. Treg expression of T-bet is also dependent on IFN-γ and leads to the expression of CXCR3. This expression allows Tregs to migrate to sites of T_H1-type inflammation and suppress the tissular T_H1 immune responses (Koch et al. 2009). Accordingly, T-bet-deficient Tregs do not accumulate at these inflammatory sites and are therefore unable to control T_H1 responses. In fact, CXCR3-deficient Tregs show a similar phenotype. These findings, along with similar results involving Bcl-6 and CXCR5, suggest that the subset-specific TFs impart specificity based on chemokine receptor expression and this allows Tregs to better co-localize with their target cell population (Linterman et al. 2011).

The second mechanism involves more intrinsic modification of Treg-mediated suppression. For example, IRF4-deficient Tregs have significant defects in the production of CTLA-4, and both IRF4- and STAT3-decifient Tregs have deficiencies in IL-10 production. Although these defects influence the suppression of all immune responses, the T_H-specific bias is established through significantly higher sensitivity of T_H2 cells and T_H17 cells to CTLA-4 and IL-10, respectively (Tian et al. 2011; Huber et al. 2011; Chaudhry et al. 2011).

6.4 *Environmental Factors Influencing Treg Subset-Specific TF Expression*

The environmental signals that polarize effector T cells into distinct subsets are similarly responsible for Treg subset differentiation. This concept used to describe Treg control of effector T cells can be expanded further to describe how Tregs interact with non-immune cells and nonlymphoid tissues. The identification of the first tissue-specific Treg subset that plays a role in controlling inflammation and maintain tissue homeostasis raises additional questions regarding the location of the environmental imprinting (Feuerer et al. 2009a). The presence of adipose-specific gene patterns suggests that this programming does not occur in the secondary lymphoid tissues but rather in the specific tissue itself. It also suggests that additional tissue-specific Treg populations might be present in tissues where controlling inflammation and maintaining homeostasis is critical.

6.5 *Treg Subset Generation and Stability*

The ability of Tregs to transition from one subset to another, with their specific suppressive mechanisms, could account for much of the plasticity observed in the Treg population. However, this raises questions regarding the stability of these Treg subsets. On one hand, every Treg could have the potential to express each TF

and expression would depend entirely on environmental signals. In contrast, individual Tregs could be predestined to differentiate into distinct populations that are able to suppress specific effector T cell populations. This predestination could be mediated by antigen-specificity, TCR sensitivity, or epigenetic factors. For example, recent evidence suggests that while on a population level, T_H cells can develop into different T helper subsets, there is strikingly limited diversity on the single cell level (Tubo et al. 2013). This propensity toward certain T_H subsets is dependent on the specific TCR of the $CD4^+$ T cell. In fact, fat-resident Tregs have a very specific TCR repertoire and a unique TF signature indicating that there may be a bias toward certain subsets (Feuerer et al. 2009a; Cipolletta et al. 2012). In addition, a distinct surface marker and DNA methylation pattern was observed on tTregs that were destined to become reprogrammed EOS-negative, Foxp3-expressing effector cells (Sharma et al. 2013).

7 Perspectives: Targeting Tregs

In addition to the well-known role of Tregs in mediating self-tolerance and immune homeostasis there is clear evidence that Tregs can impede the development of effective immune responses in cancer-bearing hosts and individuals suffering from chronic infectious diseases. In fact, several studies reported accumulation of Tregs in diverse tumor types. Such Tregs are capable of suppressing the function of effector T cells and are thereby often associated with reduced survival and poor prognosis (Ormandy et al. 2005; Chen et al. 2011a; Viguier et al. 2004). Thus, tumor-induced expansion of Tregs is a major obstacle for successful cancer immunotherapies (Pardoll 2012). A similar scenario has been described for infectious agents. Such is the case for viral infections, in which Tregs dampen virus-specific cytotoxic T cell responses in mice (Dittmer et al. 2004). Based on these findings, new immunotherapeutic strategies focus on targeting Tregs. Different approaches have been proposed to target Treg function, and initial investigations suggested that depletion of Tregs might be a valid strategy. For example, elimination of Tregs by targeting CD25 with monoclonal antibodies was considered as a promising therapeutic option (Jones et al. 2002; Ambrosino et al. 2006), and several groups could show that depletion of Tregs before inoculation of tumor cells led to their efficient rejection (Onizuka et al. 1999; Shimizu et al. 1999; Teng et al. 2010; Li et al. 2003). However, when Tregs were depleted after tumor challenge, hardly any tumor regression was observed, most likely because the administrated antibodies lack sufficiently high specificity since activated effector T cells expressing CD25 were also removed upon treatment (Onizuka et al. 1999; Quezada et al. 2008; Betts et al. 2007). A number of clinical trials using a CD25-directed diphtheria toxin (denileukin diftitox) to eliminate Tregs have been started in patients with renal cell carcinoma or melanoma (Dannull et al. 2005; de Vries et al. 2011; Mahnke et al. 2007), but have given discrepant results (Barnett et al. 2005; Attia et al. 2005), most likely due to the nonspecificity of the reagent.

A more promising and more specific Treg-targeting strategy has been recently reported, where anti-CCR4 monoclonal antibodies were shown to selectively deplete human effector/memory-like Foxp3$^+$ Tregs and to evoke potent anti-tumor immune responses (Sugiyama et al. 2013).

An alternative strategy in addition to the specific depletion of Tregs might be the selective and transient inhibition of Treg function. Since Tregs obtain immunosuppressive function by expression of Foxp3, and reduction of *Foxp3* expression results in loss of suppressive capacity (Wan and Flavell 2007; Williams and Rudensky 2007), a potential target candidate protein would be Foxp3. Recently, several Foxp3 binding peptides have been identified from a phage-displayed random peptide library (Casares et al. 2010). One of them (P60) is of great interest since this peptide was able to bind Foxp3 and inhibit Treg activity in vitro and in vivo by reducing Foxp3 nuclear translocation. Interestingly, adult mice treated with P60 peptide did not develop any inflammatory immune disease. These data suggest that in vivo inactivation of the Tregs' suppressive capacity in adult individuals might enhance the efficacy of a vaccine against tumors or infections. Indeed, in vivo treatment with P60 improves immunogenicity of AH1 peptide vaccination, protecting mice from CT26 tumor challenge. Similarly, vaccination against HCV is enhanced in individuals after administration of peptide P60 (Casares et al. 2010). These data suggest that direct targeting of *Foxp3* expression and/or function might be an attractive strategy to reduce the suppressive activity of Tregs, which may offer a considerable potential for therapeutic applications.

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Transcriptional Regulatory Networks for CD4 T Cell Differentiation

Darah Christie and Jinfang Zhu

Abstract $CD4^+$ T cells play a central role in controlling the adaptive immune response by secreting cytokines to activate target cells. Naïve $CD4^+$ T cells differentiate into at least four subsets, Th1, Th2, Th17, and inducible regulatory T cells, each with unique functions for pathogen elimination. The differentiation of these subsets is induced in response to cytokine stimulation, which is translated into Stat activation, followed by induction of master regulator transcription factors. In addition to these factors, multiple other transcription factors, both subset specific and shared, are also involved in promoting subset differentiation. This review will focus on the network of transcription factors that control $CD4^+$ T cell differentiation.

Contents

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1 Introduction

An effective immune response is vital in the protection against invading foreign pathogens. $CD4^+$ T cells play a pivotal role in host defense by secreting cytokines to drive appropriate immune responses. Classified by cytokine secretion profile, $CD4^+$ T cells are subdivided into four major subsets. Th1 cells secret IFNγ to clear intracellular pathogens while Th2 cells secret IL-4, IL-5, and IL-13 to clear helminthes and extracellular pathogens (Zhou et al. 2009). Th17 cells, originally identified as the causative cell type in the experimental autoimmune encephalitis (EAE, a mouse model of multiple sclerosis), are characterized by the secretion of IL-17 and are involved in the clearance of extracellular bacteria and fungi (Korn et al. 2009). Regulatory T cells (Tregs), including thymus derived Tregs (tTregs) and peripherally induced Tregs (pTregs), secrete anti-inflammatory cytokines including TGFβ and IL-10 and act to suppress immune responses to prevent damage to the host (Josefowicz et al. 2012). At steady state, Tregs are indispensable for maintaining self-tolerance thus preventing autoimmunity through multiple mechanisms. Besides Th1, Th2, Th17, and iTreg cells, some $CD4^+$ T cells reside within the B cell follicle and are thus named T follicular cells (Tfh); these cells express the chemokine receptor CXCR5 and produce large amounts of IL-21 (Crotty 2011). Tfh cells function by providing help to B cells. However, the relationship between Tfh cells and classical Th1, Th2, and Th17 effector cells is not certain since some Tfh cells are capable of producing either IFNγ or IL-4 (Lee et al. 2012a; Yusuf et al. 2010). In addition, regulatory T cells expressing the key transcription factor Foxp3 have been also found in B cell follicles (Chung et al. 2011; Linterman et al. 2011) and Th17 cells have been shown to convert to Tfh cells in Peyer's patches and provide help to B cells, thus increasing IgA production (Hirota et al. 2013). Thus, it remains unclear whether Tfh cells represent a separate subset or whether they differentiate from other $CD4^+$ T cell subsets. Furthermore, it has been shown that IL-21-expressing Tfh cells may give rise to memory cells that can further differentiate into conventional effector cells during recall responses

(Luthje et al. 2012). Finally, Th9 and Th22 cells have also been characterized as separate subsets recently, based on the expression of IL-9 and IL-22, respectively (Jabeen and Kaplan 2012; Duhen et al. 2009), but their relationship to Th2 and Th17 cells, respectively, requires further investigation. Together, these subsets orchestrate the clearance of pathogens while preventing damage to the host.

The induction and maintenance of each $CD4^+$ subset is controlled by the cytokine environment, which activates signal transducers and activators of transcription (Stat) pathways to induce the expression of the master regulator transcription factors. The Stat and master regulator controlling each subset have been defined as follows, Stat4/Tbet (Th1), Stat6/Gata3 (Th2), Stat3/RORγt (Th17), Stat5/FoxP3 (Treg), and Stat3/Bcl6 (Tfh), and have been widely studied (Zhu et al. 2010). Although these factors are essential for the differentiation of a particular subset, the master regulators do not act alone but are instead a component of a larger transcriptional network. Multiple transcription factors can interact directly or indirectly to control gene expression programs. Direct interaction of transcription factors can increase transcriptional activity by increasing recruitment of additional transcription factors or transcriptional machinery to target genes. Conversely, direct interaction may inhibit gene expression by blocking the binding of transcription factors to target genes. Many transcription factors also recruit chromatin and histone modifying enzymes to increase or decrease accessibility of binding sites for other transcription factors. Finally, multiple binding sites within a gene may allow for cooperation between multiple transcription factors or, conversely, allow competitive inhibition between factors, where the binding of one transcription factor may block the binding of another. In this case, the balance of transcription factors will determine the pattern of gene expression. These types of interactions allow cells to alter gene expression in response to changes in the balance of transcription factors, allowing a level of plasticity within $CD4^+$ T cell subsets. In order to fully understand the regulation of $CD4^+$ T cell differentiation and the plasticity potential within each, a thorough understanding of the entire network of transcription factors is required.

2 Signals for Differentiation

The initial steps in $CD4^+$ T cell differentiation, after activation through T cell receptors, relies upon binding of cytokines to their cognate receptors, resulting in the activation of receptor associated Janus kinases (Jaks), which phosphorylate the intracellular domain of cytokine receptors to provide specific docking sites for Stat binding (Leonard and O'Shea 1998; O'Shea and Plenge 2012). Upon recruitment to the receptor, activated Jaks phosphorylate and activate Stats, which in turn induce the expression of genes involved in the initial differentiation of each subset, including the induction of the master regulators. The master regulators activate expression of the polarizing cytokine, which induces a positive feedback loop to strengthen the differentiation toward a given subset.

2.1 Th1: Stat4, Stat1

Th1 differentiation is generally induced by IL-12 secreted from antigen presenting cells (Fig. 1). IL-12 signals through Stat4 and the importance of this pathway in Th1 differentiation is demonstrated by Stat4 deficient mice, which show impaired Th1 responses with decreased IFNγ production (Kaplan et al. 1996b; Cai et al. 2000; Thierfelder et al. 1996). Stat4 activates the expression of many Th1-specific genes, including *Ifng* (Lund et al. 2004; Hoey et al. 2003), *Il12rb2,* and *Tbx21* encoding Tbet (Hoey et al. 2003). In addition to directly inducing Tbet expression, Stat4 also indirectly upregulates Tbet by inducing IFNγ expression, which signals through Stat1 to activate Tbet expression (Lighvani et al. 2001; Afkarian et al. 2002). In turn, Tbet binds to and activates IFNγ expression (Matsuda et al. 2007; Lovett-Racke et al. 2004; Jenner et al. 2009) and the loss of Tbet expression correlates with decreased IFNγ expression (Szabo et al. 2002; Long et al. 2006). As such, during Th1 differentiation, a positive feedback loop is generated between IFNγ and Tbet, reinforcing the Th1 gene expression program. Indeed, while Stat4 deficient mice have defects in Th1 differentiation, treatment with IFNγ resulted in increased Tbet expression (Lighvani et al. 2001) and treatment of wild type cells with either IL-12 or IFNγ-induced Tbet expression (Zhu et al. 2012), indicating Th1 differentiation can occur in the absence of IL-12. However, a study of the contributions of IL-12 and IFNγ to Th1 differentiation demonstrated that while IFNγ stimulation induced Th1 differentiation, recall responses of Th1 cells required IL-12 stimulation, indicating the essential role of IL-12/Stat4 for maintaining Th1 cells (Schulz et al. 2009). Both Stat4 and Tbet are required for maximal IFNγ production and while loss of either one resulted in decreased IFNγ production, loss of both Tbet and Stat4 resulted in a complete block of IFNγ production, indicating cooperation between these factors (Zhu et al. 2012). However, the targets of Stat4 and Tbet are not completely overlapping, pointing to a wider role for Stat4-induced gene expression programs in Th1 differentiation, beyond the induction of IFNγ and Tbet expression (Thieu et al. 2008). Surprisingly, Stat4$^{-/-}$Stat6$^{-/-}$ double deficient mice cultured under nonpolarizing conditions were capable of secreting IFNγ, indicating that in the absence of Th2 differentiation, cells can activate expression of Th1 specific genes without signaling through Stat4 (Kaplan et al. 1998). However, whether such IFNγ production is Tbet dependent has not been determined.

2.2 Th2: Stat6, Stat5

Differentiation of Th2 cells requires stimulation with IL-4 (Le Gros et al. 1990; Swain et al. 1990; Hsieh et al. 1992; Seder et al. 1992), particularly in vitro, and in the absence of IL-4, cells stimulated through the T cell receptor are driven predominately toward Th1 differentiation (Seki et al. 2004). IL-4 signals through

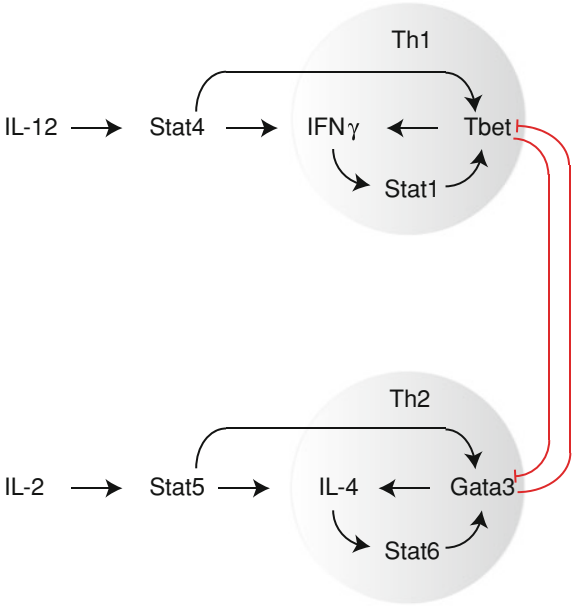

Fig. 1 Positive feedback regulation of $CD4^+$ T cell differentiation. Cytokine induced Stat activation initiates $CD4^+$ T cell differentiation by activating expression of master regulators. A positive feedback loop is generated by increased expression of activating cytokines in response to master regulation expression, thus reinforcing expression of the master regulator and the subset specific gene expression program

Stat6 and mice deficient in Stat6 show limited Th2 cell differentiation (Kaplan et al. 1996a) and Th2 cytokine production, both in response to Th2 polarizing conditions in vitro (Shimoda et al. 1996) and in response to Th2-inducing pathogens (Takeda et al. 1996). Furthermore, expression of a constitutively active form of Stat6 drives cells toward Th2 differentiation, even under conditions that strongly favor Th1 polarization (Kurata et al. 1999; Zhu et al. 2001). Th1 cells expressing constitutively active Stat6 have altered chromatin structure at both *Gata3* regulatory regions (Onodera et al. 2010) and the Th2 cytokine locus (Lee and Rao 2004) resembling that found in Th2 cells, indicating that Stat6 can induce chromatin modifications to increase expression of Th2 specific genes. Similar to Th1 cells, Th2 commitment is also driven by a positive feedback loop, in which IL-4 induces further IL-4 production (Le Gros et al. 1990; Swain et al. 1990; Hsieh et al. 1992; Seder et al. 1992). IL-4 signaling through Stat6 induces Gata3 expression (Kurata et al. 1999; Zhu et al. 2001) and Gata3 in turn, drives the expression of IL-4. Gata3 deficient cells fail to produce IL-4 at early time points after T cell stimulation and show no Th2 differentiation (Yamane et al. 2005). Although Stat6 has a clearly demonstrated role in Th2 differentiation, further study of these mice revealed that while Th2 recall responses were severely impaired (Finkelman et al. 2000), initial Th2 differentiation did occur normally (Finkelman et al. 2000; Jankovic et al. 2000). Indeed, IL-2 in combination with low peptide stimulation was shown to drive early Th2 differentiation in the absence of IL-4 signals (Yamane et al. 2005). IL-2 signals through Stat5 and Stat5 deficient mice were found to have fewer Th2 cells, demonstrating the importance of this pathway in Th2 differentiation (Kagami et al. 2001). Stat5 has been shown to bind to the

IL-4 gene (Hural et al. 2000) and to induce IL-4Rα expression (Liao et al. 2008). Furthermore, blocking IL-2 during Th2 polarization blocked Th2 differentiation (Cote-Sierra et al. 2004) while constitutively active Stat5 increased IL-4 production in the absence of IL-2 (Zhu et al. 2003). However, IL-2 and Stat5 do not upregulate Gata3 expression during initial differentiation (Yamane et al. 2005; Cote-Sierra et al. 2004; Zhu et al. 2003). Instead, IL-2 induces initial IL-4 production by naïve T cells, which in turn stimulates Gata3 expression via Stat6, thus activating the positive feedback between Gata3 and IL-4 to strengthen the commitment to the Th2 subset (Fig. 1). In fully committed Th2 cells, however, Stat5 activation is important for maintaining Gata3 expression (Guo et al. 2009).

2.3 Th17: Stat3

Th17 differentiation is induced by stimulation with IL-6 and TGFβ (Yang et al. 2007) and mice with deletions of either cytokine or associated receptors have a block in Th17 differentiation (Nishihara et al. 2007; Li et al. 2007b; Ivanov et al. 2006; Korn et al. 2007), indicating the requirement for both cytokines in Th17 differentiation. Since TGFβ also induces Treg differentiation, IL-6 is required to divert cells from becoming T regulatory cells. IL-6 signals through Stat3 and constitutively active Stat3 is capable of inducing Th17 differentiation and increasing RORγt expression, although this requires costimulation with TGFβ (Zhou et al. 2007). In contrast, Stat3 deficient mice resemble IL-6 deficient mice, showing an inability to generate Th17 cells (Yang et al. 2007; Mathur et al. 2007; Durant et al. 2010) and increased resistance to EAE induction (Harris et al. 2007). Furthermore, human patients with mutations in Stat3 also show diminished Th17 populations and RORγt production (Milner et al. 2008; Ma et al. 2008). The loss of IL-6 (Korn et al. 2007), IL-21 (Nurieva et al. 2007) or Stat3 (Yang et al. 2007) results not only in decreased Th17 populations but also increased Treg populations, demonstrating the reciprocal regulation of Th17 and Treg differentiation. Stat3 binds to and activates multiple Th17 genes, including cytokines IL-17 and IL-21 as well as transcription factors RORγt, Batf, IRF4, Ahr, and Maf (Durant et al. 2010). Although IL-17 is the hallmark cytokine produced by Th17 cells, it is not capable of driving Th17 differentiation (Korn et al. 2009). Instead, IL-21 may play this role as it is capable of replacing IL-6 in the differentiation of Th17 cells and blocking IL-21 under Th17 conditions reduces Th17 differentiation (Wei et al. 2007; Korn et al. 2007). Furthermore, while IL-6 induces initial IL-21 expression, subsequent IL-21 expression is under autoregulation as IL-6 deficient cells were induced to express IL-21 by treatment with IL-21 (Zhou et al. 2007). The importance of IL-21 in Th17 differentiation is demonstrated by IL-21R deficient mice, which had decreased Th17 differentiation (Zhou et al. 2007). Interestingly, although initial induction of IL-21 expression by IL-6 required only Stat3, the maintenance of Th17 differentiation by IL-21 required both Stat3 and RORγt (Nurieva et al. 2007). Furthermore, the induction of Th17 differentiation by

constitutively active Stat3 was diminished in the absence of RORγt (Zhou et al. 2007), indicating cooperation between Stat3 and RORγt in Th17 differentiation, either at overlapping targets or by activation of unique targets required for Th17 differentiation. Together, these studies indicate that, similar to Th2 cells, Th17 differentiation also proceeds through an initiation stage of Th17 differentiation followed by the maintenance of the Th17 expression program. Initial IL-6 stimulation activates Stat3, resulting in the increased expression of Th17 cytokines, including IL-21 as well as Th17 transcription factors. In turn, IL-21, signaling through Stat3, is required for the maintenance of Th17 differentiation.

IL-23 is also involved in Th17 differentiation as treatment of cells with IL-23 induces IL-17 production (Aggarwal et al. 2003) while IL-23 receptor deficient mice produce less IL-17 and show decreased susceptibility to EAE induction (McGeachy et al. 2009). Although naïve $CD4^+$ T cells do not express IL-23 receptor, IL-6 induces IL-23R expression, which is further augmented by treatment with both IL-6 and IL-23 (Ghoreschi et al. 2010). Both of these cytokines signal through Stat3 and stimulation with IL-6 and IL-23 resulted in increased binding of Stat3 to the *Il23r* locus. IL-23 treatment in combination with IL-6 and IL-1β can also drive Th17 differentiation in the absence of TGFβ stimulation (Ghoreschi et al. 2010). Th17 cells induced by IL-23 have a unique phenotype compared to TGFβ-induced Th17 cells as IL-23-induced Th17 cells express both RORγt and Tbet and are more pathogenic when transferred into $Rag2^{-/-}$ mice. Th17 cells generated with TGFβ produce IL-10, which may be the reason for low pathogenicity of such cells (Lee et al. 2012b). Furthermore, addition of TGFβ to IL-23 driven Th17 differentiation resulted in decreased expression of the IL-23 receptor. Thus TGFβ may also limit Th17 pathogenicity by downregulating IL-23 responsiveness. Interestingly, it has been recently reported that Th17 cells produce TGFβ3, which is IL-23 dependent, and TGFβ3 induces pathogenic Th17 cells (Lee et al. 2012b).

2.4 Treg: Stat5

Differentiation of iTregs is induced by stimulation with IL-2 and TGFβ (Josefowicz et al. 2012). Both IL-2Rα deficient mice and Stat5 deficient mice had decreased Treg populations while constitutively active Stat5 rescued Treg populations in IL-2Rα deficient mice (Burchill et al. 2007). Stat5 binds directly to the *FoxP3* promoter to induce the Treg differentiation program (Burchill et al. 2007; Yao et al. 2007). Stat5 requires histone deacetylase activity to induce Treg differentiation (Burchill et al. 2008), indicating that in addition to directly activating transcription, Stat5 also acts to remodel chromatin structure to change accessibility of additional factors. IL-2 signaling through Stat5 also blocks Th17 differentiation and the loss of IL-2 signaling resulted in decreased Treg populations while Th17 populations were increased (Laurence et al. 2007). Stat5 binds directly to the *Il17* promoter in the same region where Stat3 binds, resulting not only in decreased

Stat3 binding but also in loss of permissive histone modifications within the *Il17* gene (Yang et al. 2011). Interestingly, Stat3 can also bind to the *Foxp3* promoter to inhibit expression (Xu et al. 2010), indicating that the balance between Stat3 and Stat5 levels not only induces the differentiation program of one subset but also actively inhibits the other.

2.5 Tfh: Stat3

Tfh cells are defined by their localization within germinal centers and the surface expression of the chemokine receptor CXCR5 (Crotty 2011). IL-6 signaling through Stat3 has been reported to induce Tfh differentiation (Eddahri et al. 2009; Nurieva et al. 2008). Although IL-6 is involved in both Th17 and Tfh differentiation, Th17 requires both IL-6 and TGFβ while Tfh requires only IL-6 (Nurieva et al. 2008). IL-6-induced Tfh differentiation results in upregulation of IL-21 expression (Dienz et al. 2009; Nurieva et al. 2008), which plays an important role in B cell maturation within germinal centers (Zotos et al. 2010). Indeed, the importance of IL-21 production by Tfh cells has been demonstrated by a decrease in antibody production in mice with T cell-specific deletions of IL-21 (Dienz et al. 2009; Zotos et al. 2010). However, the effect of IL-21 deficiency on the Tfh population size is unclear as some groups have shown decreased Tfh populations in IL-21 and IL-21 receptor deficient mice (Vogelzang et al. 2008; Nurieva et al. 2008) while others have shown normal induction of Tfh differentiation (Zotos et al. 2010), with an accelerated contraction of Tfh cells after stimulation (Linterman et al. 2010). As such, it is possible that similar to Th17 differentiation, IL-6 is required for the initiation of Tfh differentiation while IL-21 is involved in the maintenance of this subset. However, there may be other factors involved in Tfh differentiation, as both IL-6 deficient mice and Stat3 deficient mice showed decreased Tfh populations early in differentiation that equalized at later time points (Choi et al. 2013). Deletion of both Stat3 and Stat1 prevented Tfh differentiation at all time points, indicating the importance of multiple signaling pathways in Tfh differentiation. In addition to upregulation of IL-21 in early Tfh differentiation, IL-6 treatment is also required for downregulation of IL-2Rα expression (Choi et al. 2013). Treatment with either IL-2 or constitutively active Stat5 blocked Tfh differentiation (Ballesteros-Tato et al. 2012; Nurieva et al. 2012) while deletion of either Stat5 or IL-2Rα increased Tfh differentiation (Johnston et al. 2012). Stat5 acts to block Tfh differentiation by binding directly to the *Bcl6* promoter to block Stat3 binding and activation of Bcl6 expression (Oestreich et al. 2012).

3 Master Regulators of CD4 T Cell Differentiation

The induction of $CD4^+$ T cell differentiation is generally thought of as a two-step process. As discussed above, cytokine signaling through the Stat transcription factors is an essential first step in the differentiation process, as evidenced by the loss of specific $CD4^+$ subsets in various Stat knockout mice. However, a major role of Stat activity during $CD4^+$ T cell differentiation is to induce the expression of the so-called "master regulators" of $CD4^+$ T cell differentiation. In general, a master regulator is identified when the enforced expression of this factor alone can induce differentiation into a given subset while deletion of the factor prevents differentiation into this subset from naïve $CD4^+$ cells.

3.1 Tbet

The Tbox family member Tbet controls Th1 differentiation (Szabo et al. 2000), as evidenced by increased IFNγ production in response to Tbet overexpression, under both neutral and Th2 priming conditions (Szabo et al. 2000). In addition, Tbet deficient cells express very low levels of IFNγ and are unable to suppress IL-4 and IL-5 expression under neutral priming conditions (Szabo et al. 2002).

3.1.1 Autoactivation of Tbet

Tbet has been shown to bind to its own promoter (Kanhere et al. 2012), indicating a possible requirement for Tbet autoactivation under certain circumstances (Mullen et al. 2001). However, a study overexpressing Tbet in Stat1 deficient cells failed to show upregulation of endogenous Tbet expression in response to retroviral-induced Tbet expression (Afkarian et al. 2002). Tbet binding to its own promoter may cooperate with Stat1 but cannot induce expression in the absence of Stat1, as the loss of Stat1 leads to the loss of Tbet (Lovett-Racke et al. 2004). Likewise, a reporter mouse expressing ZsGreen under the control of the Tbet promoter showed similar levels of ZsGreen expression in both control and Tbet deficient cells in response to IL-12 and IFNγ stimulation, indicating a minimal role for autoactivation in Tbet expression (Zhu et al. 2012). However, the ZsGreen Tbet reporter on a $Tbet^{-/-}Stat4^{-/-}$ double deficient background showed substantial loss of reporter expression during *T. gondii* infection, indicating that Tbet may autoactivate expression under certain conditions (Zhu et al. 2012). These results indicate that while Tbet may bind its own promoter, autoregulation of Tbet expression occurs only during specific conditions possible at later stage of differentiation or at memory stage when extrinsic stimulating cytokines become limiting.

3.1.2 Mechanism of Action

Large-scale ChIP-sequencing studies have identified binding sites of Tbet throughout the genome, including many immune regulatory genes, as well as cytokine and cytokine receptor genes (Zhu et al. 2012; Kanhere et al. 2012). However, although Tbet is generally considered a transcriptional activator, Tbet has also been reported to target genes for repression, including *Socs1*, *Socs3*, and *Tcf7*, which all show increased expression in response to Tbet deficiency (Oestreich et al. 2011). Tbet overexpression alone did not inhibit these genes, indicating that Tbet does not function to directly repress these genes. Instead, Tbet interacts with Bcl6 (Oestreich et al. 2012) and recruits this repressor to target genes to decrease gene expression.

In addition to directly binding target genes to induce expression, Tbet also induces changes in chromatin structure to increase or decrease gene accessibility. Although expression of a dominant negative mutant of Tbet blocked IFNγ expression when expressed in cells during early stages of Th1 differentiation, expression of this mutant in Th1-committed cells failed to have an effect on IFNγ expression (Mullen et al. 2002). This was shown to correlate with the loss of DNase hypersensitivity site I in the early Th1 cells, whereas the Th1-committed cells maintained this site even in the presence of the mutant Tbet, indicating the importance of gene accessibility for maintenance of gene expression. Subsequent work has demonstrated multiple interactions between Tbet and various chromatin-remodeling enzymes. In naïve T cells, the histone deacetylase Sin3A was associated with the 5' CNS of *Ifng*, although as Th1 differentiation proceeded, Sin3A levels at the 5' CNS decreased and overexpression of Tbet correlated with removal of Sin3A from *Ifng* (Chang et al. 2008). Tbet has also been shown to interact with the demethylases Jmjd3 and UTX as well as the SWI/SNF remodeling complex (Miller et al. 2010). Tbet recruits the RbBp5 component of the H3K4 methyltransferase complex to induce permissive H3K4me2 marks at the promoters of *Cxcr3* and *Ifng*, as well as the Jmjd3 H3K27-demethyltransferase to remove the repressive H3K27me3 marks (Miller et al. 2008). Furthermore, loss of Tbet correlated with loss of permissive H3K4me1 marks at the control regions of *Ifng*, *Il12rb2,* and *Cxcr3* while the Th17 gene *Ccr6* had less repressive H3K27me3 marks (Zhu et al. 2012). By altering the chromatin landscape, Tbet increases accessibility of Th1 target genes to allow other factors to bind and strengthen gene expression while also decreasing accessibility of non-Th1 genes.

3.1.3 Inhibition of Other Subsets

Tbet also play an essential role in the inhibition of other subsets during Th1 differentiation. While Tbet downregulates IL-4 and Gata3 expression (Zhu et al. 2012) in Th1 cells, Gata3 is both expressed and bound to target genes in Th1 cells (Jenner et al. 2009; Kanhere et al. 2012; Wei et al. 2011). Genome-wide analyses indicate that a number of genes are bound by Gata3 in both Th1 and Th2 cells, and

include both Th1 and Th2-specific genes. In Th1 cells, many of these sites are also bound by Tbet, which acts to control gene expression as expression of co-occupied genes normally expressed in Th1 cells were increased in response to Tbet over-expression in *Ifng*$^{-/-}$*Tbet*$^{-/-}$ double deficient cells while genes that are normally expressed in Th2 cells showed decreased expression (Jenner et al. 2009). Although some targets are shared in both Th1 and Th2 cells, the overall pattern of Gata3 distribution in Th1 cells is different from Th2 cells, where Gata3 shows decreased binding to Th2-specific genes and increased binding to Th1-specific genes (Kanhere et al. 2012). However, many of the Th1-specific genes bound by Gata3 in Th1 cells do not have a Gata motif at the binding sites but contain binding motifs for either Tbet or Runx family proteins, indicating that Tbet and Runx3 contribute to the localization of Gata3 in Th1 cells. Indeed, Runx3 is able to interact with Gata3 (Yagi et al. 2010), as does phosphorylated Tbet, and this interaction inhibits Th2 differentiation by blocking the binding of Gata3 to target genes (Hwang et al. 2005b). Increased levels of phosphorylated Tbet correlated with increased IFNγ expression while inhibition of Tbet phosphorylation resulted in increased Th2 differentiation (Chen et al. 2011a). Tbet also binds directly to the *Gata3* gene and increases restrictive histone modifications to repress Gata3 expression (Zhu et al. 2012). Thus, inhibition of Th2 differentiation by Tbet involves both inhibition of Gata3 expression and sequestering Gata3 from many, but not all, Th2-specific genes. In addition, since Tbet also recruits Gata3 to many genes, including those expressed in Th1 cells, perhaps Tbet commandeers the activating functions of Gata3 to drive part of the Th1 gene program.

Tbet also interacts with many transcription factors to inhibit differentiation of the other CD4$^+$ subsets. Tbet deficient cells express elevated levels of IL-17, indicating a role for Tbet in the inhibition of Th17 differentiation (Mathur et al. 2006). This has been proposed to occur via the binding of Tbet to Runx1 and Runx3, which prevents the binding of these transcription factors to their targets, including the *Rorc* gene (encoding RORγt) (Lazarevic et al. 2011). Furthermore, Runx1 also forms a complex with RORγt, which is required for full activity of RORγt (Zhang et al. 2008). In this way, binding of Runx1 to Tbet blocks the formation of active RORγt complexes required for Th17 differentiation. Indeed, coexpression of Tbet with RORγt resulted in decreased RORγ activity (Villarino et al. 2010), likely by sequestering Runx1 away from an interaction with RORγt. Finally, as described above, Tbet also binds to Bcl6, which blocks Bcl6-mediated repression of genes required for Tfh differentiation (Oestreich et al. 2011). Taken together, Tbet blocks the differentiation of other subsets by binding to and inhibiting multiple transcription factors.

3.2 Gata3

Th2 differentiation requires the transcription factor Gata3, which, in addition to expression during T cell development, is also expressed at substantial higher levels in Th2 cells (Zhang et al. 1997; Zheng and Flavell 1997). Gata3 deficiency leads to

loss of Th2 cytokine expression (Pai et al. 2004; Zhu et al. 2004) and humans heterozygous for *GATA3* have decreased frequencies of Th2 cells (Skapenko et al. 2004). In contrast, Gata3 overexpression drives Th2 differentiation (Ouyang et al. 2000; Lee et al. 2000), characterized by increased Th2 cytokine expression and increased endogenous Gata3 expression (Lee et al. 2000). Furthermore, Gata3 overexpression also inhibits Th1 differentiation, as cells cultured under Th1 polarizing conditions continue to express Th2 cytokines (Zheng and Flavell 1997) while Gata3 transgenic mice show decreased responses to delayed type hypersensitivity, a Th1 immune response (Nawijn et al. 2001).

3.2.1 Mechanism of Action

Gata3 binds directly to the *Il4* enhancer to drive IL-4 production (Agarwal et al. 2000) and also to its own promoter to autoactivate expression (Ouyang et al. 2000). In this way, the positive feedback loop within Th2 differentiation is driven by both Gata3-induced expression of IL-4, which signals through Stat6 to activate Gata3 expression and by autoactivation of Gata3. Although Gata3 autoactivation plays a minimal role when a sufficient amount of IL-4 stimulation is present, as demonstrated by comparable expression levels of Gata3 in both wild type cells and cells expressing mutant Gata3 lacking the exon 4 encoded zinc fingers (Wei et al. 2011), autoactivation may be important for maintaining Gata3 expression when IL-4 signaling ceases.

Gata3 is required continuously for maintenance of Th2 differentiation as loss of Gata3 even after Th2 differentiation leads to decreased Th2 cytokine production and cell proliferation as well as increased IFNγ expression (Zhu et al. 2004). In addition to Th2 cytokines, Gata3 also binds to control regions of many transcription factors and many genes involved in T cell signaling to promote Th2 differentiation (Wei et al. 2011). Surprisingly, ChIP-Seq analysis identified Gata3 binding sites within multiple $CD4^+$ T cell subsets, indicating a role for Gata3 beyond Th2 differentiation. These Gata3 binding sites were shown to be in close proximity to a number of other transcription factor binding sites, including Runx, Ets and AP-1, indicating cooperation of Gata3 with other transcription factors. In Th2 cells, Gata3 binding colocalizes with the binding of Ets family member Fli1 to the DNA and the loss of Gata3 resulted in loss of Fli1 binding at 75 % of the shared binding sites. These results indicate that while Gata3 targets many genes in Th2 cells, it may also play a role in regulation of other subsets by acting within larger transcription factors complexes. Indeed, Tregs lacking Gata3 expression have reduced suppressive activity during inflammation (Wohlfert et al. 2011) and Gata3 has also been shown to be involved in CD8 T cell memory (Wang et al. 2013).

Similar to Tbet, Gata3 also exerts control over transcription programs by modifying chromatin structure. Indeed, overexpression of Gata3 correlated with increased DNase hypersensitivity sites at the *Il4* promoter (Ouyang et al. 2000; Lee et al. 2000), indicating increased accessibility for transcriptional activation.

Furthermore, deletion of Gata3 resulted in decreased histone acetylation at the *Il5* promoter, increased methylation of the *Il4* gene and increased histone acetylation at the *Ifng* promoter (Yamashita et al. 2004). Both Gata3 and Stat6 associate with the *Ifng* promoter in Th2 cells to alter chromatin structure as the loss of Stat6 correlated with the loss of repressive H2K27me2 histone modifications at *Ifng* while overexpression of Gata3 increased H3K27me2 marks and decreased permissive H3K9me marks (Chang and Aune 2007). The loss of Gata3 also resulted in decreased permissive H3K4me2 marks and increased restrictive H3K27me3 marks at Th2-specific genes and decreased repressive H3K27me3 marks at both *Tbet* and *Ifng* control regions under Th2 conditions (Wei et al. 2011). At the genome level, although only $\sim$10 % of Gata3-bound genes have altered expression upon Gata3 deletion, $\sim$50 % of all Gata3-bound genes have epigenetic changes around the Gata3 binding sites indicating a direct role of Gata3 in chromatin remodeling.

The control of the Th2 cytokine cluster requires a three-dimensional conformation of chromatin to bring the promoters for *Il-4*, *Il-5* and *Il-13* into close proximity with the locus control region (Spilianakis and Flavell 2004). Gata3 binds to a region within the LCR to induce long-range intrachromosomal interactions of the Th2 locus, as overexpression of Gata3 in nonlymphoid cells-induced permissive chromatin conformation at the Th2 locus, with the LCR in close proximity to the IL-4 cytokine cluster, resembling the structure found in Th2 cells (Spilianakis and Flavell 2004). Thus, by modulating repressive and permissive marks on histones and DNA as well as inducing permissive chromatin interactions, Gata3 controls transcriptional activation and repression of genes in Th2 cells.

3.2.2 Regulation of Other Subsets

Gata3 also plays a role in inhibiting Th1 differentiation, as Gata3 overexpressing cells show decreased Th1 polarization (Zheng and Flavell 1997). Gata3 acts to block Th1 differentiation by decreasing Stat4 expression, as Gata3 expression during Th2 differentiation correlated with decreased expression of Stat4 (Usui et al. 2003). In addition to Tbet and Stat4, Runx3 also plays a role in inducing IFNγ expression (Djuretic et al. 2007; Kohu et al. 2009; Yagi et al. 2010) and Gata3 binds directly to Runx3 (Yagi et al. 2010; Kohu et al. 2009) to inhibit Runx3-mediated IFNγ expression (Yagi et al. 2010). Furthermore, deletion of Gata3 resulted in increased expression of Th17-related genes (Wei et al. 2011). Both the downregulation of gene expression and the direct inhibition of other transcription factors are important functions of Gata3 in inhibiting the differentiation of other subsets while inducing the differentiation of Th2 cells.

3.2.3 Plasticity of Th2 Cells

Although Gata3, when expressed at higher levels, suppress Th1 and Th17-related transcription factors, it has been reported that Gata3 expressed at intermediated levels may tolerate the expression of Tbet. There is evidence that committed Th2 cells are capable of upregulating Tbet and producing IFNγ without losing their capacity to produce IL-4 (Hegazy et al. 2010). After 3 weeks of in vitro Th2 differentiation, LCMV-specific $CD4^+$ T cells were transferred into naïve C57BL/6, followed by LCMV infection 30 days post-transfer. Upon LCMV infection, these in vitro differentiated Th2 cells gained the capacity to produce IFNγ in addition to IL-4 to become IFNγ^+IL-4^+ double producing cells. These cells also upregulated Tbet expression to similar levels found in Th1 cells and Th2 cells differentiated from Tbet deficient mice failed to produce IFNγ in this model, indicating the importance of activating the Th1 transcriptional program. Indeed, although these Th2 cells initially produced high levels of Gata3, upon transfer and LCMV infection, Gata3 levels decreased while Tbet levels increased and this pattern was maintained up to 60 days post-infection.

A subset of cells has also been identified that produce both Th2 cytokines IL-4, IL-5, and IL-13 as well as IL-17 and express high levels of both Gata3 and RORγt (Wang et al. 2010). Th2 cells stimulated with IL-1β, IL-6, and IL-21 resulted in the induction of IL-17 expression. This plasticity between Th2 and Th17 plays a role in disease settings as patients with asthma had higher expression of IL-17 and IL-22 from sorted Th2 cells upon restimulation. In addition, intranasal exposure to allergens-induced IL-17 producing Th2 cells specifically in the lung and these cells persisted in the lung after exposure.

3.3 RORγt/RORα

Th17 differentiation is controlled by the orphan nuclear receptor RORγt. Mice deficient in RORγt have limited Th17 differentiation (Ivanov et al. 2006; Volpe et al. 2008) while overexpression of RORγt induced IL-17 expression in the absence of Th17 polarizing cytokines (Ivanov et al. 2006). As Th17 cells are the causative agents of EAE, the loss of RORγt and subsequent loss of Th17 cells was also protective against EAE induction. RORγt binds directly to the *Il17a/Il17f* gene to induce expression (Ichiyama et al. 2008). In addition to RORγt, another family member, RORα, is also expressed in Th17 cells and overexpression of RORα also increased IL-17 production (Yang et al. 2008). RORα binds to the CNS2 of *Il17* and overexpression of both RORα and RORγt acted synergistically to increase IL-17 expression, while deletion of both RORγt and RORα resulted in a complete block in Th17 differentiation. Although RORγt is clearly required for Th17 differentiation, it functions within a larger complex of transcription factors (Ciofani et al. 2012). Genome-wide ChIP analysis demonstrated that RORγt functions in cooperation with Batf, IRF4, and Stat3; RORγt appears to regulate

gene expression driven by these factors, both by reinforcing Th17-specific genes such as *Il17a* and *Il17f* and by attenuating expression of a number of genes induced by Batf, IRF4, and Stat3. However, genes attenuated by RORγt remain expressed in Th17 cells, indicating that RORγt plays a role in fine-tuning the expression of many target genes during Th17 differentiation. RORγt is also involved in the inhibition of both Th1 and Treg differentiation as overexpression of RORγt resulted in decreased IFNγ production (Ivanov et al. 2006) while the loss of RORγt resulted in increased FoxP3 expression (Burgler et al. 2010).

3.3.1 Plasticity of Th17 Cells

Although Th17 cells are considered a unique $CD4^+$ T cell subset, Th17 cells have been shown to produce IFNγ (Mathur et al. 2006; Shi et al. 2008; Lee et al. 2009; Bending et al. 2009). After in vitro differentiation, both Th1 and Th17 cells were transferred into NOD/Scid mice and diabetes development was monitored (Bending et al. 2009). Although Th17 cell transfer showed a slight lag in the induction of diabetes, Th17 transfer resulted in the development of diabetes and analysis of cells isolated from peripheral lymph nodes showed increased IFNγ production and loss of IL-17 production compared to original transplanted Th17 cells. Although these transplanted cells were cultured under Th17 inducing conditions, the possibility remained that a small population of undifferentiated cells could be responsible for diabetes induction. To demonstrate that Th17 cells can convert to IFNγ producers, a subsequent in vitro study used an IL-17f reporter mouse to sort IL-17 expressing Th17 cells and demonstrated that treatment of these cells with IL-12 increased IFNγ production (Lee et al. 2009). These cells also lost RORγt expression while gaining Tbet expression and Th17 cells deficient in either Stat4 or Tbet failed to upregulate IFNγ in response to Th1 polarizing conditions, indicating a requirement for the activation of the Th1 transcriptional program (Lee et al. 2009).

The switch from IL-17 to IFNγ production appears to be important during EAE as suppression of Tbet expression by siRNA after EAE induction resulted in decreased disease severity (Gocke et al. 2007). The use of the IL-17 fate reporter mouse has shown that the majority of the CNS infiltrating IFNγ-producing cells in MOG-induced EAE arises from IL-17 producing cells, and cells that have produced IL-17 produce the highest levels of cytokines upon restimulation (Hirota et al. 2011). Th17 cells have also been shown to become $IFN\gamma^+IL17^+$ double producers (Ivanov et al. 2006; Lee et al. 2009; Lexberg et al. 2010; Villarino et al. 2010), which express both Tbet and RORγt (Lexberg et al. 2010). Interestingly, cells isolated from spinal cords of EAE mice show a larger population of $IFN\gamma^+IL\text{-}17^+$ compared to IL-17 single positive. Potentially, this double positive population is composed of those Th17 cells in the process of converting to Th1 cells. This switch of Th17 cells to Th1 cells in the CNS appears to drive the pathogenesis of EAE and demonstrates the importance of $CD4^+$ T cell subset balance in the maintenance of a healthy individual.

3.4 *FoxP3*

Mutations in the *FOXP3/Foxp3* gene were identified in X-linked neonatal diabetes mellitus, entropathy and endocrinopathy syndrome (IPEX) patients (Wildin et al. 2001) and in the scurfy mouse, respectively (Brunkow et al. 2001), representing a human condition and a mouse model characterized by severe autoimmunity. FoxP3 is expressed in $CD4^+CD25^+$ T cells and cells overexpressing FoxP3 show poor proliferation and very little cytokine production in response to T cell receptor stimulation (Hori et al. 2003). In addition, FoxP3 overexpressing cells are capable of suppressing the activation of $CD4^+CD25^-$ T cells and preventing the development of inflammatory bowel disease (IBD) induced by transferring naive $CD4^+CD25^-$ T cells into lymphopenic hosts (Hori et al. 2003; Fontenot et al. 2003). In contrast, $CD4^+CD25^+$ cells deficient in FoxP3 expression failed to suppress $CD4^+CD25^-$ T cells (Fontenot et al. 2003; Gavin et al. 2007; Williams and Rudensky 2007) and a mixed bone marrow chimera experiment demonstrated that development of suppressive T regulatory cells required FoxP3 expression (Fontenot et al. 2003).

3.4.1 Mechanism of Action

Genome-wide analysis has identified a large number of FoxP3 target genes, including genes involved in regulating transcription as well as genes involved in epigenetic modification (Zheng et al. 2007). Furthermore, analysis of FoxP3 binding partners identified interactions with a large number of transcription factors, as well as candidates involved in chromatin organization and modification (Rudra et al. 2012). Not only does FoxP3 bind to these factors at the protein level, but ChIP-Seq analysis of FoxP3 binding sites showed that FoxP3 also binds to the regulatory regions of these factors, thus controlling the expression of many of its binding partners. In agreement with these genome-wide studies showing interactions of FoxP3 with chromatin modifying enzymes, many FoxP3 target genes were found to have permissive histone modifications. Furthermore, FoxP3 interacts with the histone acetyltransferase TIP60 as well as histone deacetylase 7 (Li et al. 2007a) and disruption of this interaction leads to increased acetylation at the *Il2* promoter, which is normally repressed in T regulatory cells (Bettini et al. 2012). However, a study comparing chromatin modifications in naïve T cells with those in FoxP3 expressing cells found no major changes in chromatin modifications before and after FoxP3 expression, indicating that other factors, such as FoxO proteins, are likely required to modify the chromatin landscape in T regulatory cells prior to FoxP3 binding (Samstein et al. 2012).

FoxP3 also exploits direct interaction with NFAT to target specific genes during Treg differentiation (Wu et al. 2006). Expression from an IL-2 reporter construct containing NFAT:AP-1 binding sites was inhibited in the presence of FoxP3. This inhibition required NFAT, as FoxP3 bound to the *Il2* promoter sequence only in

the presence of the NFAT DNA binding domain. Furthermore, mutation of FoxP3 residues to disrupt NFAT binding resulted in increased IL-2 expression in response to T cell stimulation. The importance of this interaction between NFAT and FoxP3 was demonstrated by a failure of FoxP3 mutant cells to protect against diabetes when transferred into NOD mice.

3.4.2 Regulation of Other Subsets

FoxP3 also plays an active role in inhibiting both Th2 and Th17 differentiation by inhibiting Gata3 and RORγt to block IL-4 and IL-17 expression (Zeng et al. 2009). FoxP3 also binds to phosphorylated Stat3, resulting in the recruitment of FoxP3 to *Il6* regulatory regions (Chaudhry et al. 2009). This results in the repression of IL-6 expression, which further blocks Th17 differentiation. In addition, FoxP3 binds to RORγt (Zhou et al. 2008; Lochner et al. 2008; Ichiyama et al. 2008) to inhibit IL-17 expression (Ichiyama et al. 2008; Zhou et al. 2008) by decreasing RORγt binding to the *Il17* promoter, which correlates with decreased permissive histone modifications (Ichiyama et al. 2008). FoxP3 also binds to the RORγt: Runx1 complex to inhibit IL-17 expression, as mutation of the RORγt or Runx1 binding site within FoxP3 blocks IL-17 inhibition (Zhang et al. 2008). RORγt in turn has been shown to inhibit Treg differentiation by binding directly to the *Foxp3* promoter to inhibit FoxP3 expression (Burgler et al. 2010).

3.4.3 Plasticity of Treg Cells

Stimulation of Treg cells with IL-6 resulted in increased IL-17 expression and the development of both IL-17$^+$FoxP3$^+$ double positive cells and IL-17 single positive cells, indicating that the conversion of Treg cells to Th17 cells includes a double positive population (Xu et al. 2007). Furthermore, two populations of FoxP3 expressing cells have been reported based on the expression level of FoxP3 (Tartar et al. 2010). Those cells expressing high levels of FoxP3 had no detectable RORγt while cells expressing intermediate levels of FoxP3 also expressed high levels of RORγt. These FoxP3 intermediate cells upreguated IL-17 production in response to Th17 polarizing conditions while FoxP3 high expressing cells did not. Furthermore, polarization of the FoxP3 intermediate population under Th17 conditions led to increased RORγt expression while Treg polarization led to increased FoxP3 expression, indicating that FoxP3$^+$RORγt$^+$ have the capacity to become either Th17 or Treg cells, depending on the cytokine environment.

An analysis of proteins interacting with FoxP3 identified Gata3, which both interacts with and is upreguated by FoxP3 (Rudra et al. 2012). Gata3 and FoxP3 are coexpressed in Treg cells in the lamina propria and T cell receptor stimulation of Treg cells resulted in upregulation of Gata3 expression (Wohlfert et al. 2011). Gata3 promotes Treg differentiation by binding directly to the *Foxp3* enhancer to increase FoxP3 expression (Wohlfert et al. 2011; Wang et al. 2011). Competitive

transfer experiments of wild type and Gata3-deficient bone marrow cells in *Rag1*$^{-/-}$ mice resulted in much fewer FoxP3 expressing cells arising from Gata3 deficient cells, indicating the importance of Gata3 in Treg differentiation. These Gata3 deficient Treg cells showed decreased suppressive activity and failed to protect against a transfer model of inflammatory bowel disease (Wohlfert et al. 2011; Wang et al. 2011). Gata3 deficient cells also expressed lower levels of both FoxP3 and FoxP3 target genes, including *Cd25*, *Ctla4*, and *Gitr* (Wang et al. 2011) and had decreased FoxP3^{+}CD25^{+} populations under conditions of inflammation (Wohlfert et al. 2011). Gata3 also binds to the Tbet and *Rorc* control regions in Treg cells to repress these genes, inhibiting Th1 and Th17 differentiation (Wohlfert et al. 2011). Finally, FoxP3 and Gata3 cooperate to control gene expression as both factors target a number of shared genes in Treg cells and the specific deletion of either factor resulted in altered expression of the shared target genes (Rudra et al. 2012). Thus, Gata3 controls expression of transcription factors within Treg cells to maintain Treg phenotype while also preventing Th1 and Th17 differentiation from Tregs.

3.5 Bcl6

The debate concerning the validity of characterizing follicular T cells as a unique subset was strengthened by the identification of the master regulator Bcl6 (Chtanova et al. 2004). A microarray analysis of Tfh cells, defined as CD57^{+}CXCR5^{+}CD4^{+} T cells, identified Bcl6 as being preferentially expressed in Tfh cells compared to Th1 and Th2 cells (Chtanova et al. 2004). Subsequent work demonstrated that Bcl6 deficient T cells fail to differentiate into CXC5^{+}PD-1^{+} Tfh cells while overexpression of Bcl6 increased Tfh differentiation (Yu et al. 2009a; Nurieva et al. 2009; Johnston et al. 2009). Since Bcl6 is a transcriptional repressor, the mechanism of action to induce Tfh differentiation is not likely to rely on activation of transcription. Instead, Bcl6 has been shown to inhibit expression of various microRNAs, which are likely responsible for suppressing Tfh differentiation (Yu et al. 2009a). Indeed, predicted binding sites for microRNAs downregulated by Bcl6 were identified in the 3' UTR of *Cxcr5*, *Cxcr4*, and *Pd1*, all genes expressed in Tfh cells.

In addition to promoting Tfh differentiation, Bcl6 overexpression also blocks Th1, Th2, and Th17 differentiation by decreasing expression of *Tbet*, Gata3, and RORγt (Yu et al. 2009a; Nurieva et al. 2009). Bcl6 binds directly to *Tbet* and *Rorc* promoters to inhibit expression of these genes. However, Bcl6 is negatively regulated by Blimp1, as Blimp1 overexpression leads to decreased Bcl6 expression and CXCR5^{+} Tfh differentiation (Johnston et al. 2009). Blimp1 is expressed in non-Tfh subsets (Crotty et al. 2010), where expression is upreguated in response to T cell stimulation, indicating a mechanism by which other subsets inhibit Tfh differentiation.

3.5.1 Plasticity of Tfh Cells

Although Tfh genes are expressed during early Th1 differentiation, IL-2 production by Th1 cells inhibits expression of Tfh genes at later time points (Oestreich et al. 2012). While Bcl6 levels are decreased after Th1 polarization, removal of IL-2 resulted in elevated expression of both Bcl6 and CXCR5 and decreased Blimp1 expression. Furthermore, after culture of Th1 cells under low IL-2 levels to induce Tfh gene expression, the addition of higher IL-2 levels resulted in decreased Bcl6 and CXCR5 expression with increased Blimp1 expression, indicating plasticity in the expression of the Tfh genes. This change in Blimp1 expression in response to IL-2 correlated with increased binding of Blimp1 to *Cxcr5* in high IL-2 conditions. In this way, there is a balance between Th1 and Tfh cells, where they appear capable of converting in response to changes in the cytokine environment.

4 Additional Factors Involved in $CD4^+$ T Cell Differentiation

As discussed above, much of the work on the transcriptional regulation of $CD4^+$ T cell differentiation has centered on the idea of a two-step process with the initiation of differentiation induced by Stat activation in response to cytokine stimulation followed by the activation of the master regulator for each subset. However, many studies have identified roles for additional transcription factors within $CD4^+$ T cell differentiation programs and suggest that rather than two factors controlling differentiation, a network of factors are likely involved in fine-tuning the expression and repression of various genes to determine subset gene control.

4.1 Factors Induced by T Cell Stimulation

Although cytokine-induced Stat activation plays a critical role in the induction of master regulators, signaling through T cell receptor is also essential for T cell differentiation. Stimulation through the T cell receptor results in the activation of the NFAT, NFκB, and AP-1 families of transcription factors. These factors cooperate with Stat-induced expression of subset-specific cytokines and master regulators. A summary of the interaction of transcription factor activation by T cell signaling with cytokine-mediated Stat activation and the resulting differentiation program is shown in Fig. 2.

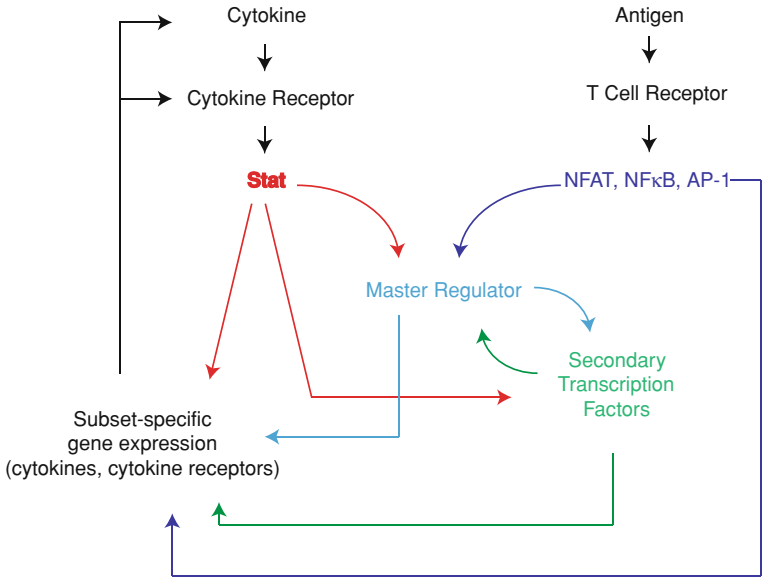

Fig. 2 Cooperation between cytokine signaling and T cell receptor signaling during $CD4^+$ T cell differentiation. NFAT, NFκB, and AP-1 activation in response to T cell stimulation activates expression of master regulators and cytokines to drive $CD4^+$ T cell differentiation. The strength of antigenic stimulation results in altered patterns of transcription factor activation, which cooperates with cytokine-induced signaling to drive subset-specific differentiation

4.1.1 NFAT Proteins

There are five members of the NFAT family of transcription factors, NFAT1 (NFATp or NFATc2), NFAT2 (NFATc or NFATc1), NFAT3 (NFATc4), NFAT4 (NFATc3), and NFAT5 (Macian 2005). In response to stimulation of the T cell receptor, phospholipase C is activated and cleaves phosphatidylinositol 4, 5 bisphosphate into inositol 1, 4, 5 triphosphate (InsP3) and diacylglycerol. InsP3 in turn triggers the release of intracellular calcium stores, which activates the calcium release activated calcium channels at the cell surface, resulting in elevated cytoplasmic calcium levels. Intracellular calcium binds to calmodulin, resulting in the activation of calcineurin, which dephosphorylates NFATs, allowing nuclear translocation of these transcription factors. Among the targets of NFAT are the cytokines involved in driving $CD4^+$ T cell differentiation, as the deletion of NFATc1 and NATc2 resulted in decreased production of IL-2, IFNγ, IL-4, and IL-5 in response to T cell stimulation (Peng et al. 2001).

NAFT binding sites have been identified within the *Ifng* promoter and supershift analysis demonstrates NFAT binding at these sites (Sica et al. 1997). Furthermore, overexpression of a constitutively active form of NFATc1 resulted in increased IFNγ expression and decreased IL-4 expression, thus polarizing cells to become Th1 (Porter and Clipstone 2002). Similarly, deletion of NFAT1 on an

IL-4-deficient background also resulted in decreased IFNγ production accompanied by increased susceptibility to *L. major* infection resulting in increased lesion size and parasite count (Kiani et al. 2001). NFAT also appears to negatively regulate Th2 responses as mice deficient in NFAT1 (Kiani et al. 1997) or both NFATc2 and NFATc3 (Rengarajan et al. 2002b) produce higher levels of IL-4 and decreased levels of IFNγ in response to nonpolarizing T cell stimulation, and IL-4 expression is maintained for prolonged periods of time after stimulation. *Nfat c2*$^{-/-}$*Nfat c3*$^{-/-}$ double deficient mice also produce higher levels of the Th2 cytokine IL-5 even on an IL-4 deficient background, indicating that NFAT regulates Th2 differentiation independently of IL-4 expression. Similarly, deletion of NFATp and NFAT4 resulted in increased IL-4, IL-5, IL-6, and IL-10 production, while IFNγ and IL-2 production was reduced (Ranger et al. 1998b). These results indicate a role for NFAT family members in the induction of IFNγ production and suppression of Th2 cytokine expression during Th1 differentiation.

In addition to cytokine environment, the strength of T cell stimulation is thought to contribute to Th1/Th2 differentiation pathways, where higher signal strength induces Th1 differentiation while weaker signaling induces Th2 differentiation (Constant and Bottomly 1997). The level of T cell stimulation is translated through intracellular signaling cascades into unique patterns of transcription factor activation. Stimulation with a low affinity peptide was shown to increase the ratio of NFATc to NFATp in differentiating T cells, which corresponded with increased IL-4 production (Brogdon et al. 2002). In agreement with these results, deletion of NFATc resulted in decreased IL-4 production, likely due to the loss of NFAT binding to the *Il4* promoter (Yoshida et al. 1998; Ranger et al. 1998a).

NFAT family members have also been shown to bind to the *Il17* promoter (Liu et al. 2004; Gomez-Rodriguez et al. 2009; Hermann-Kleiter et al. 2012) and expression of a hyper-activatable NFAT1 resulted in increased IL-17 production in response to Th17 polarizing conditions. Although these mice produced increased levels of IL-17 in vitro, they were less susceptible to EAE induction, which may be due to an increased Treg population, indicating a role for NFAT in Treg differentiation. Indeed, NFAT has been shown to drive FoxP3 expression and Treg differentiation by binding directly to the *Foxp3* promoter and enhancer regions (Mantel et al. 2006; Tone et al. 2008). Deletion of NFAT2, NFATc2, and NFAT1 or NFAT1 and NFAT4 resulted in decreased Treg populations, although the Treg cells that were produced remained functional (Vaeth et al. 2012).

Together, these results demonstrate roles for the NFAT family members in driving the expression of multiple subset-specific cytokines as well as FoxP3. Whether NFAT proteins play an important role in regulating the expression of other master transcription factors is understudied. In addition to signal strength inducing specific NFAT family members, subset-specific functions of NFAT family members may arise from cooperation with other factors expressed during differentiation. NFAT1 has been shown to have CD4^{+} subset-specific target binding, binding to either the *Il4* or *Ifng* promoter in Th2 or Th1 cells, respectively (Agarwal et al. 2000). This specificity in binding is likely dependent on the

chromatin structure at these genes and the relative accessibility of binding sites, thus relying on other factors for subset-specific transcriptional activity.

4.1.2 NFκB

The NFκB family of transcription factors is composed of 5 family members, RelA (p65), NFκB1 (p50/p105), NFκB2 (p52/p100), c-Rel, and RelB (Hayden and Ghosh 2012). Stimulation of the T cell receptor leads to the activation of PKCθ, which activates a signaling cascade resulting in NFκB activation [reviewed in (Hayden and Ghosh 2012)], which in turn regulates expression of subset-specific cytokines, cytokine receptors, and transcription factors. To test the role of NFκB during T cell differentiation, transgenic mice were generated to express a mutant form of IκBα, which is resistant to degradation and acts as a dominant inhibitor of NFκB activity (Aronica et al. 1999). These mice showed decreased delayed type hypersensitivity responses, decreased IFNγ production, and increased IL-4 production. These mice also showed decreased levels of phosphorylated Stat4 and decreased Tbet expression (Corn et al. 2003). Similarly, deficiency in RelB also resulted in decreased IFNγ production along with loss of Tbet expression and decreased levels of phosphorylated Stat4 (Corn et al. 2005), indicating the importance of NFκB activation during Th1 differentiation. Indeed, p50 has been shown to bind directly to the *Ifng* promoter to drive cytokine expression (Lai et al. 2011). However, in contrast to this cooperation between cytokine-induced transcription factors and T cell receptor-induced factors, Tbet has been shown to inhibit members of the NFκB family (Hwang et al. 2005a). Tbet binds to c-Rel to block binding of c-Rel to target genes, resulting in altered gene expression patterns. One target of c-Rel is *Il2*, which is downregulated in Th1 cells, allowing a switch to IFNγ production. In this way, Tbet acts to block expression of non-Th1 genes by activated NFκB.

Mice deficient in the p50 subunit of NFκB show decreased Th2 differentiation, with decreased IL-4, IL-5 and IL-13 production as well as decreased Gata3 expression with no change in Tbet expression (Das et al. 2001). Similarly, deficiency in the IκB family member Bcl3 also resulted in decreased IL-4 and IL-5 production along with decreased Gata3 expression. Analysis of the *Gata3* promoter demonstrated that activated T cell extracts were capable of binding to a putative NFκB binding site and this was blocked by addition of unlabeled NFκB probe and was shifted by an antibody against p50, thus verifying the ability of p50 to bind to *Gata3* regulatory regions. However, NFκB appears to be required only for early induction of Gata3 expression, as inhibition of nuclear translocation of NFκB after 4 days of Th2 polarization showed no effect on Gata3 expression (Das et al. 2001).

NFκB family members also play a role in Th17 differentiation as deletion of c-Rel resulted in decreased expression of IL-17 along with decreased susceptibility to EAE induction (Chen et al. 2011b). These mice also had decreased RORγt expression. Two NFκB binding sites were identified upstream of the *Rorc* transcription start site and a reporter assay using the *Rorc* promoter demonstrated

cooperation between c-Rel and RoRγt in driving expression from the *Rorc* promoter. Similarly, deletion of the NFκB adapter Carma1 resulted in decreased expression of IL-17a, IL-17f, IL-21, IL-22, IL-23R accompanied by decreased susceptibility to EAE induction (Molinero et al. 2012). The IκB family member IκBζ was shown to be expressed at the highest levels in Th17 cells and deletion of this gene resulted in decreased expression of IL-17f, IL-21, IL-22, and IL-23R while overexpression resulted in increased IL-17f, IL-21, and IL-23R expression (Okamoto et al. 2010). While there was no change in the expression of RORγt or RORα in these mice, RORγt cooperates with IκBζ in driving cytokine expression as there was decreased binding of IκBζ to CNS2 of the *Il17* promoter in the absence of RORγt. Together, these results demonstrate a requirement for NFκB activation to drive optimal expression of Th17 cytokines, cytokine receptors, and transcription factors.

In addition to decreased Th17 differentiation in Carma1 deficient mice, there was also an increase in Treg populations, indicating a role for NFκB in Treg differentiation. Indeed, c-Rel deficient mice show decreased Treg populations in the thymus, spleen, and lymph nodes (Isomura et al. 2009; Ruan et al. 2009). NFκB drives FoxP3 expression as both c-Rel and p65 increased expression of a reporter construct containing the *Foxp3* promoter, although neither p50 nor RelB increased expression of the reporter (Ruan et al. 2009). Deletion of the putative Rel-NFAT binding sites in the FoxP3 reporter construct inhibited the c-Rel driven expression. Thus, NFκB induces FoxP3 expression to promote Treg differentiation.

Taken together, these results demonstrate roles for NFκB family members in driving expression of subset-specific transcription factors, cytokines, and cytokine receptors. While some genes are controlled by specific family members, such as p65 and c-Rel driving FoxP3 expression while neither p50 nor RelB can, NFκB family members do not necessarily show subset specificity as p50 has been shown to play a role in both Th1 and Th2 differentiation. In this case, it is likely that the NFκB family members activated by the T cell receptor cooperate with those factors induced by the cytokine environment, either through gene accessibility induced by Stats and master regulators or by regulating expression of genes also targeted by Stats and master regulators.

4.1.3 AP-1

The AP-1 transcription factor is a heterodimeric protein composed of members of the Jun (cJun, JunB, JunD), Fos, Maf, and ATF (including Batf) families (Shaulian and Karin 2002). These factors are activated in response to the induction of the MAP kinase cascade upon T cell stimulation. The pattern of AP-1 formation appears to play a role in $CD4^+$ T cell differentiation as strong T cell stimulation resulted in the formation of Fos-Jun dimers while partial ERK inhibition resulted in formation of Jun-Jun dimers and increased IL-4 production (Jorritsma et al. 2003). JunB in particular has been shown to drive IL-4 expression in cooperation with c-Maf (Li et al. 1999).

Batf is a member of the AP-1 family of transcription factors and is expressed in Th1, Th2, and Th17 cells (Schraml et al. 2009). However, generation of Batf deficient mice resulted in a specific defect in Th17 differentiation, while Th1 and Th2 differentiation proceeded normally. These mice showed increased resistance to EAE and microarray analysis demonstrated decreased expression of multiple Th17 associated genes, including *Rorc*, *Rora*, *Ahr*, *Il21*, and *Il17*. Surprisingly, overexpression of RORγt in Batf deficient cells failed to rescue IL-17 expression under Th17 polarizing conditions, indicating a specific requirement for Batf during Th17 differentiation, independent of RORγt expression.

Batf has been shown to activate transcription as part of a larger complex, including interferon regulatory factor 4 (IRF4) and JunB (Li et al. 2012; Glasmacher et al. 2012). As IRF4 binds weakly to DNA on its own, IRF4 ChIP-Seq analysis was performed in T cells to identify transcription factor binding sites associated with IRF4 binding. This analysis identified AP-1 binding sites as the motif most highly associated with IRF4 binding. EMSA analysis of DNA sequences containing tandem IRF4 and AP-1 binding sites demonstrated binding by Th17 extracts while supershift analysis identified binding of IRF4, JunB, and Batf. These three factors bind in a cooperative manner to target many genes during Th17 differentiation, including *Il17a*, *Il21*, *IL23r*, and *IL12rb1* (Li et al. 2012; Glasmacher et al. 2012) and the loss of any one of these factors inhibits Th17 differentiation. Similar to Batf deficient mice, IRF4 deficient mice also show a block in Th17 differentiation, with decreased IL-17 production and RORγt expression (Brustle et al. 2007). The loss of either Batf or IRF4 resulted in decreased DNA binding of the partner factor, indicating the requirement for both factors for efficient DNA binding and target gene expression (Li et al. 2012; Glasmacher et al. 2012). Furthermore, overexpression of IRF4, Batf, or JunD alone showed limited binding to target DNA while coexpression of all three showed strong binding (Li et al. 2012). Thus, Th17 differentiation requires cooperation among these factors, as the loss of any one prevents the binding of the others, resulting in decreased gene expression.

Batf is negatively regulated by early growth response factor-2 (Egr-2), a member of the Egr zinc finger transcription factor family (Miao et al. 2013). Cells deficient in Egr-2 showed a specific increase in IL-17 production, while IFNγ and IL-2 production remained unchanged in response to nonpolarizing T cell stimulation. This function of Egr-2 was specific to Th17 differentiation, as loss of Egr-2 did not increase IL-17 production under either Th1 or Th2 polarizing conditions. Although nuclear extracts from Egr-2 deficient cells had an elevated level of Batf capable of binding to the *Il17* promoter, there was no significant increase in total Batf expression in these cells. Instead, Egr-2 was shown to physically interact with Batf and this interaction is proposed to block Batf binding to the *Il17* promoter, thus decreasing IL-17 production. Egr-2 expression is upreguated in response to IL-6 and TGFβ-induced Th17 differentiation and acts to negatively regulate the Th17 differentiation. This plays an important role in vivo, as Egr-2 deficient mice had an increased susceptibility to EAE induction and MS patient $CD4^+$ T cells

showed both decreased Egr-2 expression and increased IL-17 and Batf expression, indicating the importance of Egr-2 expression for regulating proper Th17 responses.

4.2 Interferon Regulatory Factor 4

IRF4 is expressed in both B cells and T cells and expression of IRF4 is upreguated in response to T cell stimulation (Biswas et al. 2010). Cells deficient in IRF4 produce lower levels of IL-4 under conditions of nonpolarizing T cell stimulation and when polarized toward Th2 (Rengarajan et al. 2002a; Lohoff et al. 2002). These cells also show limited Gata3 upregulation in response to IL-4 stimulation, although Stat6 was activated, indicating normal IL-4 signaling. However, overexpression of Gata3 rescued IL-4 production in IRF4 deficient cells. In a study of the ability of IRF4 to drive expression from the *Il4* promoter, it was shown that overexpression of IRF4 only induced expression of a reporter when coexpressed with NFATc2, while coexpression of IRF4, NFATc2, and c-Maf drove the greatest activation of reporter expression. As such, IRF4 acts as a component of a larger transcription factor complex to support IL-4 production and the importance of IRF4 in this complex demonstrated by the greatly diminished IL-4 production in IRF4-deficient cells (Rengarajan et al. 2002a; Lohoff et al. 2002). IRF4 also interacts with PU.1 and this interaction acts to block binding of IRF4 to target genes, including *Il4* and *Il10*, to decrease IRF4-driven expression of these genes (Ahyi et al. 2009). While PU.1 is expressed in Th2 cells, there is heterogeneity in the expression of PU.1 across the Th2 population, allowing for differential regulation of IL-4 and IL-10 within the subset (Chang et al. 2005).

An additional $CD4^+$ T cell subset has recently been defined as Th9, characterized by the production of IL-9, induced by stimulation with IL-4 and TGFβ (Chen et al. 2011b). Both IRF4 and PU.1 have been shown to play a role in Th9 differentiation. Stimulation under Th9 promoting conditions leads to the upregulation of IRF4 expression, which correlates with increased IL-9 production, likely facilitated by the direct binding of IRF4 to the *Il9* promoter (Staudt et al. 2010). IRF4 deficient cells fail to produce IL-9 but produce elevated IFNγ after treatment with IL-4 and TGFβ. Similarly, PU.1 also binds directly to the *Il9* promoter and PU.1 deficient cells show greatly decreased IL-9 production in response to IL-4 and TGFβ treatment, with no effect on Th17 or iTreg induction (Chang et al. 2010). Furthermore, overexpression of PU.1 resulted in increased IL-9 production in the absence of polarizing cytokines. Although both PU.1 and IRF4 are involved in Th9 differentiation, these two factors are activated independently, as IRF4 expression requires functional Stat6, indicating a reliance on IL-4 signaling for expression, while PU.1 is expressed independent of IL-4 signaling (Goswami et al. 2012). In the absence of functional Stat6, PU.1 bound the *Il9* promoter normally, indicating no requirement for IRF4 in PU.1 binding, however, the decreased IL-9

expression in the absence of IRF4 demonstrates cooperation between these two factors in IL-9 expression and Th9 differentiation.

IRF4 is also involved in both Th17, as discussed above, and Treg differentiation. IRF4 deficient cells show limited differentiation into IL-17 producing cells under Th17 polarizing conditions with decreased RORγt expression. IRF4 deficient mice are resistant to EAE induction (Brustle et al. 2007). Furthermore, IRF4 deficient cells fail to suppress FoxP3 expression under Th17 polarizing conditions, indicating a role for IRF4 in the inhibition of Treg differentiation. However, generation of a Treg-specific deletion of IRF4 resulted in lymphoproliferative disease, with increased $CD4^+$ T cell populations in lymph nodes with lymphocyte infiltration in pancreas, stomach, and lung (Zheng et al. 2009). Although these results point to a loss of Treg function, IRF4 deficient mice did not fail to generate FoxP3 expressing cells and actually had elevated Treg populations in lymph nodes. However, the $CD4^+$ T cells in the lymph nodes were more activated and showed a specific increase in IL-4 production, indicating a loss of Th2 regulation with the loss of IRF4 in Treg cells. FoxP3 and IRF4 interact with each other and may bind together to regulate target genes. In the absence of IRF4, expression of 20 % of Treg-specific genes was decreased while 7 % was increased. In particular, *Icos*, *Maf*, *Ccr8*, and *Il1rl1*, all involved in Th2 differentiation, were decreased in IRF4 deficient Treg cells. However, it is unclear how the interaction between IRF4 and FoxP3 and the induction of Th2-specific genes in Treg cells acts to suppress Th2 responses.

IRF4 also plays a role in Tfh differentiation, as IRF4 deficient mice fail to form germinal centers and have significantly decreased GC B cell and Tfh cell populations [202]. The transfer of wild type $CD4^+$ T cells into IRF4 deficient mice rescued germinal center formation, indicating the requirement of IRF4 in T cells for proper Tfh differentiation and germinal center formation. During Tfh differentiation, IRF4 cooperates with Stat3 to regulate gene expression (Kwon et al. 2009). Examination of the binding sites of IRF4 and Stat3 showed a high degree of overlap between these two factors, where 76 % of Stat3 binding sites in Tfh cells were also bound by IRF4 and in the absence of IRF4, there was decreased or complete loss of Stat3 binding to these sites. Thus, although Stat3 plays an important role in Tfh differentiation, there remains a requirement for IRF4 for proper target binding.

4.3 Runx

The Runx family of transcription factors has also been shown to play a role in $CD4^+$ T cell differentiation. Runx3 binds to the *Ifng* promoter to induce expression (Djuretic et al. 2007; Yagi et al. 2010) and overexpression of Runx3 increased IFNγ production (Djuretic et al. 2007; Kohu et al. 2009; Yagi et al. 2010) while Runx3 deficiency resulted in decreased IFNγ production (Djuretic et al. 2007). In addition,

both Runx1 (Naoe et al. 2007; Kitoh et al. 2009) and Runx3 (Djuretic et al. 2007) bind to the *Il4* silencer to inhibit IL-4 expression, as demonstrated by decreased IL-4 expression in response to Runx3 overexpression (Yagi et al. 2010). Runx1 is expressed predominately in naïve T cells while Runx3 is expressed in Th1 cells (Naoe et al. 2007) and these factors have been proposed to play a role in limiting IL-4 production in these subsets. In addition to the interaction with the *Il4* silencer, Runx3 also binds to Gata3 (Yagi et al. 2010; Kohu et al. 2009) to inhibit Gata3-mediated gene expression (Kohu et al. 2009). While expression of excess Gata3 can prevent the Runx3-mediated induction of IFNγ expression, it does not prevent IL-4 silencing, indicating the importance of Runx3 in the inhibition of Th2 differentiation (Yagi et al. 2010).

The *Il17* promoter contains two binding sites for Runx1 upstream of a RORγt binding site and mutation of either site decreased expression driven by the *Il17* promoter (Zhang et al. 2008). Indeed, both RORγt and Runx1 bind directly to the *Il17* promoter and Runx1 and RORγt also coprecipitate with each other, indicating a physical interaction in addition to closely spaced binding sites. While overexpression of Runx1 increased the activity of the *Il17* promoter, this was inhibited by mutation of the RORγt site, indicating that RORγt is required for full Runx1 activity. Similarly, while overexpression of both Runx1 and RORγt induced very strong Th17 differentiation under both neutral and Th17 polarizing conditions, overexpression of a dominant negative mutant of Runx1 with RORγt decreased IL-17 differentiation below that induced by RORγt overexpression alone, indicating a requirement for cooperation between these factors during Th17 differentiation. Furthermore, Runx1 expression levels correlate with RORγt expression levels, as overexpression of Runx1 resulted in increased RORγt expression while siRNA knockdown of Runx1 resulted in decreased RORγt. This indicates a possible role for Runx in the regulation of RORγt expression.

The Runx family also plays a role in FoxP3 expression as Runx1, Runx3, and the co-factor Cbfβ bind to the *Foxp3* promoter. Deletion of either Runx1 or Cbfβ resulted in decreased FoxP3 expression (Kitoh et al. 2009; Bruno et al. 2009; Rudra et al. 2009; Klunker et al. 2009) while Runx3 overexpression increased FoxP3 expression (Bruno et al. 2009). Furthermore, Runx deficient mice showed signs of lymphoproliferation (Rudra et al. 2009) and these cells failed to prevent colitis when transferred into Scid mice (Kitoh et al. 2009), indicative of a loss of functional Treg cells. The effect of Runx deficiency on Treg populations appears to primarily depend on the loss of FoxP3 expression in the absence of Runx, as cotransfer of FoxP3-expressing cells from Cbfβ-deficient mice or control mice resulted in accelerated loss of FoxP3 expressing cells from Cbfβ-deficient cells compared to control cells, while the remaining *Cbf*$\beta^{-/-}$ FoxP3 cells retained suppressive function (Rudra et al. 2009).

Runx1 also associates with the nuclear orphan receptor Nr4a2 in Treg cells and the expression of both factors act synergistically in FoxP3 reporter assays (Sekiya et al. 2011). Nr4a2 binds directly to the *Foxp3* promoter and enhancer in Treg cells and is capable of driving *Foxp3* promoter and enhancer reporter constructs. Furthermore, loss of Nr4a2 resulted in increased susceptibility to inflammatory bowel

disease and failed to be protective in a transfer model of colitis. Both Runx and Nr4a2 are involved in modifying the chromatin landscape of the *Foxp3* gene. The deletion of Cbfβ in T regulatory cells resulted in the loss of H3K4me3 and H3K9me3 permissive chromatin modifications (Rudra et al. 2009), while overexpression of Nr4a2 resulted in increased histone 4 acetylation and increased levels of H3K4me3. While FoxP3 has also been shown to bind to its own promoter in a Runx-dependent manner, this binding is dependent on DNA demethylation (Zheng et al. 2010). However, neither Runx nor Nr4a2 removes DNA methylation at the *Foxp3* gene, indicating that additional factor(s) are required for the initial demethylation to allow DNA binding at the *Foxp3* promoter.

FoxP3 binds to Runx to alter the binding of Runx factors to target genes (Ono et al. 2007). In effector cells, Runx1 binds to and activates expression of *Il2*, which is suppressed in Treg cells. FoxP3 inhibits Runx1 activity as coexpression of Runx1 and Foxp3 decreased the Runx1-mediated expression of an *Il2* promoter-driven reporter construct. Furthermore, the deletion of the region of FoxP3 required for Runx1 binding also resulted in Treg cells that failed to suppress effector cells, indicating the importance of the Runx1-FoxP3 interaction in regulating Treg function. Similarly, the coexpression of FoxP3 with Runx1 decreased the induction of IL-17 expression under Th17 polarizing conditions (Zhang et al. 2008). Thus, by binding to Runx1, FoxP3 inhibits the expression of a number of Runx1 target genes. The mechanism of repression may occur by blocking Runx1 binding to target genes, or conversely, by utilizing Runx1-depending binding to target FoxP3 to genes, resulting in direct repression.

4.4 Ets

Ets1 deficient mice have decreased cytokine production under Th1 polarizing conditions, along with decreased Stat4 levels (Grenningloh et al. 2005). Although the expression of Tbet during Th1 differentiation remained unchanged in Ets1 deficient cells, Tbet was upreguated to a lesser extent during Th1 recalls responses resulting in decreased IFNγ production. To determine if elevated Tbet expression could rescue the defect in recall IFNγ production, these cells were transduced to express Tbet. However, this failed to rescue IFNγ production, indicating a requirement for both Tbet and Ets1 for optimal IFNγ expression. Both Tbet and Ets1 bind to the *Ifng* promoter and coexpression of both factors acted synergistically to upregulate expression of IFNγ. In this way, Ets1 cooperation with Tbet is important to drive IFNγ production for proper Th1 differentiation and recall responses.

Ets1 also plays a role as a negative regulator of Th17 differentiation, as Ets1 deficient cells produce more IL-17, IL-22, IL-23R, and RORγt in response to Th17 polarizing conditions (Moisan et al. 2007). This effect was reversed by introduction of Ets1 after Th17 differentiation. The proposed mechanism of Th17 inhibition by Ets1 involves the IL-2-dependent downregulation of Th17 differentiation.

While addition of IL-2 to wild type cells resulted in approximately 5-fold reduction in *Il7* producing cells, Ets1-deficient cells treated with IL-2 resulted in only a 2-fold reduction in IL-17 producing cells. As these cells do not show any deficiency in Stat5 signaling, Ets1 was proposed to act downstream of Stat5 to inhibit Th17 differentiation in response to IL-2 treatment.

Ets-1 also binds to the *Foxp3* promoter to drive FoxP3 expression (Polansky et al. 2010). In addition, Ets-1 has also shown a broader role in Treg differentiation by binding to many FoxP3 targets (Samstein et al. 2012). To identify accessible promoters in T regulatory cells, $FoxP3^-$ and $FoxP3^+$ cells were subjected to DNase digest followed by high throughput sequencing to identify areas of accessible DNA. More than 99 % of DNase hypersensitivity sites identified were accessible in both subsets. Likewise, 98 % of FoxP3 binding sites were accessible in $FoxP3^-$ cells, indicating FoxP3-independent opening of chromatin. To look for other factors that might play a role in FoxP3 mediated transcription, FoxP3 binding sites were examined for other transcription factor binding sites and an enrichment of Ets and Runx binding sites was found, with an enrichment score higher than the FoxP3 binding site, indicating a possible role in the recruitment of FoxP3 to target genes. Indeed, binding of these factors to target genes preceded FoxP3 binding as Ets and Runx were bound to target regions in both $FoxP3^+$ and $FoxP3^-$ cells. Since the DNase hypersensitivity of these sites was similar before and after FoxP3 expression, it was hypothesized that another factor may bind in place of FoxP3, thus protecting DNA from digest. In $FoxP3^-$ cells, Foxo1 was shown to bind to FoxP3 binding sites, with a subsequent loss of Foxo1 binding in $FoxP3^+$ cells. The displacement of Foxo1 by FoxP3 corresponded to genes that were downregulated in FoxP3 cells. Those few regions of FoxP3 binding that were not accessible in $FoxP3^-$ cells were found to have increased accessibility after T cell stimulation and expression from these regions was decreased in calcineurin deficient cells, indicating that FoxP3 relies on other transcription factors to regulate chromatin accessibility and binds to regions already accessible. Interestingly, in addition to binding to FoxP3 target genes, Foxo proteins also play a role in the regulation of FoxP3 expression as both Foxo1 and Foxo3 were shown to bind directly to the *Foxp3* promoter to regulate FoxP3 expression, with loss of either resulting in decreased Treg populations (Harada et al. 2010; Ouyang et al. 2010).

4.5 Ikaros

The Ikaros family of zinc finger DNA binding proteins is involved in Th2, Th17, and Treg differentiation. Ikaros appears to play a role in the inhibition of Th1 responses during Th2 differentiation as Ikaros deficient cells cultured under Th2 conditions produced increased levels of IFNγ (Thomas et al. 2010; Umetsu and Winandy 2009). Ikaros binds directly to the *Tbet* and *Ifng* promoters (Thomas et al. 2010; Quirion et al. 2009) to repress the expression of these genes, as expression of a dominant negative mutant of Ikaros resulted in increased expression of both Tbet

and IFNγ (Thomas et al. 2010). Furthermore, Ikaros also binds directly to the *Il4* promoter, potentially activating IL-4 expression, as Ikaros deficient cells produced less IL-4 under both nonpolarizing T cell stimulation (Thomas et al. 2010; Umetsu and Winandy 2009) and during Th2 differentiation (Quirion et al. 2009), although with time IL-4 expression returned to normal levels (Thomas et al. 2010). Ikaros deficient cells show decreased histone acetylation at the Th2 locus both in naïve cells and under Th2 polarizing conditions, indicating that Ikaros may function by altering chromatin accessibility of the Th2 locus during Th2 differentiation (Quirion et al. 2009). Finally, Ikaros deficient cells primed under Th2 inducing conditions also show altered expression patterns of multiple transcription factors involved in Th differentiation, including decreased Gata3 and c-Maf expression, both involved in Th2 differentiation, while Tbet and Stat1, both involved in Th1 differentiation, were upreguated. This suggests a broad role for Ikaros in controlling the expression of multiple genes to strengthen Th2 commitment while inhibiting Th1 differentiation, likely by interacting with unique factors to either activate or repress expression of target genes.

A second member of the Ikaros family, Aiolos, is specifically expressed in Th17 cells (Quintana et al. 2012). In Aiolos deficient cells, there was decreased expression of the Th17 cytokines IL-17a, IL-17f, and IL-21 along with decreased expression of the transcription factors Maf and Ahr. However, Aiolos overexpression was not sufficient to induce Th17 differentiation under non-Th17 polarizing conditions. Ahr and Stat3, both of which bind to the *Aiolos* promoter, induce Aiolos expression in Th17 cells as overexpression of Ahr or a constitutively active Stat3 increased expression of an Aiolos expression construct. Similar to Ikaros deficient cells, Aiolos deficient cells also show an altered pattern of chromatin modifications, with fewer restrictive histone modifications and more permissive modifications at the IL-2 promoter, indicating a role for Aiolos in modifying the chromatin landscape of target genes. The importance of Aiolos in the regulation of $CD4^+$ T cell differentiation is demonstrated in a transfer model of colitis where mice receiving Aiolos deficient cells developed a more severe wasting disease compared to control mice, due to increased IFNγ^+, IL-2^+ and IFNγ^+IL17^+ cells.

The Ikaros family members, Helios and Eos, are expressed selectively in T regulatory cells. Helios is highly expressed by tTregs and thus may be a marker for these cells (Thornton et al. 2010). However, since Helios expression is detected in inducible Tregs under certain conditions (Gottschalk et al. 2012), Helios expression may be associated with the maturity of Tregs. Eos interacts with FoxP3 and knockdown of Eos in cells overexpressing FoxP3 resulted in loss of FoxP3-mediated IL-2 suppression, indicating a requirement of Eos in FoxP3-mediated gene repression (Pan et al. 2009). Eos carries out this suppressive function by interacting with the transcriptional corepressor C-terminal binding protein-1 (CtBP1), which is found in a complex with FoxP3 and Eos, where depletion of Eos prevented association of CtBP1 with FoxP3. The loss of Eos also resulted in changes in histone acetylation and methylation, as well as DNA methylation at the *Il2* promoter, resulting in a pattern that more closely resembled that found in effector cells. A similar result was found with the knockdown of CtBP1, indicating

the importance of the recruitment of CtBP1 by Eos to the FoxP3 containing complex. The role of Eos was not limited to the *Il2* promoter, as analysis of genome-wide expression changes in Eos deficient cells showed that many genes normally suppressed by FoxP3 were not suppressed in Eos deficient cells while genes normally upreguated by FoxP3 were unchanged in the absence of Eos. Thus, this interaction of Eos and FoxP3 is specifically required for repression of gene expression.

5 Transcription Factor Networks

Given the large number of transcription factors involved in the differentiation of $CD4^+$ T cell subsets, studies have started to look at transcription factor networks in the regulation of differentiation. To examine the interaction of transcription factors during Th17 differentiation, ChIP-Seq was performed with antibodies against Stat3, IRF4, Batf, c-Maf, RORγt, and p300 in cells undergoing Th17 differentiation (Ciofani et al. 2012). There was a high degree of co-localization of these factors at genes expressed in Th17 cells, indicating cooperation during Th17 differentiation. By grouping transcription factor binding sites that clustered in close proximity with each other, it was found that regions where all five of these factors bound showed the highest fold change in expression during Th17 differentiation. Of these factors, IRF4 and Batf were also bound to these regions in nonpolarized cells, leading to speculation that they may be required for the formation of the larger five factor containing complex. Indeed, IRF4 and Batf are codependent, where loss of one prevents binding of the other and this correlates with decreased chromatin accessibility at regions where all five factors bind.

To understand the unique function of each transcription factor during Th17 differentiation, RNA-Seq was performed on control and transcription factor knockout mice to look at genome-wide expression changes. By comparing these results, a network was created to map the interactions of these factors with each other and with target genes during Th17 differentiation. This analysis demonstrated that IRF4, Batf, and Stat3 form a positive feedback loop to strengthen their expression during Th17 differentiation while c-Maf forms a negative feedback loop, being activated by Batf, Stat3, and IRF4, but acting to repress Batf. By comparing the targets identified by ChIP-Seq analysis with the changes in expression profile in knockout mice, it is also possible to identify novel factors involved in subset differentiation. In this study, Etv6, Ncoa2, Skil, Trib3, and the AP-1 family member Fosl2, are each predicted to be involved in Th17 differentiation, although further study is needed to understand how they fit into the defined network of transcription factors.

A second genome-wide analysis of Th17 cells made use of microarray analysis performed at three time points during Th17 differentiation, which allowed for the grouping of genes based on the timing of upregulation after Th17 differentiation as well as the length of upregulation (Yosef et al. 2013). Genes upreguated at the

early time point included the transcription factors Batf, IRF4, Stat3, Stat1, and IRF1, which showed sustained expression during Th17 differentiation, while RORγt and Ahr were induced at the intermediate time point. These results correspond well with the positive feedback loop between Batf, IRF4, and Stat3 and the requirement for Batf and IRF4 for the binding of the other transcription factors. Together, these results demonstrate the major transcriptional regulators during Th17 differentiation and allow identification of additional regulators.

Many transcription factors besides the ones described above have been identified for the differentiation of each $CD4^+$ T cell subset including Hlx (Mullen et al. 2002; Martins et al. 2005; Mikhalkevich et al. 2006), Gfi-1 (Zhu et al. 2002, 2006, 2009), Dec2 (Yang et al. 2009), Hif1a (Dang et al. 2011), Ahr (Veldhoen et al. 2008; Quintana et al. 2008; Kimura et al. 2008), Tcf7 (Yu et al. 2009b, 2011; van Loosdregt et al. 2013) and Notch (Shin et al. 2006; Bailis et al. 2013; Minter et al. 2005; Fang et al. 2007; Amsen et al. 2007; Mukherjee et al. 2009; Samon et al. 2008; Auderset et al. 2013; Elyaman et al. 2012). However, their contributions to T cell differentiation in the context of the transcriptional network are poorly understood. Figure 3 shows a schematic outline of the transcription factor networks as currently known for Th1, Th2, and Th17 cells. In order to gain a more thorough understanding of the transcriptional networks controlling the differentiation of all $CD4^+$ T subsets, similar large-scale ChIP-Seq analyses of known transcription factors coupled with knockout studies should be performed for each subset. Based on the studies of the Th17 differentiation pathway, it is likely that these results will also show widespread cooperation between a number of transcription factors to induce subset-specific transcription programs.

6 Long Intergenic Noncoding RNA

The role of long intergenic noncoding RNA (lincRNA) in T cell differentiation has become a subject of interest recently as lincRNA have been shown to play a role in many cellular functions, including chromatin remodeling, as well as both transcriptional repression and activation (Pagani et al. 2013). Mice overexpressing the NeST lincRNA show increased IFNγ expression and while they show increased resistance to *Salmonella*, they have increased susceptibility to Theiler virus (Gomez et al. 2013). NeST was found predominately in the nucleus of these overexpressing cells and was shown to interact with WDR5, a core subunit of the MLL1-4 and SET1A/1B complexes, which act to methylate H3K4 residues. Indeed, NeST overexpressing mice show increased H3K4me3 marks at the *Ifng* gene. Thus, by increasing permissive histone methylation, NeST increases IFNγ expression. The expression of lincRNA may be subset specific as NeST showed higher expression in Th1 cells compared to Th2 cells (Collier et al. 2012). This subset-specific expression was driven by the Th1 transcription factors Stat4 and Tbet, as mice deficient in either factor showed decreased NeST expression. A large-scale study of lincRNA expression during T cell development and differentiation recently identified over

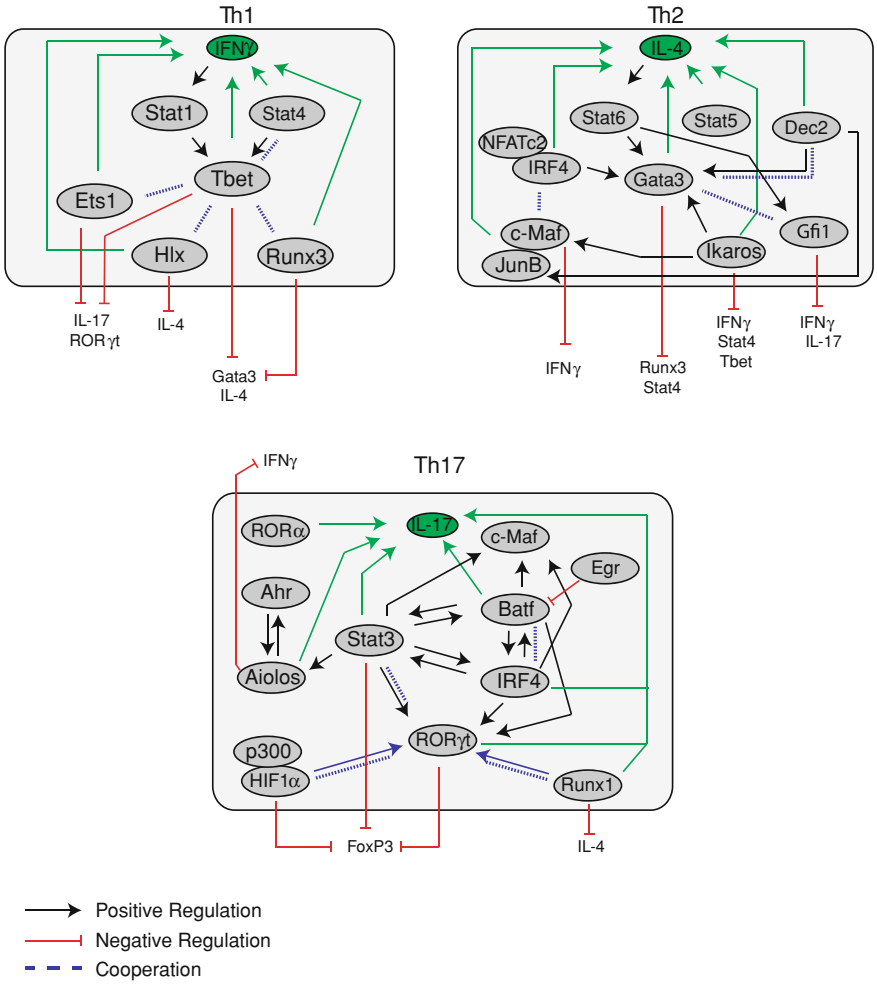

Fig. 3 Schematic summary of transcription factor networks involved in $CD4^+$ T cell differentiation. The differentiation of each $CD4^+$ T cell subset is controlled not only by Stats and master regulators but also by secondary transcription factors that activate subset-specific gene expression, including both cytokines and master regulators. There are many cooperative interactions between the transcription factors expressed within a given subset to strengthen gene expression and inhibition between factors to either regulate gene expression within a subset or to inhibit the differentiation of other subsets

1500 lincRNA clusters and found that almost 50 % of those expressed in $CD4^+$ T cells showed subset-specific expression (Hu et al. 2013). A number of these lincRNA were bound by subset-specific transcription factors Stat4, Tbet, Stat6, and Gata3, indicating that these factors control the expression not only of protein coding genes but also noncoding regions to regulate T cell differentiation. Stat4 showed

stronger binding at lincRNA genes preferentially expressed in Th1 cells and these targets were downregulated in Stat4 deficient cells. Similarly, Stat6 also showed higher binding at lincRNA genes preferentially expressed in Th2 cells and these targets were downregulated in Stat6 deficient cells. Both Tbet and Gata3 bind to subset-specific lincRNA clusters, but the loss of either transcription factor resulted in both upregulation and downregulation of target lincRNA genes, indicating that Tbet and Gata3 both activate and repress lincRNA expression during Th1 or Th2 differentiation, respectively. These results demonstrate that subset-specific transcription factors drive the expression of noncoding genes. More work is required to understand the function lincRNA in the regulation of T cell differentiation.

7 Conclusion

Early work on transcriptional regulation of T cells has focused on the two-factor model of differentiation, in which cytokine stimulation activates the Stat family of transcription factors, which in turn activate the master regulators of $CD4^+$ T cell differentiation. However, it has become clear that these factors do not act alone to induce differentiation but instead interact with a number of other transcription factors for optimal $CD4^+$ T cell differentiation and function. The use of large-scale, genome-wide studies is beginning to shed light on when and how transcription factors are up- or downregulated during development as well as providing information on the interactions between transcription factors at multiple target genes during differentiation. In this way, a picture of the network of transcription factors is beginning to emerge, although more work is needed to generate and validate transcriptional networks for all $CD4^+$ T cell subsets during differentiation. The essential role of transcriptional regulation in maintaining proper $CD4^+$ T cell function is demonstrated by mutations of various transcription factors and the resulting diseases. Mutations of *Foxp3* results in the development IPEX autoimmunity, while mutation of *STAT3* leads to hyper IgE syndrome, characterized by recurrent staphylococcal skin abscesses, eczema, and pulmonary infections (Yong et al. 2012) and MS patients have been reported to express decreased levels of Egr2, resulting in increased Th17 responses (Miao et al. 2013). These disorders illustrate the importance of transcriptional regulation during $CD4^+$ T cell differentiation and highlight the need for a more thorough understanding of these transcriptional networks to provide insight into the balance of protective immunity versus autoimmunity in humans.

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Transcriptional Control of NK Cell Differentiation and Function

Victoria Male and Hugh J. M. Brady

Abstract Natural killer (NK) cells are crucial to mounting an effective immune response. They have a significant role in cancer immunosurveillance and function as a bridge between innate and adaptive immunity. However, until recently, surprisingly little was known about the molecular basis of NK cell development as compared to the impressive body of knowledge on B- and T-cell development. Here we outline the key transcription factors known to influence NK cell development and at what stages they function. The recent progress in understanding allows us to speculate on the nature of the network of interactions between transcription factors that ultimately facilitate the production of mature functional NK cells.

Contents

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1 Introduction

Natural killer (NK) cells are innate lymphocytes which recognise and kill virally infected and malignant cells and produce cytokines, such as IFNγ, which polarise the adaptive immune response (Vivier et al. 2008). NK cells are most closely related to other members of the lymphoid lineage, including T cells, B cells and other more recently described members of the innate lymphoid cell (ILC) family, all of which are derived from common lymphoid progenitors (CLPs) (Kondo et al. 1997; Possot et al. 2011; Yang et al. 2011; Wong et al. 2012). NK cells develop both in the bone marrow and at some extra-medullary sites such as the thymus and liver (Huntington et al. 2007), but the developmental progression from CLPs to mature NK cells is currently best defined in the bone marrow (Fig. 1). Here, haematopoietic stem and progenitor cells differentiate to lymphoid-primed multipotent progenitors (LMPPs) which, although they have lost the ability to self-renew, are still able to give rise to a number of immune cell lineages (Adolfsson et al. 2005). Some LMPPs differentiate to CLPs, which can produce all subtypes of lymphoid cell. CLPs in turn give rise to NK progenitor cells, which have lost the ability to produce cells of any lineage other than NK cells (Rosmaraki et al. 2001; Carotta et al. 2011; Fathman et al. 2011), and these cells continue to develop into immature and mature NK cells (iNK and mNK, respectively), which are exported from the bone marrow (Kim et al. 2002; Chiossone et al. 2009).

Originally, NK progenitors were defined as Lin-NK1.1-DX5-CD122+ bone marrow cells (Rosmaraki et al. 2001). However, only 1 in 12 cells defined in this way is able to produce NK cells and more recently the NK progenitor phenotype has been refined. Using an Id2-GFP reporter mouse, Id2+ cells were identified both within the CLP and the Lin-CD122+ populations. These cells were unable to give rise to T or B cells, but gave rise to NK cells at a frequency of one in 2.4 and one in 3, respectively (Carotta et al. 2011). Two NK progenitor populations that seem to largely overlap with these subsets were independently identified by examining Lin- bone marrow cells expressing the NK cell markers 2B4 and CD27 (Fathman et al. 2011). These populations were designated ‘preNKP’ and ‘rNKP’ and have an NK progenitor frequency of one in 2.4 and one in 1.7, respectively.

A number of transcription factors play a role in NK cell development but the developmental stage at which most of these act has been defined using the original phenotype of NK progenitor cells (Lin- NK1.1-DX5-CD122+). Using the new, more refined definitions, it is now be possible to identify at higher resolution the points in the earliest stages of NK cell development at which these transcription factors are required.

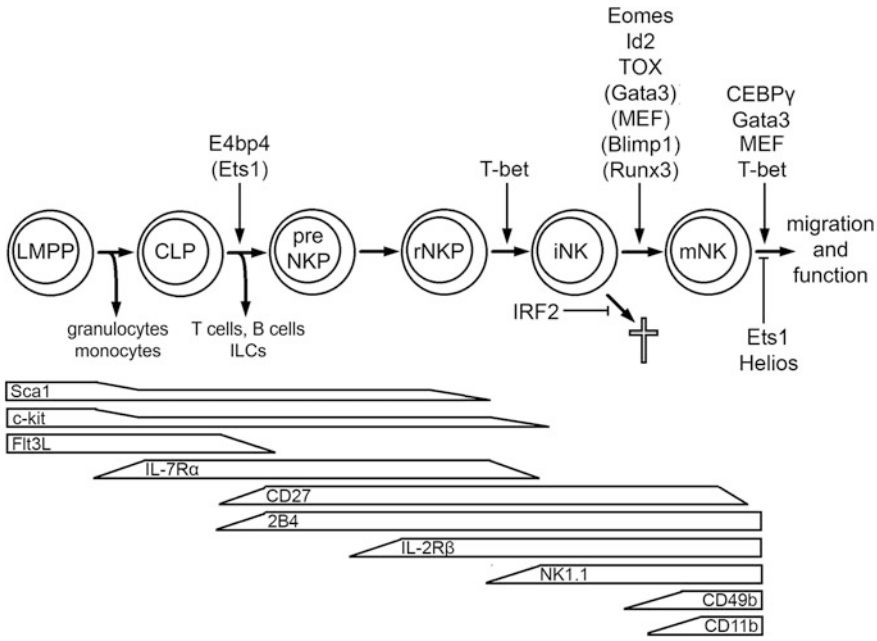

Fig. 1 Transcriptional control of NK cell development in mouse bone marrow. The stages of NK cell development in mouse bone marrow are shown, together with the phenotypes of the developmental intermediates. Transcription factors are shown at the earliest transition that they have been definitively shown to mediate. Those transcription factors that are shown in brackets display a partial, or 'leaky', phenotype at the transition indicated. *LMPP* lymphoid-primed multipotent progenitor; *CLP* common lymphoid progenitor; *preNKP* pre-NK progenitor; *rNKP* refined NK progenitor; *iNK* immature NK cell; *mNK* mature NK cell

2 Commitment to the NK Lineage

Commitment to the NK lineage occurs as CLPs, which can give rise to all members of the lymphocyte family, become NK progenitor cells, which can only differentiate to NK cells (Rosmaraki et al. 2001; Carotta et al. 2011; Fathman et al. 2011). This process occurs inefficiently in the absence of the transcription factors E4bp4 and Ets1. Some transcription factors that are currently thought to act later in NK cell development may also act at this point, but until these are re-examined in the light of the refined NK progenitor phenotype, it will be impossible to know this with any certainty. Here we discuss transcription factors as acting at the earliest point in NK cell development for which there is currently evidence (Fig. 1).

The basic leucine zipper transcription factor E4bp4 (also known as Nfil3) was originally identified by its ability to transactivate the IL-3 promoter in human T cells, but has since been shown to have roles in a number of immune cell lineages, including CD8α+ cDC development and in regulating T helper cell cytokine production (Male et al. 2012). However, the most striking feature of the E4bp4

knockout mouse is its almost complete absence of NK cells (Gascoyne et al. 2009; Kamizono et al. 2009). The E4bp4 knockout mouse was originally described as having NK progenitors but not immature NK cells (Gascoyne et al. 2009; Kamizono et al. 2009), but this work was done using the original definition of NK progenitors (Rosmaraki et al. 2001). More recently, the mouse has been re-examined for the presence of preNKP and rNKP (Fathman et al. 2011) and displays an approximately tenfold reduction in these cells, although CLP numbers are normal (Male et al. 2014). For NK cell production to occur in vitro, E4bp4 must be expressed specifically at the CLP stage and, consistent with a requirement for E4bp4 prior to the NK progenitor stage, wild-type LMPPs and CLPs express E4bp4 transcript at moderate levels (Male et al. 2014). These data suggest that E4bp4 expression is required in CLPs in order to mediate the transition from CLP to NK progenitor, and thus commitment to the NK lineage.

Mature NK cell numbers have long been known to be reduced in the absence of Ets1 (Barton et al. 1998), although the deficiency is not as pronounced as that seen in the absence of E4bp4. More recent works have shown that the Ets1 knockout mouse also displays small but significant reductions in the frequencies of preNKP and rNKP cells (20 and 50 %, respectively) (Ramirez et al. 2012). Similar to E4bp4, Ets1 transcript is detectable in wild-type LMPPs and CLPs, suggesting that its expression is also required before NK lineage commitment. (Ramirez et al. 2012) Therefore, like E4bp4, Ets1 is likely to have a role promoting commitment to the NK lineage, although the more subtle phenotype of the knockout may suggest that it is less important than E4bp4, or that there is some redundancy with other factors that act at the same stage.

Lineage commitment is defined not only by the ability to produce cells of a given lineage, but equally by the inability to give rise to cells of other lineages. Therefore, some transcription factors affect NK cell production primarily by their actions to promote or repress commitment to other lineages. One such factor is the zinc finger transcription factor Bcl11b: if is deleted in developing thymocytes, even in T cell-committed DN3 cells, they become NK, rather than T cells (Li et al. 2010). Similarly, if the helix-loop-helix transcription factor Id3 is overexpressed in human fetal liver-derived CD34+ haematopoietic stem cells, they produce fewer T cells and more NK cells in fetal thymic organ culture (Heemskerk et al. 1997). Since overexpression of Id3 does not affect the frequency of NK cells produced in tissue culture conditions optimised for NK cell production, this suggests that the effect of Id3 is mainly to repress T-cell production, rather than to promote NK cell production. It has also been suggested that Id3 may act co-operatively or redundantly with its close relative Id2, which seems to be required later in NK cell development (Boos et al. 2007).

3 Development of Immature NK Cells

NK-committed progenitors differentiate to mNK cells via an iNK cell stage, which expresses NK1.1 but does not express the integrins CD11b or CD49b (DX5). The T-box transcription factor T-bet (also known as Tbx21) is best known for its roles in Th1 and CD8+ T-cell differentiation, but also plays a role in directing the production of iNK cells. T-bet is expressed in both iNK and mNK (Gordon et al. 2012; Male et al. 2014) and the T-bet knockout mouse has a reduced frequency of NK cells, but normal numbers of preNKP and rNKP, in its bone marrow (Gordon et al. 2012; Male et al. 2014). This suggests that T-bet is not required for NK commitment but is required for the production of iNK. In support of this, T-bet transcript is first detectable just prior to the iNK cell stage, in rNKPs (Male et al. 2014).

Interestingly, although both iNK and mNK numbers are reduced in the absence of T-bet (Gordon et al. 2012; Male et al. 2014) the majority of NK1.1 cells that persist in the bone marrow of the T-bet knockout mouse are mNK (Gordon et al. 2012) indicating that T-bet expression is more important for iNK than for mNK cells. Consistent with this hypothesis, temporal deletion of T-bet leads to loss of iNK cells, but mNK cells are unaffected, suggesting that T-bet is required specifically to stabilise the iNK population (Gordon et al. 2012). The finding that T-bet-deficient NK cells both divide and undergo apoptosis at a higher rate than wild-type NK cells suggests that this could be due to increased turnover of iNK cells in the absence of T-bet (Townsend et al. 2004).

4 NK Cell Maturation

The final stage of NK cell development is the transition from iNK to mNK. This is most often defined by the acquisition of either CD11b or CD49b, but also involves increased expression of receptors such as Ly49 and increased effector function (Kim et al. 2002; Chiossone et al. 2009). A number of transcription factors act at this stage.

The T-box transcription factor Eomes (also known as Eomesodermin) is highly homologous to T-bet and in some situations, such as the differentiation of CD8+ effector T cells, there is some redundancy between the two (Intlekofer et al. 2005, 2008). Indeed, a more severe defect in NK cell production is observed when both T-bet and Eomes are absent than in the absence of either transcription factor alone (Intlekofer et al. 2005; Gordon et al. 2012). However, Eomes seems to act later in NK cell development than T-bet, mediating the development of mNK from iNK. Eomes protein is not expressed in iNK, but is expressed in mNK at increasing levels as the cells become increasingly mature (Gordon et al. 2012). The Eomes knockout mouse is embryonic lethal, but the effects of Eomes deficiency on haematopoiesis can be examined using a conditional knockout in which Eomes is

deleted only in cells expressing the immune cell signalling molecule Vav. In this model, iNK cells are present but there is a severe reduction in mNK cells, supporting the idea that Eomes is required for the production of mNK cells (Gordon et al. 2012). Furthermore, Eomes seems to be required for the maintenance of a mature phenotype in NK cells: if Eomes is temporally deleted from mNK cells, they lose their expression of the maturation marker CD49b and regain expression of TRAIL, which is characteristic of iNK (Gordon et al. 2012). Interestingly, these cells continue to express Ly49 molecules, suggesting that Eomes is not required for the expression of all the surface molecules associated with NK cell maturation.

The helix-loop-helix transcription factor Id2 and the HMG-box transcription factor TOX are both required for mNK cell production: mice deficient in either of these have normal numbers of iNK cells but a marked reduction in mNK cells (Boos et al. 2007; Aliahmad et al. 2010). These transcription factors also share a role in lymphoid tissue organogenesis: lymph nodes and Peyer's patches are absent in the Id2 knockout and lymph nodes are absent and Peyer's patches either absent or reduced in size in the TOX knockout (Yokota et al. 1999; Aliahmad et al. 2010). In the TOX knockout, this phenotype has been shown to be due to the absence of CD4+ lymphoid tissue inducer (LTi) cells, which are themselves members of the ILC family (Aliahmad et al. 2010). The finding that these transcription factors affect the development of ILCs other than NK cells has led to the suggestion, particularly of Id2, that they may act very early in innate lymphoid development, perhaps in a hypothetical innate lymphoid progenitor cell (Spits and Di Santo 2011). However, the observation that iNK cells can develop in the absence of these transcription factors would argue against this scheme, suggesting that it is more likely that these transcription factors act at separate points in the development of NK cells and other innate lymphocytes.

The transcription factors MEF (also known as Elf4), GATA-3, Blimp1 and Runx3 are also required for efficient mNK cell development: in their absence, mNK cells do develop, but in numbers that are somewhat reduced compared to the wild-type (Lacorazza et al. 2002; Samson et al. 2003; Ohno et al. 2008; Kallies et al. 2011). The MEF knockout has defects in a number of lymphoid lineages, including iNKT cell production and T-cell activation, but NK-cell development in this model is still poorly characterised: total NK cells are reduced by 60 % compared to the wild-type, but it is unclear if this includes mNK only, mNK and iNK, or even NK cells and NK progenitors (Lacorazza et al. 2002). In the GATA-3 knockout mouse, the total number of NK cells is normal; however, the NK population contains a higher proportion of iNK cells, suggesting that there is a block in the development of mNK cells and a corresponding build-up in iNK precursors (Samson et al. 2003). In the absence of Blimp1, total NK cell numbers are only very slightly reduced, but those NK cells that are present express the maturation markers CD11b and KLRG1 at lower levels than their wild-type counterparts (Kallies et al. 2011). This is consistent with the finding that Blimp1 expression is higher in CD11b+ mNK than in CD11b− iNK (Kallies et al. 2011). Those NK cells produced in the absence of Blimp1 are still able to kill NK targets, produce

IFNγ in response to cytokine stimulation and in fact proliferate more in response to challenge with tumour cells or IL-15 than wild-type NK cells (Kallies et al. 2011).

NK cells express both Runx1 and Runx3, but Runx3 is expressed at higher levels than Runx1 and its expression increases as NK cells mature (Ohno et al. 2008). Runx family gene knockout mice are embryonic lethal, but the effects of Runx family members can be assessed by expressing a dominant negative form of Runx1 (Runx dn) that interferes with the function of all Runx proteins (Ohno et al. 2008). When haematopoietic progenitor cells are transduced with Runx dn in culture, NK cells are still produced, but at a lower frequency than cultures transduced with a control vector (Ohno et al. 2008). Furthermore, when Runx dn is expressed under the CD2 promoter, reducing the levels of Runx proteins in iNK and mNK cells, the total number of NK cells produced does not differ from the wild-type, but fewer of these cells are mNK and fewer express Ly49 receptors (Ohno et al. 2008). However, cytotoxicity is unaffected and the Runx dn cells actually produce more IFNγ in response to cytokine stimulation than wild-type cells (Ohno et al. 2008). The nature of these experiments makes it difficult to say with any certainty which Runx family member is responsible for the phenotype, but the high expression of Runx3 by NK cells suggests that this is the most likely candidate.

5 NK Cell Survival

IRF2 was originally thought to be required for mNK cell development, since the IRF2 knockout mouse has a modest reduction in mNK cells but normal numbers of iNK cells (Lohoff et al. 2000). However, more recent works have shown that the lack of mNK in the IRF2 knockout is actually due to a defect in iNK survival: IRF2-deficient iNK cells express the activation marker CD69 at higher levels than wild-type cells, cycle faster and undergo apoptosis at a higher rate (Taki et al. 2005). Therefore, the reduction in mNK in this model seems to be due to the poor survival of the iNK which would otherwise give rise to them. The phenotype of the IRF2 knockout is interesting when compared to that of the T-bet knockout since both seem to have a survival defect at the iNK cell stage, yet in the T-bet knockout this manifests as a reduction in both iNK and mNK cells, with iNK cells more affected, whereas in the IRF2 knockout, iNK numbers are normal but the effect is seen at the mNK stage. This could be consistent with a model in which T-bet promotes survival somewhat earlier in NK cell development than MEF, but that in the absence of T-bet some other factor, perhaps Eomes, can substitute for it.

6 NK Cell Migration and Function

A number of transcription factors have roles in NK cell migration and function. Some, such as Ets1, T-bet, MEF and GATA-3, also have roles earlier in NK cell development while others, such as CEBPγ and Helios, act for the first time at this point.

The Ets1 knockout mouse first displays a defect in NK cell development at the preNKP stage, but the reduction in NK progenitors, iNK and mNK cells is modest so that the mouse still has a substantial number of NK cells (Barton et al. 1998; Ramirez et al. 2012). Those NK cells that develop independently of Ets1 are functionally defective in that they degranulate less well than their wild-type counterparts in response to activation via NK1.1, although their production of IFNγ is unaffected. However, they also seem to display some characteristics of chronic cytokine stimulation, such as higher expression of the activation marker CD69, higher granularity, higher expression of granzyme B and more proliferation in response to IL-15 stimulation than wild-type NK cells (Ramirez et al. 2012). Therefore, in mNK cells Ets1 seems to both mediate functional degranulation and limit activation in response to cytokine stimulation.

The zinc finger transcription factor Helios (also known as Ikzf2) also has a role in limiting NK cell activation. Noé mice have a point mutation in Ncr1 (NKp46), which abolishes its expression at the cell surface. NK cells from these mice are hyperresponsive: they produce more IFNγ and degranulate more effectively than wild-type NK cells in response to NK targets, and are more responsive in MCMV infection (Narni-Mancinelli et al. 2012). Helios is expressed at higher levels in Noé mNK than in wild-type cells and the hyperresponsive phenotype can be reversed by expressing an shRNA targeting Helios in Noé cells (Narni-Mancinelli et al. 2012). This suggests that Helios inhibits activation in mNK cells, and that NKp46 signalling in turn inhibits Helios activity. The finding that Helios is overexpressed in Ets1-deficient mNK cells also suggests that Ets1 may limit NK activation in part by acting through Helios (Ramirez et al. 2012).

NK cells that develop in the absence of MEF are defective in both IFNγ production and cytotoxicity (Lacorazza et al. 2002). This poor cytotoxicity results from their lack of granules: the cells contain little perforin and chromatin immunoprecipitation (ChIP) assays have shown that MEF directly binds and transactivates the perforin promoter (Lacorazza et al. 2002). CEBPγ knockout mice die as neonates, but the requirement for CEBPγ in NK cell development can be studied in bone marrow chimeras. CEBPγ-deficient bone marrow produces both iNK and mNK cells in normal numbers but, similar to MEF-deficient NK cells, these are poorly cytotoxic against NK targets and less able to produce IFNγ in response to cytokine stimulation than wild-type cells (Kaisho et al. 1999).

NK cells that are produced in the absence of GATA-3 display normal cytotoxicity but are defective in IFNγ production (Samson et al. 2003). It has also been suggested that GATA-3 is required for the migration of NK cells specifically to the liver: in wild-type/GATA-3 knockout bone marrow chimeras, GATA-3-deficient

NK cells reconstitute the spleen with the same efficiency as wild-type NK cells, but are significantly less efficient at reconstituting the liver and significantly enriched in the bone marrow (Samson et al. 2003). These observations could be consistent with a defect in NK cell homing from the bone marrow to the liver, but the study did not rule out the possibility that GATA-3 is required for the development of a specialist population of liver NK cells in situ.

Although recent studies have shown that T-bet knockout mice have reduced numbers of NK cells in the bone marrow (Gordon et al. 2012; Male et al. 2014), earlier work found somewhat increased NK cell numbers in T-bet-deficient bone marrow (Townsend et al. 2004; Jenne et al. 2009). Together with the decrease in NK cell numbers observed in spleen, liver and blood, this suggested that T-bet might be required for NK cell egress from the bone marrow (Townsend et al. 2004; Jenne et al. 2009). Duane mice have a point mutation in T-bet which causes the protein to be expressed at levels three to fourfold lower than the wild type. When irradiated mice were reconstituted with a mixture of wild-type and Duane bone marrow cells, Duane NK cells preferentially reconstituted the bone marrow and lymph nodes, but not blood, spleen or lymph (Jenne et al. 2009). The enrichment of Duane NK cells in lymph nodes was observed even in the presence of integrin and selectin blocking antibodies, which interfere with lymph node ingress, suggesting that T-bet affects egress from the lymph nodes. Indeed, Duane NK cells express sphingosine-1-phosphate receptor 5 ($S1P_5$), which cells must express in order to exit bone marrow and lymph nodes, at lower levels than wild-type NK cells and overexpression of T-bet in cell lines or in primary T cells resulted in increased expression of $S1P_5$ (Jenne et al. 2009). Therefore, in addition to its role in iNK cell development, T-bet is also required for $S1P_5$ expression, which allows NK cells to exit lymphoid organs.

7 Control of Extramedullary NK Cell Development

Although the majority of NK cells found in the periphery are likely to have developed in and been exported from the bone marrow, some organs, including the thymus (Vosshenrich et al. 2006), fetal liver (Jaleco et al. 1997; Uksila et al. 1983; Takeda et al. 2005) and lymph nodes (Freud et al. 2006; Warner et al. 2012) have their own populations of NK cells that develop in situ. These alternative pathways of NK cell development differ in their transcriptional control from the bone marrow pathway. Because the lymph node pathway of NK cell development has only recently been characterised in mice, there is currently little understanding of the transcription factors it requires, but more is known of the transcriptional control of thymic and liver NK cell development.

Thymic NK cells are phenotypically distinct from their bone marrow-derived counterparts, express the IL-7 receptor CD127 and, unlike bone marrow NK cells, require IL-7 for their efficient production (Vosshenrich et al. 2006). Unlike the NK cells found in other organs, thymic NK cells express high levels of GATA-3 and

irradiated mice reconstituted with GATA-3-deficient bone marrow cells do not produce thymic NK cells (Vosshenrich et al. 2006). Therefore, in contrast to the situation in bone marrow (Samson et al. 2003), GATA-3 is essential for NK cell development in the thymus. On the other hand, thymic NK cells do not require Id2 (Boos et al. 2007), although this may reflect the fact the Id2 is required for the final stage of NK maturation and thymic NK cells have a largely immature phenotype (Vosshenrich et al. 2006). Further research to discover whether other transcription factors required for medullary NK cell development, particularly E4bp4, Ets1 and T-bet, are also required for thymic NK cell development, will be of interest.

Fetal and neonatal liver contains a population of TRAIL+ NK cells, most of which also display a CD49b− immature phenotype (Takeda et al. 2005). As mice age, the proportion of liver NK cells which are TRAIL+ decreases, but some TRAIL+ liver NK cells do persist into adulthood (Takeda et al. 2005). Adult TRAIL+ liver NK cells are IFNγ-dependent (Takeda et al. 2005) but also require the transcription factor T-bet: in T-bet knockout mice, TRAIL+ NK cells are present in fetal but not adult liver, suggesting that T-bet is required for the stabilisation of TRAIL+ NK cells in the liver (Gordon et al. 2012). In contrast, TRAIL+ NK cells do not express Eomes and do not require Eomes for their development (Gordon et al. 2012). Therefore Eomes is absolutely required for the production of mNK in the bone marrow, but is dispensible for the development of TRAIL+ liver NK cells, while T-bet is required for both the bone marrow and the liver pathways of NK cell differentiation.

8 Transcriptional Control of NK Cell Development: Towards a Unified Scheme?

Although rapid progress has been made in defining the points in NK cell development at which various transcription factors act, our understanding of how these transcription factors interact with each other is still in its infancy. Some recent studies have identified downstream targets of a single transcription factor, either by examining changes in expression of the putative target in the absence of the transcription factor of interest, or by ChIP. By considering these studies in aggregate, we can begin to build up a picture of the network of transcription factors that control NK cell development (Fig. 2).

Id2 was originally identified as a target of E4bp4 by complementation: retroviral expression of Id2 in E4bp4-deficient HPCs can partially rescue their ability to produce NK cells (Gascoyne et al. 2009). This approach has recently been extended, and a number of transcription factors that can rescue NK cell development in the absence of E4bp4 have been identified, including Eomes, Id2, T-bet and Ets1 (Male et al. 2014). Eomes and Id2, but not T-bet, are underexpressed in the absence of E4bp4 and by ChIP E4bp4 has been shown to bind directly to Eomes and Id2 promoters (Male et al. 2014). Therefore, there is strong evidence

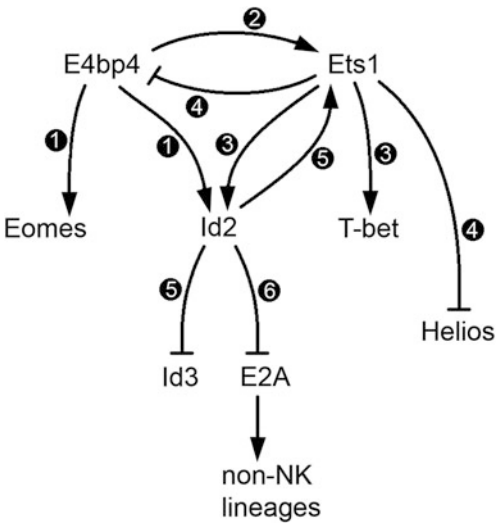

Fig. 2 Summary of known interactions between transcription factors required for NK cell development. *1* Eomes and Id2 can rescue NK cell development in the absence of E4bp4, are underexpressed in the absence of E4bp4 and by ChIP, E4bp4 binds to the promoter regions of their genes (Male et al. 2014); *2* Ets1 can rescue NK cell development in the absence of E4bp4 (Male et al. 2014); *3* Id2 and T-bet are underexpressed in the absence of Ets1 and by ChIP, Ets1 binds to the promoter regions of their genes (Ramirez et al. 2012); *4* E4bp4 and Helios are overexpressed in the absence of Ets1 (Ramirez et al. 2012); *5* Ets1 is underexpressed and Id3 is overexpressed in the absence of Id2 (Boos et al. 2007); *6* NK cells do not develop in the absence of Id2, but this phenotype is reversed in the absence of both Id2 and E2A (Boos et al. 2007)

that Eomes and Id2 are direct targets of E4bp4, but the relationship between E4bp4 and T-bet is less clear. Id2 and T-bet are underexpressed in Ets1-deficient NK cells and ChIP in these cells has also shown that these genes are direct transcriptional targets of Ets1 (Ramirez et al. 2012). Ets1-deficient NK cells also overexpress E4bp4 and Helios, suggesting that Ets1 may repress the transcription of these genes (Ramirez et al. 2012). E4bp4 and Ets1, then, both act at the CLP to NK progenitor transition and both promote the expression of Id2, but while the ability of Ets1 to rescue NK cell production in the absence of E4bp4 suggests that Ets1 expression may be promoted by E4bp4 (Male et al. 2014), Ets1 also inhibits the expression of E4bp4 (Ramirez et al. 2012).

There is also some evidence that Id2 may promote the production of Ets1 since Ets1 is underexpressed in Id2-deficient Lin-CD122+ NK progenitors cells (Boos et al. 2007). However, since this is now known to be a very heterogenous population, it is possible that the relationship between these transcription factors occurs mainly within the non-NK lineage cells rather than the true NK progenitors in this population. In contrast, Id3 is overexpressed in the absence of Id2, consistent with the idea that there may be some redundancy between these highly homologous transcription factors (Heemskerk et al. 1997; Boos et al. 2007). The transcription

factor E2A promotes the differentiation of cells to non-NK lymphoid lineages, particularly to B cells (Bain et al. 1994; Zhuang et al. 1994) and one role of Id2 is to inhibit its action (Sun et al. 1991; Boos et al. 2007). Id2 knockout mice lack mNK cells, but in the E2A/Id2 double knockout, production of CD49b+ mNK cells in the bone marrow is restored (Boos et al. 2007). These cells still fail to express CD11b, which is expressed in slightly more mature NK cells, and mNK cells continue to be absent in the spleen, suggesting that Id2 also promotes NK cell development through other pathways.

Id2 and TOX knockout mice are strikingly similar: both suffer a block in NK cell development at the same point, and both display defective development of lymphoid architecture (Yokota et al. 1999; Boos et al. 2007; Aliahmad et al. 2010). This has led to the suggestion that the two transcription factors may act in the same pathways. Retroviral expression of Id2 in TOX-deficient haematopoietic progenitor cells is unable to rescue NK cell development, suggesting that Id2 is unlikely to be downstream of TOX but this observation does not rule out the possibility that Id2 acts upstream of TOX (Aliahmad et al. 2010). However, the more recent finding that Id2, but not TOX, can rescue NK cell development in the absence of E4bp4 suggests that Id2 and TOX may in fact act in different pathways (Male et al. 2014).

Although it is now apparent that T-bet acts earlier in NK cell development than Eomes (Gordon et al. 2012) whether or not these two closely related transcription factors act in the same pathway is not yet clear. T-bet-deficient NK cells express Eomes at normal levels, suggesting that T-bet does not promote Eomes expression (Townsend et al. 2004). This could be because Eomes can substitute for T-bet, a hypothesis that is supported by the more severe defect in NK cell production that occurs in the absence of both of these transcription factors, compared to either one alone (Gordon et al. 2012). Further evidence that T-bet and Eomes may act in separate pathways comes from the differential requirements for these transcription factors at different anatomical locations. While Eomes is absolutely required for bone marrow NK cell development, it is dispensible for the production of TRAIL+ NK cells in the liver, which instead require T-bet (Gordon et al. 2012). Similarly, NK-like cells in the gut are not produced in the absence of T-bet, but do not require Eomes for their development (Sciumé et al. 2012; Klose et al. 2013; Rankin et al. 2013). Therefore, it seems that although Eomes and T-bet can, to some extent, substitute for each other in medullary NK cell development, Eomes is primarily required in the bone marrow pathway of NK cell development, while T-bet is most important for the production of NK and NK-like cells at extramedullary sites.

9 Conclusions

The identification and detailed characterization of NK progenitor cells has been a recent highlight of research on NK cell development. This missing link between CLP and immature NK cells has allowed researchers to start to look for the

molecular basis of NK lineage commitment. It is now emerging that a small core of transcription factors that includes E4bp4, Id2, T-bet, Eomes and Ets1 regulate NK cell development. It appears that E4bp4 has a critical role in determining commitment to the NK cell lineage from CLPs. Future work will elucidate the precise nature of the interactions between the 'core' factors and how they contribute to the full gamut of processes leading to mature functional NK cells. A remaining mystery is how the essential role of IL-15 in NK cell development is mediated through these transcription factors. Hopefully, unequivocal evidence will appear linking the signalling pathway activated by IL-15 to the 'core' factors. Great hope still remains for the efficacy of NK cell immunotherapy as a major therapeutic intervention. Central to this is that NK cells can be expanded in vitro for transfusion to patients and that these transfused cells will be both cytotoxic and survive long enough in vivo to be highly effective. A good understanding of the molecular basis of NK cell development is important for this hope to become reality. The activity of the 'core' transcription factors may be influenced by other events such as post-translational modifications that might occur in response to extrinsic stimuli such as cytokines but could also include cell–cell interactions. NK cell numbers are remarkably heterogenous between individuals and this may influence susceptibility to various disease states. It is worth speculating that some of this heterogeneity could possibly even be due to genetic polymorphisms in the genes that regulate NK cell development.

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Transcriptional Control of Pre-B Cell Development and Leukemia Prevention

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Abstract The differentiation of early B cell progenitors is controlled by multiple transcriptional regulators and growth-factor receptors. The triad of DNA-binding proteins, E2A, EBF1, and PAX5 is critical for both the early specification and commitment of B cell progenitors, while a larger number of secondary determinants, such as members of the Ikaros, ETS, Runx, and IRF families have more direct roles in promoting stage-specific pre-B gene-expression program. Importantly, it is now apparent that mutations in many of these transcription factors are associated with the progression to acute lymphoblastic leukemia. In this review, we focus on recent studies that have shed light on the transcriptional hierarchy that controls efficient B cell commitment and differentiation as well as focus on the oncogenic consequences of the loss of many of the same factors.

Contents

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1 Introduction to Early B Cell Development

Early B cell development initiates with the gradual stepwise differentiation of multipotent hematopoietic stem cells (HSCs) to early B cell progenitors in the bone marrow (BM). The lymphoid-primed multipotent progenitors (LMPPs) are the first lymphoid specified progenitor cells downstream of the HSC that retain the full lympho-myeloid lineage potential but give rise to little or no megakaryocyte and erythrocyte progenitors (MEPs) (Adolfsson et al. 2001, 2005). LMPPs include lymphoid-biased progenitors such as early lymphoid progenitors (ELPs) that are defined by the expression of the *Rag1* gene (Igarashi et al. 2002). ELPs are the precursors of common lymphoid progenitors (CLPs) in which critical transcriptional regulated B-cell specification and commitment occur (Karsunky et al. 2008; Mansson et al. 2008, 2010; Kondo et al. 1997). CLPs have recently been further split, based on the expression of the cell surface receptor, Ly6D, into all lymphoid progenitors (ALPs) that show pan-lymphocyte developmental potential and B cell-biased lymphoid progenitors (BLPs) (Inlay et al. 2009).

BLPs give rise to pre-pro-B-cells (also known as Fraction A), which can be identified by the expression of the B cell-associated marker B220 (CD45R) (Rumfelt et al. 2006; Gounari et al. 2002; Li et al. 1996). Commitment to the B cell lineage occurs at the pro-B cell stage (Fraction B/C cells) (Rumfelt et al. 2006). Committed pro-B cells can be identified by their expression of CD19 and their lack of fms-like tyrosine kinase FLT3 (also known as Flk2 or CD135 (Rumfelt et al. 2006; Holmes et al. 2006)). Committed pro-B cells express high levels of *Rag1/2* and recombine their variable (V) gene segments to previously rearranged D_H-J_H segments at the *Immunoglobulin heavy chain* (*Igh*) locus (ten Boekel et al. 1995; Li et al. 1993). Successful rearrangement of the *Igh* locus leads to the expression of IgH at the cell surface in association with the surrogate light chain ($\lambda 5$, VpreB) and accessory signaling molecules (Igα, Igβ) to form the pre-B cell receptor (pre-BCR) (Karasuyama et al. 1990, 1994). Signaling through the pre-BCR transiently down regulates expression of *Rag1/2* (Grawunder et al. 1995), induces a proliferative burst (Rolink et al. 1994) and triggers differentiation to the

small pre-B stage (Fraction D cells) (Kitamura et al. 1991, 1992). Small pre-B cells re-express *Rag1/2* and undergo rearrangement of their Ig light chain (*Igl*) locus (ten Boekel et al. 1995; Grawunder et al. 1995). Productive *Igl* rearrangement results in the expression of the B cell receptor (BCR) and progression to the immature B cell stage (Fraction E cells); these cells exit the BM and complete their development in the periphery (Loder et al. 1999).

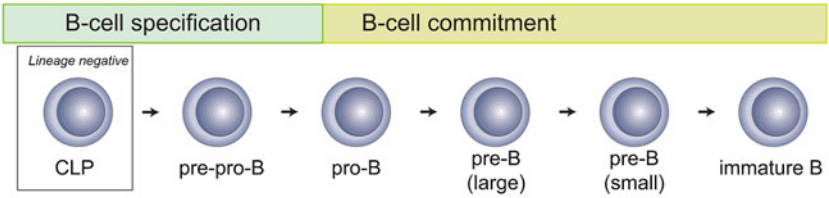
In addition to the productive rearrangements required to produce the pre-BCR, pre-B cell development in the mouse also requires signaling through the IL-7R, as mice lacking signaling components of the IL-7 receptor have few pre-B cells (Peschon et al. 1994; Clark et al. 2014). Similarly, absence of the signaling cascade components that follow IL-7R activation such as STAT5 (A and B) (Goetz et al. 2004; Yao et al. 2006), cyclin D3 (Cooper et al. 2006), and phosphoinositide 3-kinase (PI3K) (Ramadani et al. 2010; Fruman et al. 1999; Suzuki et al. 1999; Clayton et al. 2002; Jou et al. 2002; Okkenhaug et al. 2002) greatly attenuate the proliferation and survival of pre-B cells. Interestingly, mutation in ATP11c, a P4 ATPases (flippase) that is required for IL-7 signaling, results in a progressive loss of pro- and pre-B cells (Clark 2011; Pang and Nutt 2011; Siggs et al. 2011; Yabas et al. 2011). Clearly, efficient pre-B cell differentiation requires the coordination of the intrinsic cell differentiation program, appropriate recombination of the *Igh* and *Igl* genes, and responsiveness to extrinsic signals provided by cytokines (IL-7 in the mouse). In this review we highlight the key transcriptional regulators that control this process and discuss their deregulation in leukemia.

2 Transcriptional Regulation of Early B Cell Development

It is well known that the initiation of B cell development from the lymphoid progenitors relies on a transcriptional network consisting of three main transcription factors E2A (encoded by *Tcfe2a*), Ebf1 (*Ebf1*), and Pax5 (*Pax5*). These factors in turn activate a number of secondary factors that directly drive pre-B cell development. As E2A, Ebf1 and Pax5 have been studied in great detail and are the subject of numerous reviews (Cobaleda et al. 2007; Mandel and Grosschedl 2010; Murre 2007; Singh et al. 2007; Nutt and Kee 2007), here we provide only a brief introduction to these factors and concentrate on factors that have more recently been shown to be important in pre-B cell development (Fig. 1).

2.1 E2A

E2A is a basic helix-loop-helix (bHLH) transcription factor. The bHLH domain of E2A mediates dimerization and binding to the E-box motif in DNA. Alternative splicing of the E2A gene (*Tcfe2a*) gives rise to two isoforms, E12 and E47, which differ only in their bHLH domain. Mice deficient in *Tcfe2a* completely lack mature



	CLP	pre-pro-B	pro-B	pre-B (large)	pre-B (small)	immature B
PU.1	+	+	+	+	+	+
IRF8	++	+	+	+	+	+
Ikaros	+	+	+	++	+++	++
c-Myb	+	+	+	+	++	-
Runx1	+	+	+	+	+	+
Gfi1	+	+	++	+++	++	+
Gfi1b	+	+	+	+	+	+
Miz-1	++	+++	++	++	++	++
Spi-B	-	-	+	+	++	+++
IRF4	-	-	+	++	+++	+
Aiolos	-	-	+	+	++	+++
Foxo1	-	+	++	+	+++	+++
Bach2	-	-	+	+	+++	++
BLNK	-	++	+++	++	+++	++

Fig. 1 B cell development in the bone marrow of adult mice. Important progenitor stages are depicted from common lymphoid progenitor (CLP) to immature B cell, along with the expression of key transcription factors and proteins. Progenitors within the lineage-negative fractions of bone marrow are contained within the box. Expression levels are shown on an arbitrary scale; −, no or very low expression; +, ++, +++ low, intermediate or high relative expression respectively. Expression levels were derived from the Immunological Genome project (www.immgen.org) (Nutt et al. 2005; Vassen et al. 2007) and S.L.N. unpublished

B cells, indicating that E2A is essential for B cell development (Bain et al. 1994; Zhuang et al. 1994). Expression of *Tcfe2a* is upregulated at the CLP stage of development and remains high in pro-B, pre-B and immature B cells in the bone marrow (Kwon et al. 2008). Lymphoid defects in *Tcfe2a*$^{-/-}$ mice are already apparent at the LMPP and CLP stages of development, which are modestly reduced in number and display decreased priming of lymphoid gene expression (Borghesi et al. 2005; Dias et al. 2005, 2008). While E2A appears essential for the priming of the expression of many lymphoid transcripts (Mercer et al. 2011), the major development block occurs at the pre-pro-B cell stage due to a failure to upregulate a number of B cell-specific genes including *Ebf1* and *Pax5* (Bain et al. 1994; Seet et al. 2004). The progenitors from these mice do not express *Rag1* and consequently cannot undergo *Ig* gene rearrangement (Bain et al. 1994; Borghesi et al. 2005). Similarly, conditional deletion of *Tcfe2a* after B cell commitment leads to a breakdown of B cell gene expression and the loss of committed pro-B

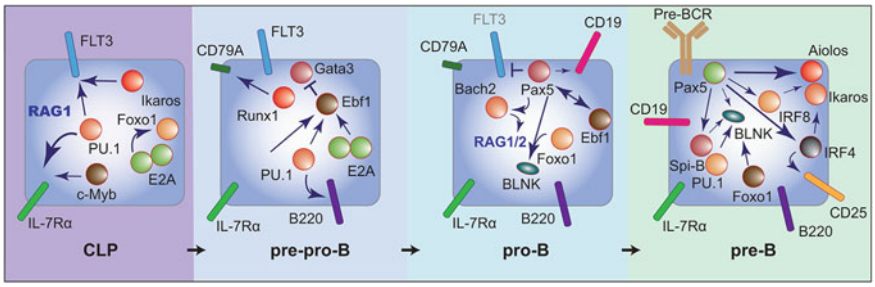


Fig. 2 Model for the transcription regulation of early B cell development. Key stages in early B cell differentiation are depicted, including the important transcription factors and cell surface receptors at each stage. *Arrows* indicate positive regulation, ⊥ indicates inhibition. *CLP* common lymphoid progenitors. Models are adapted from earlier work by (Medina et al. 2004; Nutt and Kee 2007; Singh et al. 2005)

cells (Kwon et al. 2008). E2A activity regulates a large number of genes in B cells, often in conjunction with Ebf1 and Pax5 (Lin et al. 2010). One important E2A target is Foxo1 that in turn then acts with E2A and HeLa E-box binding protein (HEB) to support B cell programming (Welinder et al. 2011) (Fig. 2). The role of Foxo1 will be discussed in further detail below.

2.2 Ebf1

Ebf1 is a zinc finger helix-loop-helix transcription factor that collaborates with E2A to initiate B cell gene expression. *Ebf1* is expressed at a low level in CLPs, but is greatly upregulated in pro-B cells (Mansson et al. 2008; Vilagos et al. 2012). Despite the phenotypic resemblance between the B cell developmental blocks of E2A and Ebf1-deficient mice (Bain et al. 1994; Lin and Grosschedl 1995), CLPs can develop in Ebf1-deficient mice but are unable to undergo B cell differentiation due to the reduced expression of B cell associated genes. Ebf1 regulates expressions of many genes that encode proteins required for B cell development including, Igα, VpreB, λ5 and Pax5 (Treiber et al. 2010; Mandel and Grosschedl 2010), while also repressing genes associated with alternative lineage fates (Nechanitzky et al. 2013). Conditional inactivation after the formation of pro-B cells confirmed the intrinsic requirement of Ebf1 in the earliest stages of B cell development (Gyory et al. 2012; Vilagos et al. 2012).

Overexpression of Ebf1 restricts the developmental potential of hematopoietic progenitors (Zhang et al. 2003; Pongubala et al. 2008) and partially rescues B cell development in the absence of E2A, PU.1, Ikaros and IL-7Rα (Dias et al. 2005; Medina et al. 2004; Reynaud et al. 2008; Seet et al. 2004). Careful investigation of the *Ebf1* promoters has revealed a complex regulatory network that acts to stabilize B cell expression (Roessler et al. 2007). Indeed, from a recent study, Ebf1

directly represses Gata3 by binding to the promoter region of *Gata3* locus, and induces recruitment of silencing modification proximal to the locus (Fig. 2). This highlights a new role of Ebf1 in suppressing T cell differentiation while allowing B cell differentiation in the presence of Pax5 (Banerjee et al. 2013).

2.3 Pax5

Unlike E2A and Ebf1, Pax5 is not required for the initial stages of B cell specification (Nutt et al. 1997, 1999). Pax5 is a member of the paired-box transcription factor family and is expressed in constant level throughout all B cell stages from the pro-B cell stage onwards until it is down regulated in plasma cells (Fuxa and Busslinger 2007). In the absence of *Pax5*, B cell development is arrested at the early pro-B cell stage of differentiation (Nutt et al. 1997). Strikingly, $Pax5^{-/-}$ pro-B cells are unable to differentiate into mature B cells but instead are capable of differentiating into a broad range of other hematopoietic cell types (Nutt et al. 1999; Rolink et al. 1999). Similar results were demonstrated by conditionally inactivating *Pax5* in pro-B cells (Mikkola et al. 2002). This was probably because Pax5 plays a crucial role in repressing genes, such as *Flt3*, *Notch1* and *Mcsfr* (*Csf1r*) that are associated with signaling in multipotent progenitors or non-B cell lineages (Delogu et al. 2006; Holmes et al. 2006, 2008; Souabni et al. 2002; Tagoh et al. 2006; Nutt et al. 1999; Pridans et al. 2008). Notably, a similar capacity for multilineage differentiation was reported for E2A and Ebf1-deficient lymphoid cell lines (Ikawa et al. 2004; Nechanitzky et al. 2013). This is because these cells lack high expression of markers of B cell specification as well as Pax5 (Ikawa et al. 2004).

Pax5 binds to thousands of genes in the B cell genome and plays an active role in regulating B cell chromatin (McManus et al. 2011; Revilla et al. 2012; Tagoh et al. 2006). The consequence of this copious DNA-binding is the direct regulation, both activation and repression, or hundreds of transcripts many of which are of central importance in B cell differentiation and function (Delogu et al. 2006; Pridans et al. 2008; Schebesta et al. 2007). Interestingly, a major function of Pax5 appear to be the activation of the expression of a suite of transcriptional regulators, including IRF4, IRF8, Spi-B, Aiolos and Bach2 (Fig. 2) that have important functions at the pre-B cell stage of development (Holmes et al. 2008; Pridans et al. 2008; Schebesta et al. 2007).

3 Other Players in Early B Cell Development

Besides the conventional co-transcriptional regulatory circuit of E2A, Ebf1, and Pax5 that functions to lock in progenitor cells to B cell fate, a number of transcription factors have been identified as playing important role in pro- and pre-B

cell development (see Fig. 1). This review highlights some of the new players involved in early B cell differentiation and malignancy.

3.1 PU.1 and Spi-B

Two members of the ETS domain transcription factor family, PU.1 and Spi-B have been implicated in early B cell development. PU.1 is encoded by the gene *Sfpi1* and is a critical regulator of hematopoiesis (reviewed by (Dakic et al. 2007)). Both germ line deletion and conditional inactivation of PU.1 in adult HSCs have demonstrated that PU.1 is required for the production of B cell progenitors from HSCs (Dakic et al. 2005; Scott et al. 1994). PU.1 is dynamically expressed throughout hematopoiesis with myeloid cell being characterized by high PU.1 expression, while B cells are uniformly PU.1 low (Back et al. 2005; Dakic et al. 2005). The relatively low expression of PU.1 is an essential requirement for B cell development, as high PU.1 diverts hematopoietic progenitors along the myeloid pathway (DeKoter and Singh 2000; Kueh et al. 2013).

PU.1 regulates FLT3 and IL-7Rα, two key cytokine receptors that are expressed by early lymphoid progenitors (Fig. 2) (DeKoter et al. 2002; Carotta et al. 2010). This regulation is likely to be important as mice lacking both receptors do not generate any B cell progenitors (Vosshenrich et al. 2003). In addition, CD45R, which encodes a common marker for B cells (B220) that is initially expressed in pre-pro B cells, is a direct target of PU.1 (Anderson et al. 2001). Despite the above-mentioned studies showing the regulatory roles of PU.1 in early B cell development, disruption of this transcription factor in CLPs demonstrated no effect (Iwasaki et al. 2005). In support of this observation, two other studies using specific deletion of PU.1 under the promoter of CD19 showed that PU.1 is not strictly essential beyond the pre-B cells in the bone marrow (Polli et al. 2005; Ye et al. 2005).

Spi-B, the ETS protein that is most closely related to PU.1 is also expressed in B cells, where it is under the control of Pax5 (Pridans et al. 2008; Schebesta et al. 2007). Absence of Spi-B only affects the maturation and the function of peripheral B cells while the early B cell development remains undisturbed (Su et al. 1997). The identical DNA binding specificity of Spi-B and PU.1 suggests that the loss of PU.1 function in B cells could be compensated for by Spi-B. In support of this idea, deficiencies in both PU.1 and Spi-B, results in a developmental block at the pre-B cells (Xu et al. 2012; Sokalski et al. 2011). While one identified target of PU.1 and Spi-B is the adaptor molecule BLNK (SLP65) that is important in pre-BCR signaling (Xu et al. 2012), the mechanism by which PU.1 and Spi-B fit into the network of B cell-specific transcription factor remains poorly understood.

3.2 *IRF4 and IRF8*

Interferon regulatory factor (IRF) 4 (also known as Pip, LSIRF, LCSAT, NF-EM5, and MUM1) is part of the IRF family of transcription factors. IRF4 plays a fundamental role in late B cell differentiation to promote *Ig* class switch recombination, germinal center formation and plasma cell differentiation (Mittrücker et al. 1997; Ochiai et al. 2013; Sciammas et al. 2006, 2011; Willis et al. 2014). Additionally, IRF4 has been demonstrated to be important for *Igκ* recombination and the attenuation of the IL-7 signaling pathway, thus promoting the transition from the pre-B to B cell stages of maturation (Clark et al. 2014; Johnson et al. 2008). The interaction between IRF4 and E2A enhances the binding affinity of E2A for the 3' *Igκ* enhancer region (Eκ3'). The knockdown of IRF4 in pre-B cells also reduces the histone acetylation at both *Eκ3'* and the intronic enhancer (*Eκi*), suggesting an important role of IRF4 in early B cell development (Lazorchak et al. 2006). IRF4 is also important for receptor editing in immature B cell stage to establish B cell tolerance (Pathak et al. 2008).

IRF8 (also known as ICSBP) is another IRF family transcription factor family member that is highly homologous to IRF4. Deficiency in IRF8 results in a reduction in CLP, which later accounted for the significant reduction in pre-pro-B cells (Wang et al. 2008). The decreased commitment of CLPs to pre-pro-B cells was found to be associated with the reduced expression of B cell-specific transcription factors such as E2A, Ebf1, and Pax5. Interestingly, IRF8 and PU.1 have been shown to synergistically regulate *Ebf1* expression (Wang et al. 2008).

IRF4 and 8 bind very weakly to DNA containing only IRF sites, but are recruited to their binding sites via interaction with other transcription factors. In particular, PU.1 and Spi-B have been shown to recruit IRF4 or IRF8 to ETS-IRF composite elements (EICE) located in the *Eκ3'* and *Igλ* enhancers (Brass et al. 1999; Eisenbeis et al. 1995; Escalante et al. 2002; Pongubala et al. 1992). Due to their extensive homology, IRF4 and IRF8 were suggested to function redundantly. Indeed, double deficiencies in IRF4 and IRF8 resulted in a development arrest at the pre-B cell stage (Lu et al. 2003). The pre-B cells in the bone marrow of these double mutant mice are hyperproliferative and express high level of pre-BCR. Interestingly, these cells are also defective in *Igl* gene rearrangement and transcription, and restoration of either IRF could rescue the early development of B cells (Ma et al. 2008; Lu et al. 2003). In keeping with the molecular interaction between the IRFs and PU.1/Spi-B, we have found that B cell development in IRF4 and PU.1 double deficient mice also blocks at the pre-B cell stage (S.H.M.P., S.C., and S.L.N. submitted). Interestingly, IRF4 and 8 have been shown to induce the expression of two closely related transcription factors, Ikaros and Aiolos, that promoted the expansion of the pre-B cell numbers (see below; Ma et al. 2008).

3.3 Ikaros and Aiolos

The zinc finger transcription factors, Ikaros (encoded by *Ikzf1*) and Aiolos (*Ikzf3*) are transcriptional regulators that play multiple roles in B cell development and function (John and Ward 2011). The absence of pre-pro-B cells in Ikaros-deficient mice and the reduction of these B cell progenitors in mice bearing hypomorphic alleles of Ikaros suggested a defect in lymphoid priming (Kirstetter et al. 2002; Wang et al. 1996). Indeed, LMPP of Ikaros-deficient mice exhibited lower levels of *Il7r* and *Rag1* expression, which is important for B cell priming and specification (Yoshida et al. 2006). Strikingly, similar to $Pax5^{-/-}$ pro-B cells, $Ikzf1^{-/-}$ pro-B cells (rescued by ectopic expression of Ebf1) are able to differentiate into myeloid cells indicating that Ikaros is restricting lymphoid progenitors to the B cell fate (Reynaud et al. 2008).

Ikaros binds to a number of genes required for pre-BCR signaling, *Ig* gene recombination, cell growth, adhesion and proliferation (Ferreiros-Vidal et al. 2013; Schwickert et al. 2014). Strikingly, by using specific deletion of Ikaros in pro/pre-B cells, Ikaros activates a transcriptional event essential for BCR signaling by attenuating IL-7 signals for B cell differentiation (Heizmann et al. 2013; Schwickert et al. 2014). Ikaros was also found, using a slightly different model, to be critical in pre-B cells during the transition from stroma-adherent proliferative stage to non-adherent differentiation stage. Loss of Ikaros locks pre-B cells with enhanced integrin signaling and highly proliferative stage (Joshi et al. 2014). Similarly, Ikaros also promotes the migration of pro-B cells and simultaneously prevents cell adhesion in early B cell development (Schwickert et al. 2014).

Aiolos is expressed throughout B cell development from the pre-pro-B cell stage where it is under the control of Pax5 (Fig. 1) (Pridans et al. 2008; Schebesta et al. 2007). Aiolos-deficient mice have relatively normal B cell development, however Aiolos has been shown to play roles in the silencing of the *Igll1* gene (encoding $\lambda 5$) in pre-B cells after pre-BCR signaling (Thompson et al. 2007; Karnowski et al. 2008). Indeed, this mechanism correlates with the expression of *Ikzf3* being highly upregulated in response to pre-BCR signals (Ferreiros-Vidal et al. 2013). Ikaros and Aiolos can form both hetero- and homodimers and in keeping with this, genome wide studies revealed that Ikaros and Aiolos share many target genes, including the B cell associated genes such as *Cd79b*, *Foxo1, Blnk* and *Syk* (Ferreiros-Vidal et al. 2013) implicated in pre-BCR signaling, cell cycle regulation and somatic rearrangement of *Ig* genes. Interestingly, there is significant enrichment of Ikaros binding sites within regulatory regions that also bind Ebf1, E2A, Pax5 and Foxo1, further reinforcing the notion that B cell development is initiated and stabilized by a combinatorial transcriptional network (Ferreiros-Vidal et al. 2013; Lin et al. 2010; Revilla et al. 2012).

3.4 c-Myb, Gfi1 and Miz-1; Regulators of IL-7 Signaling

Signaling through the IL-7R is essential for early B-lymphopoiesis in the mouse, although the mechanisms by which this signal is regulated are complex and only partially understood. In addition to the previously discussed roles of PU.1 in activating *Il7r* expression (DeKoter et al. 2002) and STAT5A/B (Malin et al. 2010) in transducing the signal three other transcription factors, c-Myb, Gfi1, and Miz-1 are implicated.

c-Myb has long been known to be essential for hematopoiesis, however, its function in B cell development has only been appreciated more recently (Greig et al. 2008). Mice bearing hypomorphic *c-Myb* alleles displayed profound reduction in the B cell compartment (Emambokus et al. 2003; Carpinelli et al. 2004; Sandberg et al. 2005; Xiao et al. 2007). In addition, conditional deletion of c-Myb specifically in the B cell lineage demonstrating a direct requirement of c-Myb in developing pro-B cells (Fahl et al. 2009; Greig et al. 2010). c-Myb was further demonstrated to be required for lymphoid priming before the CLP stage, and also to maintain normal expression of IL-7R in pro-B cells (Greig et al. 2010). A recent study has suggested that both Ebf1 and c-Myb repress *Rag1/2* transcription by negatively regulating the binding of Foxo1 to the *Rag* locus during the transition between large pre-B to small pre-B cells (Timblin and Schlissel 2013). While the role of c-Myb in early B cell development has been slowly elucidated, its collaborating role with other transcription factors such as PU.1, E2A, Ikaros, Runx1—to mention a few, remains poorly understood. Nevertheless, c-Myb has been shown to synergise with PU.1 to activate *Il7r* transcription (Greig et al. 2010).

Gfi1 is a zinc finger containing repressor that plays an important role in early lymphopoiesis (Moroy and Khandanpour 2011). Gfi1-deficient mice have a reduced CLP compartment and few pre-pro-B and pro-B cells, a phenotype that resembles both mice lacking the IL-7R or harboring c-Myb hypomorphic alleles (Moroy and Khandanpour 2011). Interestingly, Gfi1 has been shown to inhibit PU.1 activity in hematopoietic progenitors capable of both lymphoid and myeloid differentiation, thus promoting the B cell fate (Spooner et al. 2009). As Ikaros is thought to be upstream of *Gfi1*, this finding provides a mechanism by which Ikaros promotes lymphopoiesis. The highly related gene *Gfi1b* is also expressed in developing B cells and while the degree of redundancy of these factors remains to be fully determined, mice lacking both factors have a more severe block in early B cell development than that observed for Gfi1 knockouts alone (Schulz et al. 2012). Gfi1b has been implicated in pre-B cell differentiation where it represses *Rag1/2* expression through both direct binding to the shared *Rag* enhancer and indirect repression of *Foxo1* (Schulz et al. 2012).

Miz-1 is a BTB/POZ domain transcription factor that has been implicated in cell cycle control and the inhibition of apoptosis (Moroy et al. 2011). Miz-1 is an important regulator of IL-7 signaling, with B-lymphopoiesis blocked at the pre-pro-B cell stage in its absence. Miz-1-deficient CLPs express normal amounts of the IL-7R but fail to adequately transduce the required survival and proliferation signals

(Kosan et al. 2010). Interestingly, Miz1-deficient progenitors have increased SOCS1 and decreased Bcl2, potentially explaining the high apoptotic rate and inability to respond to IL-7. Ebf1 may also act downstream of Miz1, as ectopic Ebf1 and Bcl2 can partially rescue B cell development in the absence of Miz-1 (Kosan et al. 2010).

3.5 Runx1

Runx1 (also known as acute myeloid leukemia 1 (AML1)) encodes a transcription factor belonging to the highly conserved family of DNA-binding proteins that contain a *Runt* homology domain. It forms a heterodimeric complex with a core non-DNA-binding factor (Cbf) which is essential for hematopoiesis (Speck and Gilliland 2002). The expression of Runx1 remains constant throughout B cell development (Lorsbach et al. 2004). By using a conditional knockout model, Runx1 was shown to be indispensable in generating CLPs (Growney et al. 2005). The function of Runx1 was further analyzed using a conditional deletion of Runx1 specifically in B cells, pinpointing its role during the transition of pre-pro-B cells to pro-B cells (Seo et al. 2012). Interestingly, expression of Ebf1 was able to partially rescue the phenotype, indicating that Runx1 serves as an upstream regulator of *Ebf1* activation. (Seo et al. 2012) Together with this, it was also shown that Runx1 cooperates with Ebf1 and Pax5 to synergistically activate *mb1* expression (encoding Igα), thus allowing pre-B cell signaling to occur (Maier et al. 2004).

A recent study further elucidated the role of Runx1 in early B cell progenitors (Niebuhr et al. 2013a). This study suggested that Runx1 has no role in B cell specification but rather their survival or subsequent development. Strikingly, overexpression of Bcl2 rescued the survival of the B cell progenitors. *Lyn*, *Spib* and *Aiolos* were identified as target genes (with the two latter being upregulated), suggesting that the Runx1 regulation of *Lyn* was critical for IL-7 and pre-BCR stimulation in pre-B cells (Niebuhr et al. 2013b). Spi-B and Runx1 share several target genes, suggesting these two transcription factors may cooperate together to regulate the genes necessary for pre-B cell transition. Aiolos, on the other hand, is required to silence *Igll1* gene in pre-B cells after pre-BCR signaling (Thompson et al. 2007), demonstrating the importance of Runx1 repression of Aiolos during the pre-B cell transition (Niebuhr et al. 2013a).

3.6 Foxo1

Foxo1 is part of the forkhead O (Foxo) transcription factor family that acts downstream of phosphatidylinositol-3-OH kinase [PI(3)K] pathway, which is critical in both B cell development and the maturation and function of peripheral B

cells. Phosphorylation of the Foxo proteins by Akt induces their nuclear export and consequent inactivation of the transcriptional activity (Calnan and Brunet 2008), which is important for subsequent early B cell differentiation (Herzog et al. 2009). Deficiency of Foxo1 in B cells revealed a partial developmental arrest at the pro-B cells. The pro-B cells exhibited reduced expressions of *Il7r* that led to apoptosis, and of *Rag1* and *Rag2,* which led to impaired *Igh* rearrangement (Dengler et al. 2008). It has been suggested that Foxo1 and another Forkhead P transcription family member, FoxP1, both regulate *Rag1/2* expression (Amin and Schlissel 2008; Dengler et al. 2008; Herzog et al. 2009; Hu et al. 2006). It has also been recently shown that attenuation of IL-7 signaling results in induction of Foxo1, which in turns activates the expression of Blnk and Syk thus enabling the differentiation signaling functions of the pre-BCR (Ochiai et al. 2012). This suggest a feedforward mechanism whereby Blnk inhibits IL-7 signaling (Herzog et al. 2009), thereby promoting its own expression via Foxo1, Pax5 (Ochiai et al. 2012) and possibly PU.1/Spi-B (Xu et al. 2012).

3.7 Bcl6 and Bach2

Bcl6 is a transcriptional repressor that is well known for its essential role in germinal center B cells. One primary function of Bcl6 in germinal centers is to protect the cells from the pro-apoptotic effects of the DNA damage response to allow somatic hypermutation and class switch recombination (Basso and Dalla-Favera 2012). Recently, Bcl6 has also been shown to play a similar role in pre-B cells. *Bcl6* expression in the bone marrow was repressed by IL-7R signaling, but activated by successful pre-BCR recombination and signaling (Duy et al. 2010). Bcl6 then functions to protect the pre-B cells from the DNA damage induced apoptosis associated with Igκ gene recombination, as well as to promote pre-B cell quiescence (Nahar et al. 2011). In keeping with this finding, Bcl6-deficient mice showed a reduction in both the number and clonal diversity of pre-B cells.

BTB and CNC homology 2 (Bach2) is a B cell-specific transcription factor, which also is required for class switch recombination and somatic hypermutation in B cells as well as for efficient formation of germinal centers (Muto et al. 2004). Pax5 activates *Bach2* expression in developing B cells (Schebesta et al. 2007) where it plays an important role in regulating the pre-BCR checkpoint (Swaminathan et al. 2013b). Bach2 is crucial for negative selection of pre-B cells that failed to productively rearrange VDJ gene segments of the *Igh* by directly regulating the transcription of *Rag1/2* (Swaminathan et al. 2013b). Recent molecular studies demonstrate that Bach and Bcl6 have competing and opposing functions in the pre-BCR checkpoint and suggest that this interaction is important to prevent leukemogenesis (Swaminathan et al. 2013a).

4 Transcription Factors and Their Association with B Cell Acute Lymphoblastic Leukemia

Given their high proliferative potential and sequential *Ig* gene rearrangements requiring Rag1/2 activity, it is not surprising that pre-B cells are the source of one of the most common human leukemias, precursor-B/B cell acute lymphoblastic leukemia (collectively termed here B-ALL). Even though bone marrow B cell differentiation has been extensively studied for three decades, it has only more recently become apparent that mutations in the transcriptional regulators of pre-B cell development are also major players in pre-B cell malignancies.

The involvement of pre-B cell transcriptional regulators in B-ALL pathogenesis is highlighted by the finding that factors such as *PAX5, IKZF1, IKZF3, TCF3 (E2A), LEF1* and *EBF1* are commonly mutated in B-ALL (Mullighan et al. 2007). *PAX5,* for example, is mutated in 30–50 % of B-ALL cohorts through a variety of mechanisms including deletions, translocations, and point mutations. More recently, a *PAX5* mutation has also been shown to be associated with familial B-ALL (Shah et al. 2013). While the *PAX5* mutations are thought to be pivotal to the initial leukemogenesis, deletions and point mutations in *IKZF1* and *IKZF3* account for 10–15 and 2 % of the B-ALL cases respectively (Kuiper et al. 2007; Mullighan et al. 2007). Notably, genetic alterations in *IKZF1* are associated with a poor clinical outcome and act as a strong predictor of relapse (Mullighan et al. 2009; Kuiper et al. 2010). Similarly, deletions of *BACH2* have been found in 32 % of B-ALL cases (Merup et al. 1998), and lower-than-median expression levels of *BACH2* define patients with the worse clinical outcome (Swaminathan et al. 2013b). On the other hand, mutations in *FOXO1* are often associated with diffuse large B cell lymphoma (DLBCL), but have not been implicated in B-ALL (Trinh et al. 2013). Interestingly, the mutations of essentially all these factors appear to occur only on one allele, suggesting that these transcription factors are haploinsufficient tumor suppressors.

While the human studies have clearly shown that the major transcriptional regulators of pre-B cell development are also tumor suppressors, it has proven difficult to gain a molecular understanding of the process. One difficulty is that mice heterozygous for any of these factors do not spontaneously develop B-ALL, suggesting that other cooperating mutations are required. This possibility has been supported by the finding that mice that are heterozygous for either *Pax5* or *Ebf1* develop B-ALL only when they also harbor a constitutively active form of STAT5 (Heltemes-Harris et al. 2011).

The interaction between the ETS family transcription factors, PU.1 and Spi-B and the IRF family members IRF4 and IRF8 are also implicated in B-ALL. For example the compound loss of both PU.1 and Spi-B in B cell progenitors results in a developmental arrest at the pre-B cell stage. This block eventually leads to leukemia at a high frequency that closely resembles B-ALL in humans (Sokalski et al. 2011). Interestingly, *Blnk* was identified as a downstream target of both PU.1 and Spi-B (Sokalski et al. 2011; Xu et al. 2012), an important finding as Blnk-

deficiency is sufficient to induce B-ALL in mice (Jumaa et al. 2003), and mutation or aberrant mRNA splicing of *BLNK* is associated with B-ALL (Mullighan et al. 2007, 2009).

In keeping with the interaction of ETS and IRF family members during lymphopoiesis, it has been similarly demonstrated by the deficiencies of IRF4 and IRF8, which produce a developmental block at the pre-B cell stage (Lu et al. 2003), result in B-ALL at a high frequency (Jo et al. 2010). Moreover, while *Irf4*$^{-/-}$ mice do not develop B-ALL, IRF4 deficiency cooperates with oncogenes such as BCR-Abl and c-Myc to promote leukemogenesis in mouse models (Acquaviva et al. 2008; Pathak et al. 2008). We have recently extended these findings to show that mice deficient in either PU.1 and IRF4 or PU.1 and IRF8 develop B-ALL at high frequency. These B-ALLs show low expression of *Blnk*, *Spib* and *Ikaros*, suggesting that the ETS/IRF complexes function as tumor suppressors by regulating these important target genes (S.H.M.P., S.C. and S.L.N. submitted).

The importance of the ETS/IRF interaction in human B-ALL is only now emerging. Rare mutations in *SPI1* (*PU.1*) and *IRF8* have been found in human B-ALL (Mullighan et al. 2011; Zhang et al. 2011) and DLBCL (Bouamar et al. 2013), while *SPIB* expression is reduced in pre-B-ALL carrying the t(12;21) *ETV6-RUNX1* translocation (Niebuhr et al. 2013a). IRF4 has been implicated in several B cell malignancies, including chronic lymphocytic leukemia (Shukla et al. 2013) and multiple myeloma (Shaffer et al. 2008). *IRF4* was also recently reported to be 2-fold overexpressed in pediatric B-ALL compared to unfractionated healthy BM (Adamaki et al. 2013), a finding that contrasts with our own analysis of a large cohort of B-ALL samples that suggests that *IRF4*, as well as *SPI-B* expression, is uniformly reduced in B-ALL (S.H.M.P., S.C. and S.L.N. submitted).

RUNX1 is also a major target for mutation in B-ALL through translocation. The most prevalent translocation involving *RUNX1* is the *ETV6-RUNX1* (encoding the TEL-AML1 protein) that represents the most common genetic subtype in B-ALL (25 %). Genome-wide studies have identified additional genetic alterations in this subtype of ALL, including B cell-specific transcription factors, *PAX5* and *EBF1*, and deletion of second copy of *ETV6* (Mullighan et al. 2007; Parker et al. 2008; Kuiper et al. 2007). Recent analysis of gene expression in a large number of cases of B-ALL showed that reduced *IKZF3* and *SPI-B* correlated with the *ETV6-RUNX1* translocation (Niebuhr et al. 2013a).

5 Conclusions

While our understanding of the mechanisms by which the transcription factor triad of E2A/EBF1/PAX5 acts to specify the earliest stages of B cell development from hematopoietic progenitors is relatively advanced, less is known about how

committed progenitors subsequently differentiate down the B cell developmental pathway. Recent advances show that a complex mix of secondary factors, including members of the Ikaros, ETS, Runx and IRF families act often downstream of E2A, EBF1 or PAX5 to coordinate the differentiation process (Fig. 2). Further genome wide studies of the binding sites for these secondary determinants, as well as studies of the alterations in nuclear structure and the epigenetic landscape will aid in developing a robust model of the gene regulatory network for early B cell differentiation. Interestingly, for most of the past three decades research into the transcriptional controls of pre-B cell differentiation and that investigating acute leukemia formation showed little overlap, however the explosion in cancer genome information has demonstrated that mutations in most of the transcriptional regulator of pre-B cell development are key drivers of the oncogenic process. Thus the promotion of normal differentiation and tumor suppression are intimately linked at the pre-B cell stage of B-lymphopoiesis.

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Transcription Factors Controlling Innate Lymphoid Cell Fate Decisions

Christoph S. N. Klose and Andreas Diefenbach

Abstract The mucosal epithelium is in direct contact with symbiotic and pathogenic microorganisms. Therefore, the mucosal surface is the principal portal of entry for invading pathogens and immune cells accumulated in the intestine to prevent infections. In addition to these conventional immune system functions, it has become clear that immune cells during steady-state continuously integrate microbial and nutrient-derived signals from the environment to support organ homeostasis. A major role in both processes is played by a recently discovered group of lymphocytes referred to as innate lymphoid cells (ILCs) that are specifically enriched at mucosal surfaces but are rather rare in secondary lymphoid organs. In analogy to the dichotomy between CD8 and CD4 T cells, we propose to classify ILCs into interleukin-7 receptor α-negative cytotoxic ILCs and IL-7Rα^+ helper-like ILCs. Dysregulated immune responses triggered by the various ILC subsets have been linked to inflammatory diseases such as inflammatory bowel disease, atopic dermatitis and airway hyperresponsiveness. Here, we will review recent progress in determining the transcriptional and developmental programs that control ILC fate decisions.

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Contents

1 Innate Lymphoid Cells

Like B and T cells, innate lymphoid cells (ILCs) belong to the lymphoid lineage of hematopoietic cells. However, in contrast to B and T cells, ILCs lack the expression of rearranged antigen receptors and, therefore, are considered to be part of the innate immune system. Their existence has not been uncovered until recently, because ILCs are mainly localized at mucosal surfaces and, with the exception of conventional NK (cNK) cells, ILCs are very rare in secondary lymphoid organs. Although ILCs lack antigen receptor rearrangement, the basic transcriptional programs, guiding their fate decisions, are to a large degree evolutionary conserved (Tanriver and Diefenbach 2014) and they have often been compared to the one of T helper (Th) cell subsets (Spits et al. 2013; Walker et al. 2013).

Based on three lines of evidence which are described in more detail in the following chapters, we have recently proposed to subdivide ILCs into cytotoxic or

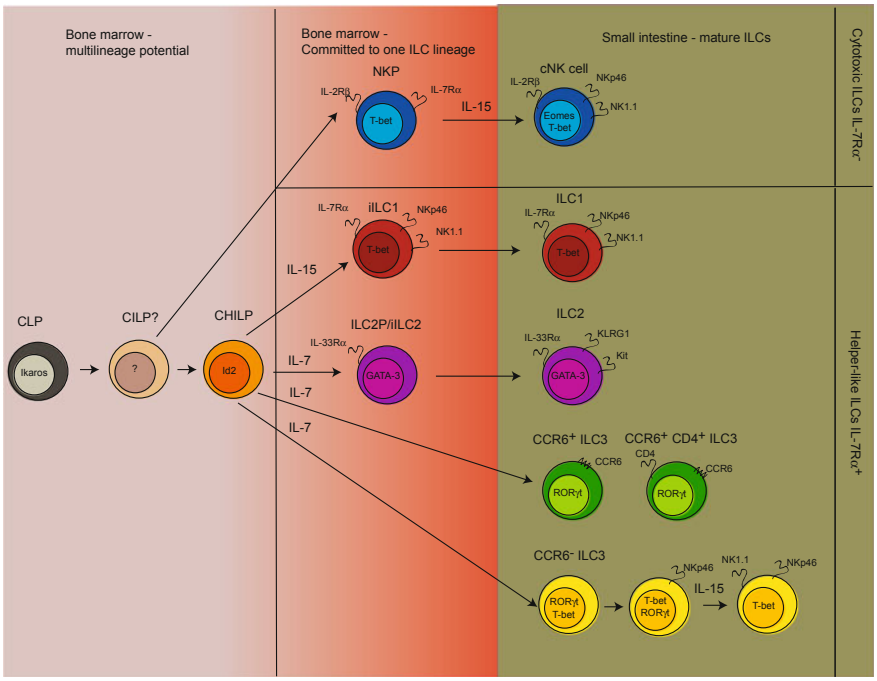

Fig. 1 ILC differentiation

killer ILCs (i.e., cNK cells) and into helper-like ILCs such as ILC1s, ILC2s, and ILC3s (Fig. 1) (Klose et al. 2014), thereby providing a mirror image among innate lymphocytes of the dichotomy between cytotoxic CD8 versus helper CD4 T cells. (1) A recently described progenitor to ILCs gives rise to all helper-like ILCs but not to cNK cells (Klose et al. 2014; Constantinides et al. 2014). (2) Helper-like ILCs but not cNK cells express the interleukin 7 receptor α chain (IL-7Rα or CD127) and depend on the transcription factor GATA-3 for development (Klose et al. 2014; Yagi et al. 2014; Samson et al. 2003; Hoyler et al. 2012; Serafini et al. 2014). (3) The expression pattern of the related T-box transcription factors Eomes and T-bet among helper-like ILCs (T-bet in ILC1s) and cNK cells (co-expression of Eomes and T-bet) (Klose et al. 2014; Daussy et al. 2014; Gordon et al. 2012) parallels the pattern found in helper ($T\text{-}bet^{+}$ $Eomes^{-}$) *versus* cytotoxic T cells ($T\text{-}bet^{+}$ $Eomes^{+}$) (Intlekofer et al. 2005; Pearce et al. 2003). Conventional NK cells are the only known member of the group of cytotoxic ILCs. Their transcriptional regulation is described in detail in Chap. "Transcriptional control of NK cell development and function" of this book and is, therefore, not discussed here.

The classification of helper-like ILCs into three groups is based on their cytokine expression profile that is controlled by key transcription factors (Fig. 1) (Spits et al. 2013). ILC1s similar to Th1 cells express the transcription factor T-bet and secrete IFN-γ. In contrast, and in parallel to Th2 cells, ILC2s express high

levels of GATA-3 which drives the secretion of cytokines such as IL-5, IL-9, and IL-13. ILC3s depend on the transcription factor RORγt. Two different ILC3 group members have been identified based on the expression of CCR6, both of which express RORγt (Klose et al. 2013; Sawa et al. 2010). $CCR6^+$ ILC3s include the previously described lymphoid tissue inducer (LTi) cells (Mebius et al. 1997). LTi cells are essential for the generation of lymph nodes and Peyer's patches and are localized in adult mice preferentially in lymphoid structures in the gut called cryptopatches (CP) and isolated lymphoid follicles (ILFs) (Eberl et al. 2004). $CCR6^+$ ILC3s secrete cytokines such as IL-17A and IL-22 but do not express activating NK cell receptors such as NKp46, NKG2D, or NK1.1 (Takatori et al. 2009; Zenewicz et al. 2008; Cupedo et al. 2009). In sharp contrast, $CCR6^{-/low}$ ILC3s co-express T-bet in addition to RORγt. T-bet expression is required for the differentiation of $CCR6^{-/low}$ ILC3s into NK cell receptor-expressing (i.e., NKp46, NKG2D) ILC3s and for their production of IFN-γ (Klose et al. 2013; Rankin et al. 2013; Sciume et al. 2012a; Bernink et al. 2013). During these differentiation steps, T-bet expression increases and guides a transcriptional program which downregulates RORγt and IL-22 expression allowing for functional plasticity. This enigmatic conversion of ILC3s and the transcriptional regulation of other ILC fates is discussed in the following chapters (Vonarbourg et al. 2010; Bernink et al. 2013).

2 Id2 and GATA-3 Are Central Transcriptional Hubs for the Early Commitment to the Helper-Like ILC Fate

2.1 Regulation of Early Lymphocyte Differentiation by Id2

Hematopoietic cell development from a hematopoietic stem cell (HSC) to immune effector cells is characterized by progressive loss of proliferative and differentiation potential and a gain in effector functions. Irreversible commitment to a certain hematopoietic cell lineage is controlled by the action of one or more transcription factors which determine cell fate by direct or indirect regulation of target gene expression and by coordinating epigenetic changes.

A crucial branchpoint in hematopoiesis is the commitment to either the lymphoid or myeloid lineage represented by the differentiation of HSC into a common lymphoid precursor (CLP) (Kondo et al. 1997) or the common myeloid precursor (CMP) (Akashi et al. 2000), respectively. The CLP has the potential to differentiate into all known lymphoid lineages (i.e., T and B cells of the adaptive immune system and cytotoxic and helper-like ILCs) (Moro et al. 2010; Yang et al. 2011; Klose et al. 2014). The further separation between adaptive and innate lymphocytes is regulated by the E-family of transcription factors and their repressors, inhibitors of DNA binding (Id) (Murre 2005; Kee 2009; de Pooter and Kee 2010). E-proteins possess a helix-loop-helix (HLH) domain, allowing for dimerization of E proteins. A basic HLH DNA binding domain and two transcriptional regulation domains allow binding to chromatin and regulate transcription of target genes.

E-proteins bind as dimers to the respective E-box sequence within their promoter or enhancer regions of genes. Id proteins lack the basic HLH domain, which allows DNA-binding but still possess a HLH domain for dimerization. Id proteins heterodimerize with E-proteins impairing their DNA binding and E-protein-mediated gene regulation (Murre 2005; Kee 2009). Expression of the E-protein family member E2A instructs specification of lymphoid progenitors to the B cell fate. E2A together with IL-7R signaling induces the expression of EBF-1 (early B cell factor-1), a pioneer transcription factor and a potent repressor of Id2 allowing for increased expression of E2A (Lin and Grosschedl 1995; Treiber et al. 2010). While repressing Id2, EBF-1 promotes expression of Pax5 widely considered as the "master regulator" of B cell fate (Nutt et al. 1999). Interestingly, deletion of EBF-1 in late pro-B cells reverted B cell commitment and reprogrammed EBF-1-deficient pro-B cells into T cells and helper-like ILCs (Nechanitzky et al. 2013). These data assign an important function to EBF-1 as a guardian of the B cell fate, which is at least in part mediated by the repression of Id2.

Another line of evidence supporting the notion that Id2 is a central hub for ILC fate came from the analysis of Id2-deficient mice. $Id2^{-/-}$ mice could not generate ILCs including cNK cells and helper-like ILCs (i.e., ILC1s, ILC2s, and ILC3s), whereas the differentiation of B and T cells was largely unperturbed (Table 1) (Yokota et al. 1999; Moro et al. 2010; Hoyler et al. 2012). Therefore, it has been proposed that ILCs may develop from a common precursor which expresses Id2 and is developmentally dependent on Id2 (Sanos et al. 2011; Spits et al. 2013; Spits and Di Santo 2011). Indeed, investigation of Id2 expression in ILCs using Id2 reporter mice revealed that Id2 is indeed expressed in all ILCs and in lineage-specified ILC progenitors (or immature ILCs), whereas B cells and T cells have no or very low level Id2 expression, respectively (Hoyler et al. 2012; Boos et al. 2007; Carotta et al. 2011; Tachibana et al. 2011; Cherrier et al. 2012). Interestingly, cNK cells depicted lower expression of Id2 compared to ILC2s or ILC3s (Hoyler et al. 2012) and the refined NK cell precursor (rNKP) was largely Id2-negative (Klose et al. 2014) suggesting that Id2 expression is engaged after commitment to the cNK cell lineage. These data provide additional evidence for an early bifurcation between the cytotoxic arm of ILCs (i.e., cNK cells) and helper-like ILCs (IL-7Rα-expressing ILC1s, ILC2s, and ILC3s).

2.2 A Common Progenitor to All Helper-Like ILCs

As all ILCs express Id2 and are developmentally dependent on it, the existence of a common ILC precursor (variably called CILP or ILCP) has been proposed (Sanos et al. 2011; Spits et al. 2013; Spits and Di Santo 2011; Mortha and Diefenbach 2011). Recently, two groups independently reported two populations of Id2-expressing lymphoid-restricted progenitors in the bone marrow and in the fetal liver that were committed to the ILC fate (i.e., could not differentiate into B cells, T cells, or myeloid cells) but, as a population in vivo and on a single cell

Table 1 Transcription factors affecting ILC development

	cNK	ILC1	ILC2	CCR6^{+} ILC3	CCR6$^{-/low}$ ILC3	References
Id2	↓	?	↓	↓	↓	(Moro et al. 2010; Yokota et al. 1999)
GATA-3	M	↓	↓	↓	↓	(Hoyler et al. 2012; Yagi et al. 2014; Klose et al. 2014)
PLZF	→	?	↓	→	→	(Constantinides et al. 2014)
Eomes	↓	→	→	→	→	(Gordon et al. 2012; Klose et al. 2014)
Nfil3	↓	↓	?	?	?	(Kamizono et al. 2009; Gascoyne et al. 2009; Klose et al. 2014)
RORγt	→	→	→	↓	↓	(Eberl et al. 2004; Satoh-Takayama et al. 2008; Sanos et al. 2009)
T-bet	M	↓	→	→	M	(Townsend et al. 2004; Rankin et al. 2013; Klose et al. 2013, 2014)
RORα	?	?	↓	→	→	(Halim et al. 2012b; Wong et al. 2012)
TCF-1	?	?	↓	→	↓	(Mielke et al. 2013; Yang et al. 2013; Malhotra et al. 2013)
Notch	?	?	?	M	M	(Lee et al. 2012; Rankin et al. 2013; Mielke et al. 2013; Cherrier et al. 2012)
Gfi1	?	?	↓	?	?	(Spooner et al. 2013)
Tox	↓	?	?	↓	?	(Aliahmad et al. 2010)
Ahr	M	→	→	M	↓	(Kiss et al. 2011; Lee et al. 2012; Qiu et al. 2011)
Runx1	?	?	?	↓	?	(Tachibana et al. 2011)

↓: Absence or strong reduction
→: No effect reported
M: Cells are present but have a maturation defect or are functionally impaired
?: Unknown

level in vitro, had the potential to differentiate into various helper-like ILC lineages (Klose et al. 2014; Constantinides et al. 2014). These populations carried markers of early hematopoietic development such as IL-7Rα (CD127), Kit (CD117), 2B4 (CD244) and CD27 but lacked expression of Flt3 and CD93, markers expressed by the CLP (Karsunky et al. 2003, 2008). In contrast to CLPs, this lymphoid progenitor population expressed Id2 and integrin $\alpha_4\beta_7$, an integrin involved in the migration to mucosal surfaces (Holzmann et al. 1989; Wagner et al. 1996) and also found on ILC2s (Hoyler et al. 2012) and ILC3 progenitors (Possot et al. 2011; Cherrier et al. 2012). Lineage^{-} Id2^{+} IL-7Rα^{+} $\alpha_4\beta_7^{+}$ Flt3^{-} CD25^{-} cells lacked expression of ILC lineage-specifying transcription factors

such as RORγt, T-bet, Eomes and had low expression of GATA-3 arguing that these cells are not yet committed to a distinct ILC lineage (Klose et al. 2014; Constantinides et al. 2014).

Upon adoptive transfer of Lin$^-$ Id2$^+$ IL-7Rα^+ $\alpha_4\beta_7^+$ Flt3$^-$ CD25$^-$ cells into alymphoid mice, this population had no appreciable T, B, or myeloid cell potential but differentiated into functional ILC2s, ILC3s, and into a peculiar, intestinal mucosa and liver-resident Eomes$^-$ T-bet$^+$ NKp46$^+$ NK1.1$^+$ ILC lineage, distinct of cNK cells (Klose et al. 2014). These data indicate that another branching point upstream of this progenitor may exist that is marked by a progenitor still having cNK cell potential, the common ILC progenitor (CILP) (Fig. 1). These data also provide evidence for partially distinct pathways of cNK cell and helper-like ILC development. Limiting dilution and single cell assays in vitro demonstrated that a single Lin$^-$ Id2$^+$ IL-7Rα^+ $\alpha_4\beta_7^+$ Flt3$^-$ CD25$^-$ cell had the potential to give rise to all three ILC lineages. Hence, Lin$^-$ Id2$^+$ IL-7Rα^+ $\alpha_4\beta_7^+$ Flt3$^-$ CD25$^-$ cells are now referred to as the common helper-like ILC progenitor (CHILP) (Klose et al. 2014; Constantinides et al. 2014). These data now create new opportunities to scrutinize the molecular signals required for the specification of the various helper-like ILC lineages from the CHILP.

Interestingly, the CHILP population contained a promyelocytic leukemia zinc finger protein (PLZF)$^-$ and a PLZF$^+$ subset (Klose et al. 2014). PLZF (also known as ZBTB16) is a transcription factor of the BTB-POZ zinc finger family of transcription factors known to be important for NKT cell development and for the innate behavior of CD4 T cells (Kovalovsky et al. 2008; Savage et al. 2008). Lineage tracing experiments revealed that PLZF is not expressed by mature ILC populations but that it is transiently expressed during ILC development (Constantinides et al. 2014). Using a PLZF reporter allele, a bone marrow and a liver-resident PLZF$^+$ lymphoid progenitor was identified which, in adoptive transfer experiments and in clonal differentiation assays in vitro, gave rise to non-NK ILC1-like cells, ILC2s and CCR6$^{-/low}$ ILC3s. Interestingly, PLZF$^+$ progenitors had lost the potential to generate LTi cells (i.e., CCR6$^+$ CD4$^+$ ILC3s) (Constantinides et al. 2014), whereas PLZF$^-$ CHILPs still had LTi cell potential (Klose et al. 2014). Id2 is a regulator of PLZF expression and it is likely that PLZF$^-$ CHILPs are progenitors of PLZF$^+$ CHILPs although this remains to be experimentally addressed (Verykokakis et al. 2013).

2.3 *Development of All Helper-Like ILCs Depends on GATA-3*

The six mammalian GATA-binding proteins (GATA-1-6) possess two zinc finger motifs, which have been proposed to originate from gene duplication events (Ho et al. 2009; Tanriver and Diefenbach 2014). GATA-1-3 are involved in the development of hematopoietic cells whereas GATA-4-6 regulate development of endodermal tissues. The two zinc fingers within the GATA proteins bind to a

consensus sequence WGATAR (W: A/T, R: A/G) in the regulatory regions of multiple genes. In addition to the two zinc finger motifs, GATA-3 contains two transactivation domains and basic regions. The two zinc finger motifs allow GATA proteins to bind to two distant consensus sequences and mediate long-range looping of DNA (Hosoya et al. 2010; Chen et al. 2012). GATA-3 is already expressed early during hematopoietic development in hematopoietic stem cells and then further modulated, for example, during T cell development (Ku et al. 2012; Frelin et al. 2013; Zhong et al. 2005). This would suggest that GATA-3 may already play an important role early during commitment to different lymphoid lineages. Indeed, mice-derived from Rag-deficient blastocysts complemented with germline deleted GATA-3 stem cells, did not develop any T cells (Ting et al. 1996). It is now becoming apparent that GATA-3 plays important and stage-specifc roles for the development and function of helper-like ILCs. Early data showed that ILC2s (similar to Th2 cells) express high levels of GATA-3 and that deletion of GATA-3 in all $Id2^+$ cells specifically affected the maintenance of ILC2s and ILC2Ps whereas ILC3s, which express intermediate GATA-3 levels, were not affected (Hoyler et al. 2012; Mjosberg et al. 2012; Yagi et al. 2014). Deletion of GATA-3 prior to the engagement of Id2 expression either by using Cre-mediated deletion in all hematopoietic cells (using Vav-Cre) or in fetal liver chimeras revealed that GATA-3 is essential for the development of all helper-like ILC lineages whereas differentiation of cNK cells was unaffected (Samson et al. 2003; Serafini et al. 2014; Yagi et al. 2014). Thus, similar to its role for helper *versus* cytotoxic T cell determination (Zhu et al. 2004), GATA-3 is an important transcriptional regulator required for the differentiation of all helper-like ILCs but not for the differentiation of killer ILCs (Table 1).

3 Group 1 ILCs Depend on T-bet for Development and Secrete IFN-γ

While group 2 and group 3 ILCs have been well-defined, group 1 ILCs have been not well characterized. It has been proposed that ILC1s may consist of T-bet-expressing "ex-RORγt$^+$" ILC3s and cNK cells (Spits and Cupedo 2012; Spits and Di Santo 2011; Spits et al. 2013; Walker et al. 2013). For a detailed review on transcriptional regulation of cNK cell development the reader is kindly referred to Chap. "Transcriptional control of NK cell development and function" of this book. NKp46$^+$ cells with a history of RORγt expression are of the ILC3 lineage and are further discussed in the section dealing with ILC3s below. However, recent data may have identified *bona fide*, non-NK cell ILC1 populations (Fuchs et al. 2013; Klose et al. 2014).

3.1 The Population of NKp46$^+$ Cells Contains Various ILC Lineages

The identification of *bona fide* members of group 1 ILCs has proven difficult because the expression of T-bet and of NKp46 and other activating NK cell receptors (such as NKG2D and NK1.1) are not specific for one ILC lineage. NKp46 and NKG2D are expressed by cNK cells and ILC3 subsets (Cella et al. 2009; Cupedo et al. 2009; Sanos et al. 2009; Luci et al. 2009; Satoh-Takayama et al. 2008). In addition, T-bet is expressed by cNK cells (Townsend et al. 2004; Gordon et al. 2012) and by the CCR6$^{-/low}$ subset of RORγt$^+$ ILC3s (Klose et al. 2013; Rankin et al. 2013; Sciume et al. 2012a; Powell et al. 2012). The fate of all these NKp46$^+$ lymphocytes is, at least in part, mediated by the related T-box transcription factors T-bet and Eomes which control production of IFN-γ and expression of cytokine receptors (e.g., IL-2Rβ) or of the immunoreceptors NKp46, NKG2D or NK1.1 (Lazarevic and Glimcher 2011; Gordon et al. 2012). Given the promiscuous expression of T-bet in various ILC subsets and the broad expression of activating NK cell receptors, it was not obvious if a non-NK ILC1 population existed. For various reasons, it was somewhat unsatisfying that cNK cells would be *bona fide* ILC1s because they do not express IL-7Rα (as all other known ILC subsets) and, in striking contrast to Th1 cells, cNK cells develop and are maintained independently of T-bet (Townsend et al. 2004; Jenne et al. 2009; Gordon et al. 2012). Conventional NK cells co-express both T-box transcription factors and Eomes but not T-bet seemed to be required for cNK cell differentiation. In fact, Eomes-deficient mice have virtually no cNK cells (Fig. 1 and Table 1) (Gordon et al. 2012).

The analysis of small intestinal lamina propria-resident NKp46$^+$ NK1.1$^+$ cells, an operative definition of NK cells, using an Eomes reporter allele (*Eomes*$^{Gfp/+}$ mice) in combination with a reporter allele allowing for lineage tracing of RORγt-expressing cells (RORγt-fate map (fm) mice) discerned three distinct populations of NKp46$^+$ NK1.1$^+$ cells, all of which expressed T-bet (Fig. 1) (Klose et al. 2014): (1) Eomes-positive RORγt-fm$^-$ cells represented cNK cells expressing class I MHC-specific inhibitory and activating Ly49 receptors. (2) Eomes-negative NKp46$^+$ NK1.1$^+$ RORγt-fm$^+$ cells were part of the ILC3 lineage, enriched in plastic ILC3s which have downregulated RORγt and upregulated T-bet expression (Vonarbourg et al. 2010; Klose et al. 2013). (3) The third population expressed T-bet and IL-7Rα (consistent with an ILC phenotype) but was Eomes-negative and did not express RORγt and had not expressed this transcription factor during lineage differentiation (RORγt-fm-negative) (Klose et al. 2014). We refer to this non-NK, non-ILC3 population as ILC1s (Klose et al. 2014). Interestingly, all three subsets of NKp46$^+$ NK1.1$^+$ cells were represented in almost every organ investigated but the composition of the NKp46$^+$ NK1.1$^+$ population differed to a great extent. In secondary lymphoid organs, cNK cells represented the major population whereas ILC1s and ILC3s dominated in mucosal organs (Klose et al. 2014).

The analysis of these double reporter mice identified an ILC1 subset (i.e., T-bet$^+$ Eomes$^-$ RORγt-fm$^-$ cells) in the bone marrow. In the past, a population of cells with a similar cell surface marker expression profile (IL-7Rα^+ NK1.1$^+$) was termed immature (i) NK cells (Huntington et al. 2007; Vosshenrich and Di Santo 2013), despite the fact, that they expressed markers such as CD51, CD69, and TRAIL which were not expressed by cNK cells before and after this developmental stage. Analysis of *Eomes*$^{Gfp/+}$ x RORγt-fm reporter mice now revealed that bone marrow IL-7Rα^+ NK1.1$^+$ cells contain an Eomes$^+$ CD49a$^-$ and an Eomes$^-$ CD49a$^+$ population. Upon adoptive transfer into lymphopenic mice, Eomes$^+$ CD49a$^-$ cells differentiated into cNK cells whereas Eomes$^-$ CD49a$^+$ cells stably displayed an Eomes$^-$ RORγt-fm$^-$ phenotype. Thus, bone marrow Eomes$^-$ CD49a$^+$ IL-7Rα^+ NK1.1$^+$ cells do not upregulate Eomes in vivo or in vitro and, therefore, do not belong to the cNK cell but rather to the ILC1 lineage (Klose et al. 2014).

Genome-wide gene expression profiling was quite informative in that it revealed that ILC1s were more closely related to NKp46$^+$ NK1.1$^+$ RORγt-fm$^+$ cells than to cNK cells suggesting a core transcriptional program common to ILCs. When compared to cNK cells, ILC1s showed striking differences in the expression of chemokines and cytokines including their receptors and in the expression of integrins (Klose et al. 2014). Interestingly, the expression of various cell surface markers and transcription factors by ILCs (CD62L, CD69, CCR7, CD27, CXCR3, Id2, Eomes, T-bet) resembled the one described for central memory and tissue-resident memory T cells (Mackay et al. 2013; Kaech and Cui 2012; Hofmann and Pircher 2011). Therefore, ILCs seem to be programmed for tissue residency.

3.2 Transcriptional Regulation of ILC1 Development by T-bet, Nfil3 and GATA-3

In further support of the notion that ILC1s constitute a lineage independent of cNK cells or other defined ILC subsets, ILC1s have unique transcriptional requirements for differentiation and/or maintenance that sets them apart from cNK cells and from other ILC lineages (Table 1).

3.2.1 T-bet

As the various helper-like ILCs share transcriptional circuitry with Th cells, an important role of T-bet for the differentiation and/or maintenance of ILC1s was expected. Indeed, ILC1s expressed only T-bet but not the related T-box transcription factor Eomes (Klose et al. 2014). In sharp contrast to the mild phenotype of T-bet deficiency on cNK cell development (Townsend et al. 2004; Jenne et al. 2009), T-bet was essential for the ILC1 fate (Klose et al. 2014). Indeed, *Tbx21*$^{-/-}$ mice had virtually no intestinal ILC1s whereas cNK cells and ILC3s were

normally represented. Interestingly, T-bet was already required for the early commitment to the ILC1 lineage because iILC1s (Eomes$^-$ CD49a$^+$ IL-7Rα^+ NK1.1$^+$ cells) in the bone marrow were affected as well. This is an interesting finding because cNK cells are overrepresented in the bone marrow of T-bet-deficient mice because T-bet directly regulates S1PR5 (sphingosine-1-phosphate receptor 5) expression required for cNK cell egress from the bone marrow (Jenne et al. 2009). The exact mechanism of how T-bet regulates ILC1 fate remains elusive. Overexpression of Bcl-2 in T-bet-deficient mice did not rescue ILC1 differentiation arguing for a role of T-bet in ILC fate decision rather than for their maintenance (C.S.N.K and A.D., unpublished data).

T-bet is required for the regulation of IL-2Rβ chain (CD122) expression, a component of the IL-15 receptor (Lazarevic and Glimcher 2011). Although ILC1s expressed IL-7Rα similar to the other, helper-like ILC subsets, but their development and/or maintenance was not affected in mice lacking the IL-7 receptor (Klose et al. 2014). Interestingly, ILC1s co-expressed IL-2Rβ and IL-7Rα but ILC1 numbers were significantly reduced in mice lacking IL-15. IL-15 dependency of ILC1s was not absolute, indicating redundant control of ILC1 maintenance by various factors (Klose et al. 2014). These data raise interesting questions about regional niches in the intestine that can be distinguished into high-IL-7 and high-IL-15 environments. It will be interesting to see if ILC1s can depend on IL-7 in organs with low IL-15 expression.

3.2.2 Nfil3 or E4BP4

Another transcription factor which might be involved in the IL-15 signaling cascade is Nfil3 (also known as E4BP4) (Gascoyne et al. 2009; Kamizono et al. 2009). Nfil3 is a basic leucine zipper transcription factor, essential for the development of cNK cells (Gascoyne et al. 2009; Kamizono et al. 2009). Recent data indicated that Nfil3 is already controlling development of the rNKP in the bone marrow (i.e., Lin$^-$ Kit$^+$ IL-7Rα^+ IL-2Rβ (CD122)$^+$ 2B4$^+$ CD27$^+$ Flt3$^-$ cells) indicating an important role of Nfil3 for the early stages of NK cell development (Male et al. 2014). In contrast, T-bet or Eomes deficiency did not affect specification toward the rNKP. Of note a population referred to as the pre-NKP (i.e., Lin$^-$ Kit$^+$ IL-7Rα^+ IL-2Rβ (CD122)$^-$ 2B4$^+$ CD27$^+$ Flt3$^-$) was also reduced in *Nfil3*$^{-/-}$ mice. As this population is largely Id2-positive (M. Flach and A.D., unpublished data) and contains the CHILP, the question arises if Nfil3 may not play broader roles for the development of various ILC lineages. This is supported by our data showing that in mice with a deletion of Nfil3 in all hematopoietic cells, both mature intestinal and immature bone marrow ILC1s as well as NKp46$^+$ NK1.1$^+$ ILC3s were lacking (Klose et al. 2014). The broader roles of Nfil3 for ILC development and a careful analysis of Nfil3 target genes need to be addressed in future studies.

3.2.3 GATA-3

ILC1s express intermediate levels of GATA-3 (Klose et al. 2014). As alluded to above, deletion of GATA-3 in all hematopoietic cells cripples the development of all helper-like (IL-7Rα$^+$) ILCs but leaves cNK cell differentiation intact (Yagi et al. 2014; Serafini et al. 2014; Samson et al. 2003). Conditional deletion of *Gata3* by using NKp46-Cre or Id2-Cre^{ERt2} had no impact on the representation of cNK cells or ILC3s including NKp46$^+$ ILC3s. However, deletion of *Gata3* in all NKp46-expressing cells led to a dramatic reduction of ILC1s (Klose et al. 2014; Hoyler et al. 2012). Thus, GATA-3 plays multiple important roles for the differentiation of ILCs. (1) Early expression (before or at the Id2-positive stage) is required for specification of the CILP or CHILP to the helper-like ILC lineages. GATA-3 marks an important bifurcation between helper-like (GATA-3-dependent) and cytotoxic ILCs (GATA-3-independent). Future studies need to address at which progenitor stage GATA-3 is required and which GATA-3-controlled genes determine commitment to helper-like ILCs (Yagi et al. 2014). (2) Deletion of GATA-3 in all Id2-expressing cells did not affect cNK cell or ILC3 differentiation or maintenance. However, both GATA-3^{high} ILC2s and GATA-3^{int} ILC1s including their bone marrow-resident progenitors (ILC2Ps and iILC1s) required GATA-3 for maintenance (Klose et al. 2014; Hoyler et al. 2012). (3) Deletion of *Gata3* in all NKp46$^+$ cells led to reduced numbers of ILC1s whereas both other NKp46$^+$ ILC subsets, cNK cells and ILC3s were normally represented (Klose et al. 2014).

3.3 Intraepithelial ILC1-Like Cells and Unusual NKp46$^+$ ILC Subsets

Apart from ILC1s in the small intestine, other NKp46$^+$ ILC subsets were described which differed in important aspects from cNK cells. These NKp46$^+$ ILC subsets are found within specific tissue niches such as the epithelium of the small intestine, the thymus, or the liver.

3.3.1 Intraepithelial ILC1s

Intraepithelial ILC1s were mainly investigated in human tonsils and the epithelium of the intestine. These cells were found within the NKp44$^+$ CD103$^+$ and NKp44$^-$ CD103$^-$ compartment of CD56$^+$ non-T cells and were potent producers of IFN-γ (Fuchs et al. 2013). NKp44$^+$ CD103$^+$ cells expressed molecules indicating TGF-β imprinting and additional surface markers not found on cNK cells such as CD160, CD49a, CXCR6, CD69, and CD39. Interestingly, they expressed the IL-2Rβ chain but not the IL-7Rα chain, which suggested that their development might be dependent on IL-15 signaling. In mice, a possibly related CD160$^+$ NKp46$^+$ ILC subset residing within the epithelium of the intestine was identified that produced

IFN-γ. *Il15ra*-deficient mice had only a very mild reduction of intraepithelial ILC1s arguing that IL-15 signaling may not be indispensable for their development. It is unknown if intraepithelial ILC1s require IL-7 receptor signaling for differentiation and/or maintenance. In contrast to cNK cells, intraepithelial ILC1s were developmentally dependent on the transcription factor T-bet. Similar to cNK cells and ILC1s of the lamina propria (Klose et al. 2014), intraepithelial ILC1 development was perturbed in *Nfil3*-deficient mice. Like cNK cells, intraepithelial ILC1s expressed Eomes which distinguishes them from ILC1s described in the lamina propria of the small intestine (Klose et al. 2014). Analysis of intraepithelial NKp46$^+$ NK1.1$^+$ ILCs (all of which express CD160) using *Eomes*$^{Gfp/+}$ x RORγt-fm double reporter mice revealed that this population is diverse and contained ILC1s, NKp46$^+$ NK1.1$^+$ ILC3s and cNK cells (Klose et al. 2014). The functional importance of intraepithelial ILC1s was demonstrated in an αCD40-triggered innate colitis model (Uhlig et al. 2006; Buonocore et al. 2010). Intraepithelial ILC1s were potent IFN-γ producers upon αCD40 injection and depletion of NKp46$^+$ lymphocytes by NK1.1 antibody depletion ameliorated colitis severity (Fuchs et al. 2013). This might also be relevant with regard to human disease because NKp44$^+$ CD103$^+$ intraepithelial ILC1s were increased in patients with Crohn's disease (Fuchs et al. 2013).

3.3.2 Thymic "NK Cells"

The IL-7Rα chain is expressed by the CLP and NKP but quickly downregulated during NK cell development. Therefore, an NKp46$^+$ innate lymphocyte subset that expressed the IL-7Rα chain and that was mainly located in the thymus of mice had attracted interest and was termed "thymic NK cells" because their development depended on the presence of the thymus (Vosshenrich et al. 2006). In addition, thymic NK cells depicted a very different phenotype compared to cNK cells as they lacked expression of Ly49s but had higher expression of Kit and CD94. In contrast to cNK cells and ILC1s they developmentally depended on IL-7 (Vosshenrich et al. 2006). Similar to ILC1s, the transcription factor GATA-3 was essential for them to develop and they were potent producers of IFN-γ and TNF. It was suggested that they might correspond to the human CD56^{bright} NK cell subset which has high capacity to produce cytokines but low cytotoxic activity (Vosshenrich et al. 2006). Even though ILC1s and "thymic NK cells" differ in their dependency on IL-15 or IL-7, respectively, it is conceivable that they are of the same lineage because ILC1s express both the IL-15 and IL-7 receptors and their maintenance may depend on the availability of these cytokines in certain tissues. Future experiments will need to more firmly establish if ILC1s and "thymic NK cells" are separate or related ILC lineages.

3.3.3 Liver "NK Cells"

The liver is home to a special NK cell population called TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)$^+$ NK cells which are very prominent in neonatal mice but then decline with the age of mice (Takeda et al. 2005). Besides TRAIL, the surface receptor by which they were identified, they also express other molecules such as CD49a and CXCR6, a chemokine receptor which recognizes CXCL16 that is secreted by endothelial cells of the liver sinusoids (Shi et al. 2011; Peng et al. 2013). TRAIL$^+$ NK cells are distinct from liver cNK cells in that they do not express DX5 (CD49b) and Eomes (Gordon et al. 2012). TRAIL$^+$ NK cells were able to kill target cells but are less cytotoxic than cNK cells. In addition to IFN-γ, which is expressed by all known NKp46$^+$ cells, TRAIL$^+$ NK cells secrete TNF, IL-13 and GM-CSF (Takeda et al. 2005; Daussy et al. 2014; Sojka et al. 2014). Because of their high representation in the liver of neonatal mice and based on in vivo differentiation studies, a model was proposed in which TRAIL$^+$ NK cells are immature progenitors of cNK cells. Indeed, TRAIL$^+$ NK cells expressed several markers which define immature cNK cells such as CD27 but not CD11b, a NK cell maturation marker. In addition, transfer of TRAIL$^+$ NK cells into alymphoid mice led to the development of TRAIL$^-$ DX5$^+$ cNK cells (Gordon et al. 2012). Development of TRAIL$^+$ NK cells depended on T-bet but not Eomes, whereas mature cNK cells strictly depended on Eomes but not on T-bet. This could be in line with a model in which T-bet is essential to maintain immature TRAIL$^+$ NK cells and Eomes to maintain mature cNK cells and that deficiency for one of these transcription factors preferentially allows for outgrowth of the NK cell population independent of that factor (Takeda et al. 2005; Gordon et al. 2012). Nevertheless, it is difficult to appreciate why Eomes$^+$ cNK cells are normally represented in T-bet-deficient mice that lack their progenitors (i.e., TRAIL$^+$ NK cells). In fact, several recent studies have called this model into question because they provided evidence that the transcriptome of TRAIL$^+$ NK cells and cNK cells is very different, making it unlikely that they represent various maturation stages of cNK cells. In further support of this view, these studies failed to differentiate cNK cells from TRAIL$^+$ NK cells (Daussy et al. 2014; Peng et al. 2013; Sojka et al. 2014). Thus, TRAIL$^+$ NK cells and cNK cells may constitute distinct ILC lineages.

Interestingly, studies concerning immunological memory of NK cells showed that the memory potential is contained within CXCR6$^+$ liver NK cells which are most likely identical to TRAIL$^+$ NK cells (Peng et al. 2013; O'Leary et al. 2006; Paust et al. 2010). These findings would be difficult to reconcile with a model in which TRAIL$^+$ NK cells are immature progenitors of cNK cells and, therefore, argue in favor of a model that would assign TRAIL$^+$ NK cells and cNK cells to different lymphocyte lineages.

Future research will need to answer the important question regarding the lineage relationship of the unusual NK cell populations. Although such unconventional NKp46$^+$ ILCs share some common markers such as expression of CXCR6 and CD49a and limited expression of Ly49 receptors, the lineage

relationship remains difficult to determine because they are present in different organs. Therefore, it is unclear if difference in gene expression or dependency on cytokines that may be differentially expressed in the various organs reflects organ-specific imprinting of gene expression or if it indicates that these NKp46$^+$ ILC subsets constitute different lymphoid lineages.

3.4 ILC1s Provide Innate Protection Against Intracellular Pathogens

IFN-γ is indispensable for the control of intracellular infections (Suzuki et al. 1988; Flynn et al. 1993; Buchmeier and Schreiber 1985; Wang et al. 1994). A central role in this process plays the cytokine IL-12 which is produced by various subsets of mononuclear phagocytes. IL-12 was initially identified as the natural killer cell stimulating factor (NKSF) (Kobayashi et al. 1989) and induces IFN-γ production by NK cells but also instructs the differentiation of IFN-γ-producing Th1 cells. IFN-γ released by NK cells and Th1 cells allows for the activation of macrophages to an antimicrobial state effectively controlling various intracellular infections. Deficiencies in components of the IFN-γ and IL-12 signaling pathways lead to susceptibility to intracellular bacterial infections (Altare et al. 1998; de Jong et al. 1998). ILC1s and ILC1-like cells express T-bet and high levels of the components of the IL-12 receptor. Interestingly, ILC1s similar to "thymic NK cells" reacted to IL-12 stimulation with IFN-γ production (Vosshenrich et al. 2006; Fuchs et al. 2013; Klose et al. 2014). This is in striking contrast to naïve cNK cells that are remarkably poor producers of IFN-γ after stimulation with IL-12 (Lucas et al. 2007; Klose et al. 2014). Thus, ILC1s may be an important immediate source of IFN-γ during infection with intracellular pathogens. While cNK cells contain granula with proteins allowing for cytotoxic function, ILC1s were relatively poor killer cells.

High IL-12 secretion is triggered by infection with the obligate intracellular parasite *Toxoplasma gondii (T. gondii)*. Resistance to *T. gondii* infection is mediated to a large degree by IFN-γ and inflammatory monocytes, recruited to the sites of infection (Suzuki et al. 1988; Dunay et al. 2008). Previously, intestinal IL-15-dependent NK1.1$^+$ or NKp46$^+$ cells ("NK cells") were shown to be an important early source of this cytokine during oral *T. gondii* infection. Furthermore, NKp46$^+$ ILCs produced CCL3, a chemokine leading to the recruitment of inflammatory monocytes (Schulthess et al. 2012). Future studies will need to dissect which of the three NK1.1$^+$ NKp46$^+$ ILC subsets are the source of CCL3. Recently however, it became clear that among all NK1.1$^+$ ILCs, ILC1s were the main producers of IFN-γ and TNF during a sublethal *T. gondii* infection in the small intestine. ILC1s were especially potent in the co-production of IFN-γ and TNF. T-bet deficient mice (lacking ILC1s) showed increased *T. gondii* titers and reduced recruitment of inflammatory monocytes during the early phases of infection consistent with an important role of T-bet-driven innate cytokines for

early parasite control. *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice that lack all lymphoid cells but have a largely normal myeloid compartment, were unable to control *T. gondii* infection and showed only very inefficient recruitment of inflammatory monocytes to the lamina propria. Interestingly, transfer of ILC1s into alymphoid mice led to increased recruitment of inflammatory monocytes and innate parasite control (Klose et al. 2014).

Collectively, ILC1s and cNK cells are distinct ILC lineages that can be discriminated on the basis of different developmental requirements for transcription factors, distinct tissue residency, and a distinct gene expression program. ILC1s build a first line defense at the mucosal barrier to quickly react with cytokine production to invading intracellular pathogens. In contrast, cNK cells are located preferentially in secondary lymphoid organs, where they require priming by mononuclear phagocytes (Lucas et al. 2007) and are able to proliferate (Sun et al. 2009) before invading inflamed tissues (Lucas et al. 2007) to combat infections through cell-mediated cytotoxicity and cytokine production.

4 ILC2s Express High Levels of GATA-3 and Secrete IL-5 and IL-13

4.1 ILC2 Differentiation

ILC2s are very potent producers of effector cytokines such as IL-5, IL-9, IL-13, and amphiregulin, the latter is a member of the expanded epithelial growth factor (EGF) family (Wilhelm et al. 2011; Turner et al. 2013; Monticelli et al. 2011; Neill et al. 2010; Moro et al. 2010; Zaiss et al. 2006). It is still controversial if ILC2s also secrete IL-4. Secretion of IL-5 from ILC2s is continuous (i.e., occurs during steady-state) (Hoyler et al. 2012) and is controlled by circadian rhythm (Nussbaum et al. 2013). ILC2s are mainly localized in the gastrointestinal tract, the respiratory tract, the skin and fat-associated lymphoid clusters, but rare in secondary lymphoid organs at steady-state. Their localization at barrier surfaces allows them to quickly react to invading helminthes (Hoyler et al. 2012; Neill et al. 2010; Moro et al. 2010; Saenz et al. 2010; Price et al. 2010; Mjosberg et al. 2011; Liang et al. 2012). Helminth larvae invade the skin, enter the heart, then the lung via the blood vessels where they are coughed up, swallowed and finally reproduce in the gastrointestinal tract where they are expelled (Camberis et al. 2003). The crucial role of ILC2s for the resistance against the nematode *Nippostrongylus brasilienses (N. brasiliensis)* was demonstrated in several studies (Moro et al. 2010; Neill et al. 2010; Hoyler et al. 2012; Wong et al. 2012). Because of the therapeutic relevance, much research has focused on the pathophysiological reaction of ILC2s in airway hyperreactivity, allergic lung disease, atopic dermatitis, and liver injury (McHedlidze et al. 2013; Salimi et al. 2013; Roediger et al. 2013; Halim et al. 2012a; Monticelli et al. 2011; Chang et al. 2011).

Epithelial cell-derived cytokines such as IL-33, IL-25, and TSLP are strong activators of ILC2s (Van Dyken et al. 2014; Saenz et al. 2013; Neill et al. 2010; Neill and McKenzie 2011; Mjosberg et al. 2011). In fact, an innate source of IL-25-responsive cells has been documented in the past and eventually led to the discovery of ILC2s (Fort et al. 2001; Hurst et al. 2002; Fallon et al. 2006). In mice, ILC2s have been characterized by high expression of IL-7Rα, Sca-1 and CD25. However, these markers are not specific for the ILC2 lineage as IL-7Rα and CD25 are expressed by all helper-like ILCs and Sca-1 is found on a subset of ILC3s (Vonarbourg et al. 2010; Buonocore et al. 2010). ICOS, the IL-33Rα-chain, and the IL-17Rβ-chain are more specific markers of ILC2s (Moro et al. 2010; Price et al. 2010; Liang et al. 2012). KLRG1 and Kit were identified as maturation markers on ILC2s (Hoyler et al. 2012).

Precursors of ILC2s (ILC2Ps) in the bone marrow lack these markers whereas KLRG1 and Kit were upregulated in mature ILC2s in mucosal tissues of the gastrointestinal tract (Fig. 1). ILC2Ps were poor producers of ILC2-derived cytokines such as IL-5 and IL-13 despite sharing a core cluster of ILC2 lineage-specific gene sets (Hoyler et al. 2012). Consistent with the progenitor phenotype of ILC2Ps, their potential to proliferate and reconstitute the ILC2 compartment of alymphoid mice was more efficient when compared to adoptively transferred mature ILC2s (Hoyler et al. 2012; Halim et al. 2012b; Brickshawana et al. 2011). Interestingly, a lymphoid progenitor population that contains the ILC2P has been first described in 1998 and was referred to as LSK^- (Lin^- $Sca\text{-}1^+$ Kit^-) cells (Randall and Weissman 1998). Because of their phenotypic resemblance of hematopoietic stem cells (LSK cells), it was speculated that the LSK^- population constitutes a hematopoietic precursor population (Randall and Weissman 1998). However, adoptive transfer of LSK^- cells into lethally irradiated hosts did not reconstitute the hematopoietic system. Later studies identified additional markers such as CD25 and IL-7Rα indicative of lymphoid fate, on a fraction of LSK^- cells but failed to recover progeny of adoptively transferred LSK^- cells from the spleen of transplanted mice (Kumar et al. 2008). More recently bone marrow ILC2Ps were identified as Lin^- $Sca\text{-}1^{high}$ $Id2^+$ $GATA\text{-}3^+$ $CD25^+$ $IL\text{-}7R\alpha^+$ cells that were largely identical to LSK^- cells (Hoyler et al. 2012). In line with the above-mentioned previous data (Randall and Weissman 1998; Kumar et al. 2008), adoptive transfer of ILC2Ps into alymphoid mice did not generate any appreciable progeny in secondary lymphoid organs (Hoyler et al. 2012). Strikingly, ILC2Ps very efficiently homed to the small intestinal lamina propria where they differentiated into mature ILC2s (Hoyler et al. 2012). Homing to mucosal sites such as the small intestine was, in part, due to expression of the chemokine receptor CCR9 (Hoyler et al. 2012). In addition, the ILC2P expressed integrin $\alpha_4\beta_7$ which is involved in homing of lymphocytes to the intestine (Holzmann et al. 1989).

4.2 GATA-3

In analogy to Th2 cells (Zheng and Flavell 1997), ILC2s have high expression of the transcription factor GATA-3 (Hoyler et al. 2012; Mjosberg et al. 2012). ILC2Ps already showed high GATA-3 expression indicating that ILC2Ps were already specified for the ILC2 lineage. The roles of GATA-3 within the ILC lineage are complex and have been discussed above (Table 1). While all ILCs express intermediate levels of GATA-3, high level GATA-3 expression is a unique characteristic of ILC2s driving a distinct gene ezxpression program (Yagi et al. 2014; Hoyler et al. 2012). Conditional deletion of GATA-3 in ILCs and T cells using Id2-Cre led to complete absence of ILC2s and ILC2Ps, whereas ILC3s were normally maintained (Hoyler et al. 2012; Furusawa et al. 2013; Mjosberg et al. 2012). Cre-mediated deletion of *Gata3* in vitro demonstrated that GATA-3 is not only important for differentiation but also for the maintenance of the ILC2 lineage (Hoyler et al. 2012; Yang et al. 2013; Yagi et al. 2014). Thus, the ILC2 lineage is characterized by high GATA-3 expression and GATA-3 is required for the maintenance of ILC2s.

4.3 RORα

RAR (retinoic acid receptor)-related orphan receptors α, β and γ form a family of steroid hormone receptors which recognize a specific DNA sequence RGGGTCA (R: A/G). They consist of an N-terminal domain, the DNA-binding domain, a hinge domain and a C-terminal DNA-binding domain. RORα is expressed in two isoforms in mice (RORα1 and RORα4) and this transcription factor is important in cerebellum and cone development (Jetten 2009). In addition, RORα is involved in regulation of circadian rhythm (McIntosh et al. 2010; Jetten 2009). In contrast to RORγ, RORα is essential for the development of ILC2s. RORα is already expressed at the ILC2P stage and is further upregulated in mature ILC2s (Hoyler et al. 2012; Wong et al. 2012; Halim et al. 2012b). Germline deletion of the *Rora* gene led to defective differentiation of ILC2s. Particularly, expansion of ILC2s upon IL-25 injection, *N. brasiliensis* infection or papain challenge was severely impaired in the absence of *Rora* (Wong et al. 2012; Halim et al. 2012b). As a consequence *Rora*-deficient mice could not control *N. brasiliensis* infection and were resistant to papain-induced airway inflammation, both processes have been shown to be ILC2-dependent (Halim et al. 2012b; Wong et al. 2012). RORα-dependent gene sets in ILC2 are not well investigated, which will be an important goal for the future. One RORα target gene in Purkinje cells of the cerebellum is *sonic hedgehog* (*Shh*), a fundamental gene in cell proliferation and differentiation (Wallace 1999; Dahmane and Ruiz i Altaba 1999; Jetten 2009). However, whether this or similar genes are also controlled by RORα in ILC2s remains to be tested.

4.4 TCF-1 Signaling

TCF-1 is a transcription factor involved in the early specification of T cell fate (Verbeek et al. 1995; Okamura et al. 1998). Recent data showed that TCF-1 is also essential for the development of the entire ILC2 lineage including the ILC2P in the bone marrow (Yang et al. 2013). Consequently, *Tcf7*$^{-/-}$ mice (*Tcf7* is the gene encoding TCF-1) had strongly reduced ILC2 numbers in many organs. As a result, mice were susceptible to *N. brasiliensis* infection and resistant to papain-induced asthma (Mielke et al. 2013; Yang et al. 2013). The *Tcf7*-gene is regulated by Notch signaling in ILC2s and itself controls expression of IL-7Rα chain, an essential cytokine receptor component for development of ILC2 and ILC3 lineages (Yang et al. 2013; Wong et al. 2012).

4.5 Gfi1

The transcription factor growth factor independent 1 (Gfi1) is broadly expressed in the hematopoietic system and has critical roles in various hematopoietic processes including T lymphopoiesis (Spooner et al. 2009; Yucel et al. 2003). Moreover, Gfi1 functions in type 2 immune responses by controlling the IL-2-dependent population expansion of Th2 cells (Zhu et al. 2006). In analogy to T helper cells, ILC2s expressed Gfi1 already at the ILC2P stage and Gfi1 was further upregulated upon ILC2 maturation (Spooner et al. 2013). Gfi1 was required for differentiation or maintenance of ILC2s because *Gfi1*$^{-/-}$ mice had reduced numbers of ILC2s and ILC2Ps. As a result, *Gfi1*-deficient mice were susceptible to *N. brasiliensis* infection and resistant to papain-induced lung inflammation.

Microarray analysis revealed that Gfi1 regulates key genes of ILC2 function and development including the expression of *Il1rl1* (IL-33R subunit), *Il17rb* (IL-25R subunit), *Il5*, *Crlf2,* and *Gata3*. In particular, Gfi1 seems to regulate the responsiveness to IL-33, one of the most important activation signals of ILC2s (Van Dyken et al. 2014; Hoyler et al. 2012). On the other hand, Gfi1 suppresses genes involved in ILC3 commitment and function such as *Rorc(γt)*, *Sox4*, *Il17a*, *Il17f,* and *Il1r1*. Indeed, deletion of Gfi1 allowed expression of ILC3-specific genes in ILC2s (Spooner et al. 2013). Thus, Gfi1 is a transcription factor that reciprocally regulates the type 2 and type 17 effector states in lymphoid cells of the innate and adaptive immune systems (Spooner et al. 2013).

5 The ILC3 Family Developmentally Depends on RORγt and Secretes IL-22

5.1 Two ILC3 Lineages: CCR6$^+$ and CCR6$^{-/low}$ ILC3s

LTi cells, the founding member of group 3 ILCs, were first described in the late 1990s as hematopoietic cells (CD45$^+$) that express CD4 but lack expression of T cell markers such as CD3 (Mebius et al. 1997). Recent data support the view that LTi cells are CCR6$^+$ Kit^{high} ILCs and CD4 is only expressed on a subset (Sawa et al. 2010; Klose et al. 2013). It is currently unclear if CD4$^+$ and CD4$^-$ CCR6$^+$ ILC3s differ in their functional or transcriptional properties. It also remains to be established if CD4 expression is stable or if CCR6$^+$ ILC3s can downregulate CD4 expression (Vonarbourg et al. 2010; Sawa et al. 2010). The CC-chemokine receptor CCR6 recognizes the ligand CCL20 expressed by stroma cells in cryptopatches and the antimicrobial protein β-defensin 3 (Yang et al. 1999). Sensing of gram-negative commensal bacteria by NOD1 stimulates CCL20 expression which regulates isolated lymphoid follicle but not cryptopatch formation (Bouskra et al. 2008). The lineage-specifying transcription for LTis cells is RORγt, an immune cell-specific alternative transcript of the *Rorc* gene (Villey et al. 1999; He et al. 1998). Targeted deletion of the *Rorc* gene or of *Rorc(γt)*-specific exons led to disappearance of LTi cells and, consequently, to a failure of lymphoid organ development with absence of lymph nodes, Peyer's patches, cryptopatches, and isolated lymphoid follicles (Eberl et al. 2004; Sun et al. 2000; Kurebayashi et al. 2000; Eberl and Littman 2004). An extensive description of how LTi cells interact with stroma cells or endothelial cells and other lymphoid cells to initiate the process of lymphorganogenesis can be found elsewhere (van de Pavert and Mebius 2010).

In adult mice, LTi cells are mainly localized in cryptopatches and isolated lymphoid follicles (ILFs), where they are in immediate contact with B cells and mononuclear phagocytes (Kanamori et al. 1996; Hamada et al. 2002; McDonald et al. 2010). ILFs are major sites for T cell-independent IgA production and LTi cells and mononuclear phagocytes supported class switch recombination and differentiation of IgA-producing plasma cells by controlling gene expression in stroma cells (Tsuji et al. 2008).

While the role of CCR6$^+$ ILC3s in supporting lymphoid organogenesis is well supported, their broader role for immunity at mucosal surfaces has only been recognized very recently. CCR6$^+$ ILC3s are an important source of the cytokines IL-22 and IL-17A (Klose et al. 2013; Takatori et al. 2009; Zenewicz et al. 2008). IL-17A expression by ILC3s in the upper respiratory tract protected mice against fungal infections with *Candida albicans (C. albicans)* (Gladiator et al. 2013). IL-22 production is essential for resistance against attaching-and-effacing types of intestinal infections such as those with *Citrobacter rodentium (C. rodentium),* a mouse model for enteropathogenic *Escherichia coli (E. coli)* infections (Zheng et al. 2008; Sonnenberg et al. 2011b). It has been documented that in the first week of *C. rodentium* infection the majority of IL-22 is secreted by ILC3s (Sawa et al. 2011;

Kiss et al. 2011; Sonnenberg et al. 2011b). $CD4^+$ ILC3s seemed to have an important role in immunity to *C. rodentium* infection (Sonnenberg et al. 2011b). IL-22 is an extraordinary cytokine because its receptor which consists of the IL-22Rα1 chain and the IL-10Rβ chain is almost exclusively expressed on nonhematopoietic cells (Wolk et al. 2004; Sanos et al. 2011). Hence, ILC3s-derived IL-22 directly interacts with the surrounding epithelium, regulating resistance, homeostasis, proliferation, and repair of epithelial cells as well as containment of bacteria (Sonnenberg et al. 2011a, 2012; Sanos et al. 2011; Hanash et al. 2012; Dudakov et al. 2012; Zenewicz et al. 2007, 2008; Zheng et al. 2008; Pickert et al. 2009).

In late 2008, five independent groups reported a cell type which, similar to LTi cells, expressed and developmentally depended on the transcription factor RORγt, and, in addition, also expressed activating immunoreceptors characteristic for NK cells such as the natural cytotoxicity receptor 1 (NCR1, also known as NKp46) and NKG2D (Satoh-Takayama et al. 2008; Luci et al. 2009; Sanos et al. 2009; Cella et al. 2009; Cupedo et al. 2009). NKp46 was considered to be a fairly specific marker of cNK cells (Walzer et al. 2007). Therefore, the expression of characteristic markers of two different ILC lineages (cNK cells versus ILC3/LTi cells) provoked questions about the lineage relationship of this newly discovered lymphoid cell type. In principle, three models have been proposed. $CCR6^-$ ILC3s could be a differentiation stage (1) of cNK cells (Cella et al. 2009), (2) of ILC3s (Sanos et al. 2009; Cupedo et al. 2009; Luci et al. 2009) or, (3) they could constitute an independent lymphoid lineage (Satoh-Takayama et al. 2008; Sawa et al. 2010).

Based on transfer experiments and fate-labeling studies it became apparent that $NKp46^+$ ILC3s constituted a lineage distinct from cNK cells (Vonarbourg et al. 2010; Satoh-Takayama et al. 2010; Sawa et al. 2010). $NKp46^+$ ILC3s differed from $CCR6^+$ ILC3s in that they were CCR6-negative and expressed low levels of Kit (Sawa et al. 2010; Klose et al. 2013). It also became clear that a population of $CCR6^{-/low}$ ILC3s existed that was NKp46-negative. Cell transfer experiments and in vitro differentiation assays documented that $NKp46^+$ $CCR6^{-/low}$ ILC3s were the progeny of $NKp46^-$ $CCR6^{-/low}$ ILC3s (Fig. 1) (Vonarbourg et al. 2010; Klose et al. 2013; Rankin et al. 2013; Cupedo et al. 2009). The lineage relationship to LTi cells was more difficult to tackle but several arguments can be taken into account collectively contending that $CCR6^{-/low}$ and $CCR6^+$ ILC3s are distinct RORγt-expressing and RORγt-dependent ILC lineages. First, $CCR6^+$ ILC3s did not appreciably differentiate into $CCR6^{-/low}$ ILC3s or $NKp46^+$ ILC3s in vitro or when adoptively transferred into alymphoid mice (Sawa et al. 2010; Klose et al. 2013; Rankin et al. 2013). Second, $CCR6^{-/low}$ ILC3s but not $CCR6^+$ ILC3s expressed the transcription factor T-bet and its target genes (e.g. NK receptors, IFN-γ) (Klose et al. 2013; Sciume et al. 2012b; Bernink et al. 2013). Third, several knockout mice show deficiency of either $CCR6^+$ ILC3s or $CCR6^{-/low}$ ILC3s (Table 1) (Klose et al. 2013; Aliahmad et al. 2010; Kiss et al. 2011; Mielke et al. 2013). Fourth, fate-labeling studies showed different labeling efficiencies for $CCR6^+$ and $CCR6^{-/low}$ ILC3s (Sawa et al. 2010). Fifth, CD4 depletion only affects $CCR6^+$ ILC3s but not $CCR6^{-/low}$ ILC3s (Sawa et al. 2010). Despite this evidence,

it remains to be determined if CCR6^{+} and CCR6$^{-/low}$ ILC3s may originate from a putative unidentified common ILC3 precursor or may have distinct progenitors (Sawa et al. 2010).

While CCR6^{+} ILC3s were a phenotypically and functionally stable RORγt^{+} T-bet^{-} ILC lineage, CCR6$^{-/low}$ ILC3s showed phenotypic and functional plasticity. CCR6$^{-/low}$ ILC3s underwent a process that led to the upregulation of T-bet and the downregulation of RORγt (Klose et al. 2013; Vonarbourg et al. 2010; Bernink et al. 2013; Rankin et al. 2013). The following stages could be distinguished that likely represent consecutive differentiation stages: (1) CCR6$^{-/low}$ ILC3s, which express high levels of RORγt and are T-bet negative. (2) CCR6$^{-/low}$ ILC3s which express high levels of RORγt and low levels of T-bet but not yet NKp46. (3) CCR6$^{-/low}$ ILC3s which have intermediate levels of T-bet and RORγt and express NKp46 (often referred to as NKp46^{+} ILC3s or "NK22" cells). (4) CCR6$^{-/low}$ ILC3s which have downregulated RORγt but now have high levels of T-bet, NKp46 and NK1.1 (Fig. 1). Despite lack of expression of RORγt in that stage, fate-labeling studies clearly demonstrated that these cells are progeny of cells with a history of RORγt expression (Vonarbourg and Diefenbach 2012; Vonarbourg et al. 2010). The co-expression of RORγt and T-bet is remarkable because they were believed to instruct opposing transcriptional programs. Interestingly, "ex-RORγt^{+}" ILC3s still express the IL-23 receptor and are now able to produce IFN-γ. IL-23-dependent IFN-γ production is crucial for intestinal inflammation in the αCD40-mediated colitis model (Uhlig et al. 2006). Depletion of ILCs in the αCD40 colitis model led to a significant reduction in pathology (Vonarbourg et al. 2010; Buonocore et al. 2010), while adoptive transfer of RORγt^{-} but RORγt fate-label^{+} ILC3s promoted pathology in the αCD40 colitis model (Vonarbourg et al. 2010).

5.2 ILC3 Precursors in the Fetal Liver

The fetal liver harbors Flt3^{+} IL-7Rα^{int} Kit^{int} CLPs (Mebius et al. 2001; Possot et al. 2011). Induction of integrin $\alpha_4\beta_7$ expression on these cells correlated with a loss of B and T cell potential (Yoshida et al. 2001). Flt3^{-} IL-7Rα^{int} Kit^{int} $\alpha_4\beta_7^{+}$ cells were RORγt^{-} (see above) and contained the CHILP (Klose et al. 2014; Constantinides et al. 2014). Finally, commitment to the ILC3 lineage is established through expression of the lineage-defining transcription factor RORγt correlating with high level IL-7Rα (IL-7Rα^{high} $\alpha 4\beta 7^{+}$ RORγt^{+}) (Yoshida et al. 2001; Cherrier et al. 2012). Alternatively, CXCR6 has been shown to be a reliable marker for ILC3 committed progenitors in the fetal liver (Possot et al. 2011). Of note, differentiation of IL-7Rα^{high} $\alpha 4\beta 7^{+}$ RORγt^{+} was perturbed in mice genetically lacking *Id2* or *Rorc(γt)* while the population of IL-7Rα^{int} Kit^{int} $\alpha_4\beta_7^{+}$ ILC progenitors was largely normal (Tachibana et al. 2011). Both Runx1 and its partner Cbfβ2 were required for the differentiation of IL-7Rα^{int} Kit^{int} $\alpha_4\beta_7^{+}$ ILC progenitors. In addition, Runx1/Cbfβ2 complexes were required for the upregulation of RORγt expression in IL-7Rα^{int} Kit^{int} $\alpha_4\beta_7^{+}$ ILC3 progenitors (Tachibana et al. 2011).

5.3 RORγt

Retinoic acid-related orphan receptor γ belongs to the same family of steroid hormone receptors as RORα (Jetten 2009). Two isoforms RORγ1 and RORγ2 are transcribed (He et al. 1998; Villey et al. 1999). While RORγ1 is expressed in multiple tissues RORγ2 (also called RORγt) was found to be expressed in the thymus and later in Th17, LTi cells and all other ILC3 populations (Eberl et al. 2004; Ivanov et al. 2006b). Targeted deletion of *Rorc* or the first (RORγ2-specifc) exon of RORγt led to the disappearance of lymphoid structures such as lymph nodes, Peyer's patches or postnatally forming cryptopatches and isolated lymphoid follicles (Eberl et al. 2004; Sun et al. 2000). Some specialized lymphoid structure such as NALT could still be found in RORγt-deficient mice arguing that LTi cells are not strictly required for their development (Ivanov et al. 2006a). Both $CCR6^{+}$ and $CCR6^{-/low}$ ILC3s are absolutely dependent on RORγt. Which fundamental processes are controlled by RORγt in ILC3s so that it is essential for development of both subsets is not well understood. In T cells, RORγt deficiency could be rescued by overexpression of Bcl-xL which might argue that anti-apoptotic signals are regulated by RORγt (Sun et al. 2000). Lineage committed precursors of ILC3s were described in the fetal liver but not the bone marrow (Yoshida et al. 2001). RORγt is essential for transition to the $IL\text{-}7R\alpha^{high}$ stage of $CCR6^{+}$ ILC3s (see above) (Tachibana et al. 2011). This leads to the question which signals may instruct lineage commitment to the ILC3 lineage through regulating the expression of RORγt. Most of our knowledge about regulation of RORγt expression is based on studies in Th17 cells in which inflammatory cytokines such as TGF-β, IL-6, IL-1, IL-23 and IL-21 induced RORγt expression in the context of T cell receptor activation (Ivanov et al. 2006b; Bettelli et al. 2006). In the fetal liver, Runx1/Cbfβ2 complexes regulate earlier steps of ILC3 development. Interestingly, deletion of Cbfβ2 but not of P1-Runx1 led to reduced RORγt expression (Tachibana et al. 2011). Therefore, Cbfβ2 is a strong candidate for regulating RORγt in ILC3 precursors which is also supported by data from Th cells where Runx1/Cbfβ2 complexes control RORγt expression (Lazarevic et al. 2011). In the bone marrow, Notch has been shown to regulate RORγt expression (Possot et al. 2011).

How RORγt is induced in ILC3s is poorly understood. Interestingly, $CCR6^{-}$ ILC3s downregulate RORγt and upregulate T-bet (Klose et al. 2013). Regulation of the RORγt locus by T-bet and Runx1 is described for T cells but has not been investigated in ILC3s (Lazarevic et al. 2011). IL-7 is a cytokine which favors expression of RORγt (Vonarbourg and Diefenbach 2012). Most of $ROR\gamma t^{+}$ lymphocytes are found in the small intestine. In contrast, IL-12 promotes loss of RORγt. In many other organs such as the colon or secondary lymphoid organs, downregulation of RORγt is predominant and therefore ex-RORγt cells are found (Vonarbourg et al. 2010).

5.4 TOX

Together with TOX2, TOX3 and TX4, thymocyte selection associated HMG box protein (TOX) forms a subfamily within the large HMG-box superfamily of transcription factors (Aliahmad et al. 2012). The various TOX proteins share a well-conserved DNA-binding domain which is composed of three α-helices. Apart from the HMG-box, all family members possess a lysine-rich region which may represent a nuclear localization sequence and a transactivation domain but have a different C-terminus (Aliahmad et al. 2012).

TOX is highly expressed in double-positive thymocytes and required for proper development of CD4 T cells and for the upregulation of Id2 (Aliahmad and Kaye 2008). In addition, TOX is essential for the development of secondary lymphoid organs such as lymph nodes and Peyer's patches (Aliahmad et al. 2010). TOX-deficient mice show a very strong decrease of ILC3s with LTi function which explains the lack of lymphoid structures. Interestingly, $Tox^{-/-}$ mice also lacked mature cNK cells. This raises the question if TOX plays a decisive role in early commitment to the ILC fate by regulating E-protein activity. However, overexpression of Id2 in lymphoid precursor did not rescue cNK development (Aliahmad et al. 2010). Therefore, the mechanism of how TOX regulates ILC fates and at which stage(s) TOX is required will need further investigation.

5.5 T-bet

Metazoans have five T-box transcription factor genes which are characterized by encoding a conserved DNA-binding domain called T-box (Tanriver and Diefenbach 2014). The T-box domain recognizes a palindromic DNA sequence and this is the only identified DNA-binding domain of the T-box transcription factors (Naiche et al. 2005). The Tbr1 subfamily comprises three members Tbr1, T-bet, and Eomes (Takashima and Suzuki 2013). The latter two possess a nonredundant role in various tissues including immune cells such as $CD8^+$ T cells und Th1 cells (Intlekofer et al. 2005; Szabo et al. 2000). In innate lymphocytes, Eomes is specifically expressed by cNK cells and is absolutely requirement for their proper development (Gordon et al. 2012). In addition, cNK cells also express T-bet, but germline deletion of *Tbx21* (the gene encoding T-bet) has only a mild effect on the distribution of NK cells and is not absolutely required for their development (Jenne et al. 2009; Townsend et al. 2004). Hence, in cells such as cNK which express two members of T-box transcription factors, Eomes could compensate for some of the functions fulfilled by T-bet. In contrast to cNK cells, helper-like ILCs (such as ILC1s and $CCR6^-$ ILC3s) only express T-bet but not Eomes and T-bet expression is, therefore, an obligate requirement for some biological processes (Fig. 1) (Klose et al. 2013). T-bet is absolutely essential for the development of ILC1s which has been discussed above (Klose et al. 2014). In contrast, ILC3s develop in the

absence of T-bet despite the fact that CCR6$^{-/low}$ ILC3s express this transcription factor (Klose et al. 2013, 2014). T-bet deficient mice had normal numbers of CCR6$^{-/low}$ ILC3s but lacked NKp46^{+} ILC3s (Table 1). Indeed, T-bet-deficient CCR6$^{-/low}$ ILC3s failed to upregulate NK receptors such as NKp46, NK1.1 and NKG2D (Klose et al. 2013; Sciume et al. 2012b; Rankin et al. 2013). CCR6$^{-/low}$ ILC3s undergo a three step differentiation process in which they first upregulate T-bet, then upregulate the expression of activating NK cell receptors (such as NKp46, NKG2D and NK1.1) and IFN-γ and finally downregulate RORγt (Vonarbourg et al. 2010; Bernink et al. 2013). T-bet deficient ILC3s fail to secrete the proinflammatory cytokines IFN-γ and TNF (Klose et al. 2013). Activation of goblet cells to release mucus during infection with *Salmonella typhimurium* (*S. typhimurium*) is strictly dependent on IFN-γ (Songhet et al. 2011). Interestingly, T-bet-deficient mice or ILC-depleted *Rag2*$^{-/-}$ mice failed to trigger mucus release from goblet cells (Klose et al. 2013). In addition, T-bet deficient or ILC-depleted *Rag2*$^{-/-}$ depicted less immunopathology and inflammation of the cecum during *S. typhimurium* infection (Klose et al. 2013).

Intestinal inflammation was also reported from *Tbx21*$^{-/-}$ mice on a *Rag2*$^{-/-}$ background, a syndrome called TRUC (T-bet Rag ulcerative colitis) (Garrett et al. 2007). These mice develop spontaneous colitis which is triggered by *Helicobacter typhlonius*. IL-17-producing CCR6^{+} ILC3s were identified as pathogenic lymphocytes population in this model because neutralization of IL-17A or depletion of ILC3s with Thy1 or IL-7Rα deficiency reduced intestinal inflammation. ILC3 activation to produce IL-17A was triggered by IL-23 derived from dendritic cells (Powell et al. 2012). Thus, T-bet expression in DCs or CCR6$^{-/low}$ ILC3s is required to restrain IL-17A production by CCR6^{+} IL-17A-producing ILC3s. A role of IL-17A-producing ILC3s as driver of colitis has also been documented for *Helicobater hepaticus* (*H. hepaticus*)-induced colitis in 129Sv mice. Thy1^{+} IL-17A producing ILCs expressing RORγt and T-bet were shown to drive colitis in this model (Buonocore et al. 2010).

5.6 AhR

The aryl hydrocarbon receptor (AhR) is basic helix-loop-helix transcription factor which belongs to the Per-Arnt-Sim family (Klose et al. 2012; McIntosh et al. 2010). Upon ligand binding, it translocates to the nucleus where it dimerizes with Arnt which allows regulation of gene transcription. Cytochrom p450 monooxidases are classical target genes and their promoters contain xenobiotic response elements. The AhR binds numerous ligands, the most famous being the environmental toxin dioxin (Fernandez-Salguero et al. 1995). Apart from toxins, endogenous ligands (such as tryptophan metabolites) or dietary phytochemicals (such as flavonoids, polyphenols, and glucosinolates) can activate the AhR (Stevens et al. 2009; Rannug et al. 1987; Opitz et al. 2011).

Ahr$^{-/-}$ mice have a strongly diminished ILC3 compartment. As a consequence, AhR-deficient mice failed to develop cryptopatches and ILFs, whereas the development of fetal lymphoid organs such as Peyer's patches and peripheral lymph nodes was normal (Kiss et al. 2011; Qiu et al. 2011; Lee et al. 2012). While Peyer's patches form during fetal development, cryptopatches appeared 2 to 3 weeks after birth. Therefore, the question arose if AhR was involved in the differentiation of a specific ILC3 subset. Interestingly, CCR6^{+} ILC3s were only mildly affected in *Ahr*-deficient mice, whereas CCR6$^{-/low}$ ILC3s were dramatically reduced (Klose et al. 2013). Increased apoptosis has been proposed as a mechanism (Qiu et al. 2011) whereas others have found a failure of CCR6$^{-/low}$ ILC3s to proliferate (Kiss et al. 2011). On a molecular level, AhR binds to the *Kit* promoter and regulates Notch2 expression (Kiss et al. 2011; Lee et al. 2012). Interestingly, mice with an impairment of Kit signaling also have reduced numbers of intestinal patches (Kiss et al. 2011; Chappaz et al. 2011). In addition, conditional deletion of RBP-Jκ, an essential component of the Notch signaling pathway, did not lead to a reduction in intestinal patches, but to a decrease in NKp46^{+} ILC3s (Lee et al. 2012).

While the frequency of CCR6^{+} ILC3s was less affected by AhR deficiency and CCR6^{+} ILC3s were only mildly reduced (Klose et al. 2013), AhR regulated expression of IL-22 and probably other key molecules in CCR6^{+} ILC3s. AhR physically interacted with RORγt and bound to the *Il22* locus (Qiu et al. 2011). Reduced IL-22 expression caused reduced resistance against *C. rodentium* infection (Kiss et al. 2011; Lee et al. 2012).

As AhR is a ligand-activated transcription factor, the question arose which AhR ligands lead to the postnatal expansion of CCR6$^{-/low}$ ILC3s. Interestingly, dietary AhR ligands influenced the ILC3 pool size (Kiss et al. 2011). Strikingly, mice fed with a synthetic diet which contained low amounts of dietary AhR ligands (phytochemicals such as glucosinolates) had a phenotype similar to AhR-deficient mice (i.e., reduced expansion of ILC3s and diminished formation of cryptopatches and ILFs). Interestingly, addition of the tryptophan-derived phytochemical indol-3-carbinol to synthetic phytochemical-deprived diets led to a normal development of ILC3 compartment and intestinal patches (Kiss et al. 2011; Klose et al. 2012). Dietary-derived AhR ligands have also been shown to promote maintenance of intraepithelial lymphocytes (IELs) and promote homeostasis in the gut which protects mice to recover from DSS-colitis (Li et al. 2011). Hence, dietary-derived metabolites such as the glucosinolate glucobrassicin, which is contained in cruciferous vegetables, are involved in the regulation of intestinal immune homeostasis by regulating the pool size of ILC3s and IELs.

5.7 Notch and TCF-1 Signaling

T-bet directly binds to the *Notch* locus in Th1 cells (Germar et al. 2011; Weber et al. 2011). This signaling pathway regulates differentiation of CCR6$^{-/low}$ ILC3s. Differentiation on OP9 stroma cells, which express the Notch ligand delta-like 1,

promoted differentiation of NKp46$^+$ ILC3s (Rankin et al. 2013; Possot et al. 2011). Overexpression of constitutively active intracellular Notch partially rescued differentiation of NKp46$^+$ ILC3s in T-bet-deficient mice. Deletion of signaling mediator RBP-Jκ leads to an impaired differentiation to NKp46$^+$ ILC3s. AhR could be a regulator of Notch signaling in ILC3s (Rankin et al. 2013; Lee et al. 2012).

Notch signaling also regulated development of CCR6$^+$ ILC3s in the fetal liver. Notch signaling was required for commitment to the ILC3 lineage and for the upregulation of RORγt. However, at later developmental stages, Notch blocked the differentiation of ILC3s with LTi function (Cherrier et al. 2012).

A target gene of Notch signaling is the HMG-box transcription factor TCF-1 (encoded by the *Tcf7* gene). TCF-1 is an effector protein of the Wnt signaling pathway. Although expressed by all ILC3s, *Tcf7*-deficient mice have a selective defect in NKp46$^+$ ILC3s. *Tcf7*$^{-/-}$ mice were susceptible to *C. rodentium* infection (Mielke et al. 2013; Malhotra et al. 2013).

5.8 Runx1

Vertebrates have three genes of Runt-related transcription factors (Runx1-3), which are transcribed from two promoters and have several isoforms. All Runx proteins build heterodimers with CBFβ and recognize a common DNA motif TGPyGGTPy (Py: pyrimidine). DNA binding and heterodimerization is mediated by the Runt domain (Cohen 2009). Apart from regulating the development of multiple hematopoietic cells, Runx1 controls the early differentiation of ILC3 progenitors in the fetal liver. Mice with specific deletion of Runx1 from the proximal promoter or of CBFβ2 have impaired development of Peyer's patches (Tachibana et al. 2011). These mice had reduced numbers of $\alpha_4\beta_7^+$ IL-7Rα^{mid} cells in the fetal liver and subsequent stages of ILC3 development. Id2 and RORγt acted later in development because $\alpha_4\beta_7^+$ IL-7rα^{hi} cells were affected but not IL-7rα^{mid} cells. Interestingly, RORγt expression was reduced in mice with impaired Runx1 signaling, arguing that commitment to ILC3s lineage through upregulation of RORγt is in part mediated by Runx1 (Tachibana et al. 2011).

6 Conclusions and Perspectives

Much progress has been made during the last years to understand the basic composition of ILC lineages and subsets as well as to identify the transcriptional programs guiding their development. However, the exact molecular mechanism of their action and precise target genes are in many cases poorly understood and require more investigation.

ILCs constitute a potent first line of defense at mucosal surfaces where many pathogens invade the host's tissues. ILCs mediate protective immune responses against bacteria, parasites, and fungi (Klose et al. 2013, 2014; Diefenbach 2013; Gladiator et al. 2013; Sonnenberg et al. 2011b; Neill et al. 2010; Moro et al. 2010). However, it remains incompletely understood how ILCs sense invading pathogens. ILC2s strongly react to epithelial cell-derived cytokines such as IL-33 whereas ILC3s are activated by myeloid cells surrounding cryptopatches or ILFs that can produce IL-23 or IL-1β (Mjosberg et al. 2011; Satpathy et al. 2013; Van Dyken et al. 2014). While activation by cues from neighboring cells may be the major route to activate ILCs, precedent from cNK cells and T/B cells would indicate that lymphocytes express immune recognition receptors allowing them to discriminate between "healthy" and "pathologically altered" cells (Diefenbach and Raulet 2003). Clearly, ILC1s and ILC3s express activating immunoreceptors previously characterized as stimulatory NK cell receptors such as NKp46, NKp44, NKG2D, and NK1.1. Genetic deletion of *Ncr1* (the gene encoding NKp46) did not lead to impaired development of ILC3s and did not diminish resistance to *C. rodentium* infection (Satoh-Takayama et al. 2009). Interestingly, it has been documented that triggering of NKp44 on human ILC3s activates TNF production, whereas IL-22 release was mainly affected by cytokine cues (Glatzer et al. 2013). Human ILC3s may also express TLR2 and could be activated by directly sensing bacterial cell wall components (Crellin et al. 2010). However, the functional significance of direct sensing of pathogen-associated molecular patterns by ILCs remains elusive (Crellin et al. 2010). For ILC2s, immune recognition receptors have yet to be characterized. In addition to providing protection against various infections, inappropriate stimulation of ILCs has been tied to various inflammatory diseases such as inflammatory bowel diseases, psoriasis, allergic diseases, and airway hyperresponsiveness (Buonocore et al. 2010; Powell et al. 2012; Chang et al. 2011; Monticelli et al. 2011; Salimi et al. 2013). Future research into the signals leading to improper activation of ILCs may provide insights into therapeutically relevant pathways.

Another important line of research is how ILCs contribute to organ homeostasis. As mentioned throughout this review, ILCs produce soluble factors (e.g., IL-22, amphiregulin and other EGF family members) that control epithelial cell function (Sanos et al. 2011). In addition, ILCs (in particular ILC3s) have been documented to modify the function of stroma and endothelial cells (Eisenring et al. 2010; Dudakov et al. 2012). However, it remains unclear which molecular programs within nonhematopoietic cells is controlled by ILCs. Such ILC-controlled molecular pathways may be harnessed to improve organ homeostasis and tissue repair in the context of chronic and degenerative diseases.

A further crucial question is how ILCs influence adaptive immune responses. A recent study showed that T cells obtain restraining signals from class II MHC expressed on ILC3s (Hepworth et al. 2013). Specific deletion of class II MHC expression by ILC3s resulted in an autoinflammatory syndrome caused by inappropriately activated T cells. It was also demonstrated that ILC3s are required for maintenance of $CD4^+$ memory T cells (Withers et al. 2012). In what way ILCs can

shape T cell responses and how they interact with T cells requires more investigation (Hepworth et al. 2013). Another line of research has documented that ILCs may shape T cell function by affecting mononuclear phagocytes. ILC3s produced GM-CSF that conditioned mononuclear phagocytes in the intestinal lamina propria for priming of regulatory T cells (Mortha et al. 2014). In the papain-induced asthma model, IL-13 derived from ILC2s instructed dendritic cells to migrate to the lymph nodes in order to stimulate naïve T cells (Halim et al. 2014). Future research needs to flesh out the molecular circuitry by which ILCs crosstalk with components of the adaptive immune system.

Transcriptional control of helper-like ILC lineage commitment has often been compared to the one of $CD4^+$ T helper cells. Are ILCs providing help for B cells? It has been demonstrated that ILC3s in cryptopatches and ILFs helped B cells to produce IgA (Tsuji et al. 2008). In addition, ILC3s in the marginal zone of the spleen supported T cell-independent IgA production through diverse mechanisms one being the production of GM-CSF and recruitment of neutrophils (Magri et al. 2014).

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Transcriptional Control of Dendritic Cell Differentiation

Izumi Sasaki and Tsuneyasu Kaisho

Abstract Dendritic cells (DCs) are professional antigen presenting cells involved critically not only in provoking innate immune responses but also in establishing adaptive immune responses. Dendritic cells are heterogenous and divided into several subsets, including plasmactyoid DCs (pDCs) and several types of conventional DCs (cDCs), which show subset-specific functions. Plasmactyoid DCs are featured by their ability to produce large amounts of type I interferons (IFNs) in response to nucleic acid sensors, TLR7 and TLR9 and involved in anti-viral immunity and pathogenesis of certain autoimmune disorders such as psoriasis. Conventional DCs include the DC subsets with high crosspresentation activity, which contributes to anti-viral and anti-tumor immunity. These subsets are generated from hematopoietic stem cells (HSCs) via several intermediate progenitors and the development is regulated by the transcriptional mechanisms in which subset-specific transcription factors play major roles. We have recently found that an Ets family transcription factor, SPI-B, which is abundantly expressed in pDCs among DC subsets, plays critical roles in functions and late stage development of pDCs. SPI-B functions in cooperation with other transcription factors, especially, interferon regulatory factor (IRF) family members. Here we review the transcription factor-based molecular mechanisms for generation and functions of DCs, mainly by focusing on the roles of SPI-B and its relatives.

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1 Introduction

Dendritic cells (DCs), in response to signaling through pathogen sensors such as Toll-like receptors (TLRs), produce various cytokines or augment expression of costimulatory molecules, thereby playing critical roles in linking innate and adaptive immunity (Banchereau and Steinman 1998; Akira et al. 2006). Dendritic cells are heterogenous and consist of several subsets, including pDCs and various types of conventional DCs (cDCs) (Liu and Nussenzweig 2010). In addition to their common properties as antigen presenting cells, each subset exhibits a subset-specific function. The DC subsets express a distinct set of pathogen sensors and respond to their signaling in a subset-specific manner. Dendritic cells development is also regulated in a DC-subset-specific manner. The development requires subset-specific transcriptional programs, in which the transcription factors expressed in a subset-specific manner are mainly involved (Belz and Nutt 2012; Moore and Anderson 2013).

2 DC Subsets and Their Development

In murine spleen, DCs are divided into pDCs and cDCs by the expression of several surface molecules. Conventional DCs can be further divided into two major subsets, $CD8\alpha^{+}CD11b^{-}$ and $CD8\alpha^{-}CD11b^{+}$ cDCs.

DCs are originated from hematopoietic stem cells (HSCs) in the bone marrow (BM) via intermediate progenitors (Fig. 1) (Geissmann et al. 2010). HSCs first differentiate into common myeloid progenitors (CMPs). Common myeloid

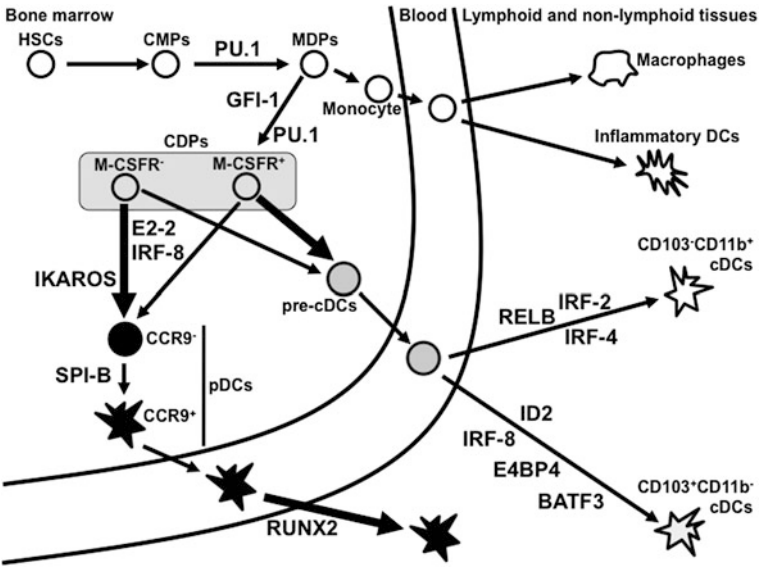

Fig. 1 *DC development in mice* DCs are generated from HSCs through several intermediate progenitors. Transcription factors involved in the developmental steps are indicated

progenitors then give rise to macrophage/DC precursors (MDPs), which retain the ability to differentiate into monocytes and DCs, but not to granulocytes. Monocytes enter the blood and reach peripheral non-lymphoid tissues, where they differentiate to macrophages or inflammatory DCs, although not to cDCs or pDCs. Macrophage/DC precursors develop to common DC precursors (CDPs), which lose the ability to differentiate into monocytes. A zinc finger-containing transcriptional repressor, GFI-1, is required for the transition from MDPs to CDPs, because GFI-1-deficient mice show reduction of all cDCs and pDCs. GFI-1-deficient hematopoietic progenitors are unable to develop into DCs, but instead differentiate into macrophages (Rathinam et al. 2005). Common DC precursors further give rise to pre-classical DCs (pre-cDCs) and pDCs. Pre-cDCs leave the BM, circulate in the blood and reach the lymphoid or non-lymphoid peripheral tissues. In the lymphoid tissues such as spleen or lymph nodes, pre-cDCs give rise to several cDC subsets, including two main subsets, $CD8\alpha^{+}CD103^{+}CD11b^{-}$ and $CD8\alpha^{-}CD103^{-}CD11b^{+}$ cDCs. Conventional DC subsets related to these two cDCs can be seen also in the non-lymphoid tissues such as skin, where CD103 is a more reliable marker to distinguish these two cDCs than CD8α. Therefore, hereafter, we refer them as $CD103^{+}CD11b^{-}$ and $CD103^{-}CD11b^{+}$ cDCs. Notably, in the intestinal tissues including mesenteric lymph nodes and lamina propria, $CD103^{+}CD11b^{+}$ cDCs are also detected as a main cDC population.

A receptor tyrosine kinase, Fms-like tyrosine kinase receptor 3 (FLT3), is expressed in DC progenitors including MDPs and CDPs. Ablation of FLT3 or FLT3 ligand (FLT3L) leads to loss of pDCs and all cDCs (McKenna et al. 2000;

Waskow et al. 2008). Furthermore, mice injected with FLT3L shows increase of all DCs (Maraskovsky et al. 1996). Thus, DC development depends on the interaction of FLT3L with FLT3.

3 Roles of SPI-B in Functions of pDCs

Plasmactyoid DCs can be distinguished from cDCs according to several surface markers including a B cell marker, B220, an I-type lectin, SIGLEC-H, or an integral membrane protein, BM stromal cell antigen 2 (BST-2) (Colonna et al. 2004). Plasmactyoid DCs are present in the BM and lymphoid tissues. Plasmactyoid DCs express nucleic acid sensing TLRs, TLR7 and TLR9, exclusively among pathogen sensors and produce vast amounts of type I IFNs, including IFN-α and IFN-ß, in response to TLR7/9 signaling (Blasius and Beutler 2010). This function of pDCs is a hallmark of pDCs. Plasmactyoid DCs detect viral infection through TLR7/9 and are involved in the anti-viral defense by producing type I IFNs. TLR7/9 can also sense host-derived endogenous nucleic acids and this sensing confers the potential to cause autoimmunity on pDCs. In homeostatic conditions, host-derived nucleic acids are unstable and degraded by tissue-derived nucleases. When nucleic acids are bound by anti-nucleic acid antibodies or anti-microbial peptides, they become stable molecular complexes, which are incorporated by pDCs and lead to pDCs activation. This is supposed to contribute to the pathogenesis of certain autoimmune disorders such as psoriasis or systemic lupus erythematosus (Gilliet et al. 2008). Thus, pDCs have both protective and pathogenic roles in immune responses.

Molecular mechanisms for pDC-specific functions to produce type I IFNs in response to TLR7/9 signaling have been progressively clarified (Fig. 2) (Honda and Taniguchi 2006; Akira et al. 2006; Kaisho and Tanaka 2008). TLR7 and TLR9 are localized in the endoplasmic reticulum in the resting state. Upon activation, they are guided by a chaperone protein, UNC93B1, to the endosome, where they bind their ligands (Beutler et al. 2007; Fukui et al. 2009). TLR7/9 have similar intracytoplasmic portions and can activate indistinguishable signal transduction cascades. The cascade depends on a cytoplasmic TLR adaptor, MyD88, and leads to activation of transcription factors, NF-κB and IRF-7 via MyD88. NF-κB and IRF-7 are critical for induction of proinflammatory cytokine and type I IFN gene expression, respectively (Honda et al. 2005; Honda and Taniguchi 2006). Several molecules are involved in IRF-7 activation (Fig. 2) (Uematsu et al. 2005; Hoshino et al. 2006; Kaisho and Tanaka 2008). IRF-7 activation requires its phosphorylation and phosphorylated IRF-7 translocates to the nucleus, where it binds to the target DNA. Thus, IRF-7 functions as a master regulator for TLR7/9-mediated type I IFN gene induction in pDCs. IRF-7 is expressed at constitutively high levels in pDCs (Izaguirre et al. 2003). This expression is further augmented rapidly and strongly upon TLR7/9 signaling. This expression pattern of IRF-7 drives positive feedback mechanisms and should contribute to the high ability of pDCs to produce

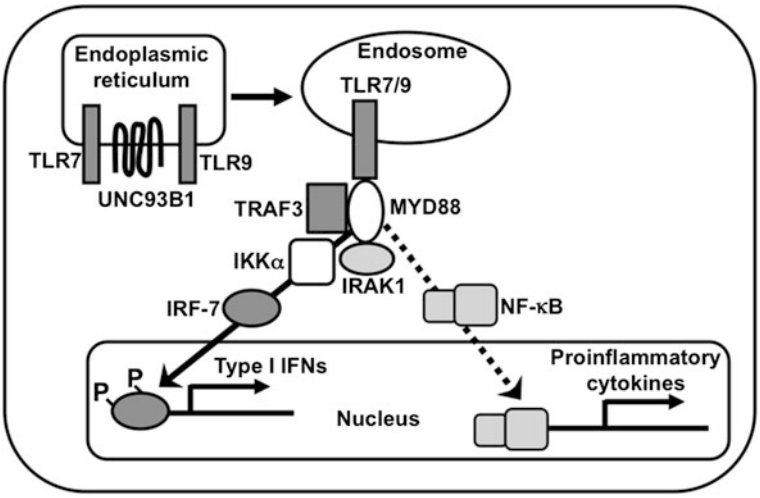

Fig. 2 *Signaling cascades of TLR7/9 in pDCs* TLR7/9 in the endoplasmic reticulum are transported with UNC93B1 to the endosome, where they meet the ligands. TLR7/9 signaling leads to induction of type I IFNs and proinflammatory cytokines via a TLR adaptor, MYD88. An adaptor, TNF receptor-associated factor 3 (TRAF3), a serine threonine kinase, IκB kinase-α (IKKα) and IL-1 receptor-associated kinase 1 (IRAK1) are involved in IRF-7 activation

type I IFNs. But IRF-7 expression is prominently induced also in cDCs stimulated by the stimuli through various TLRs including TLR4, TLR7 or TLR9 and these stimulated cDCs still fail to produce so much amount of type I IFNs as pDCs. Therefore, pDCs should have some other pDC-specific mechanisms to produce high amounts of type I IFNs.

Although a number of molecules have been shown to phosphorylate and activate IRF-7, their expression is not specific to pDCs, but rather ubiquitous. So we have analyzed gene expression profiles of DC subsets and focused on an ETS-family transcription factor, SPI-B, which is highly and constitutively expressed in pDCs (Sasaki et al. 2012).

ETS family consists of around 30 members in mammals (Oikawa and Yamada 2003). The first member, *v-ets,* was identified as a fusion oncogene of the avian transforming retrovirus E26 that can induce leukemia in chickens (Leprince et al. 1983). Then *Ets1* and *Ets2* were identified as the first cellular homologues of *v-ets* (Watson et al. 1985). Thereafter a number of cellular homologues have been revealed. All ETS family members possess an ETS domain which consists of about 85 amino acids and can bind to a purine-rich motif with a GGAA/T core consensus sequence. ETS family members regulate a variety of cellular functions, including proliferation and differentiation. Several members are involved in carcinogenesis by activating the transcription of angiogenesis-inducing genes.

Among ETS family members, SPI-B, PU.1 and SPI-C show closely related overall structures and ETS domains and form an SPI-B-related subfamily (Fig. 3).

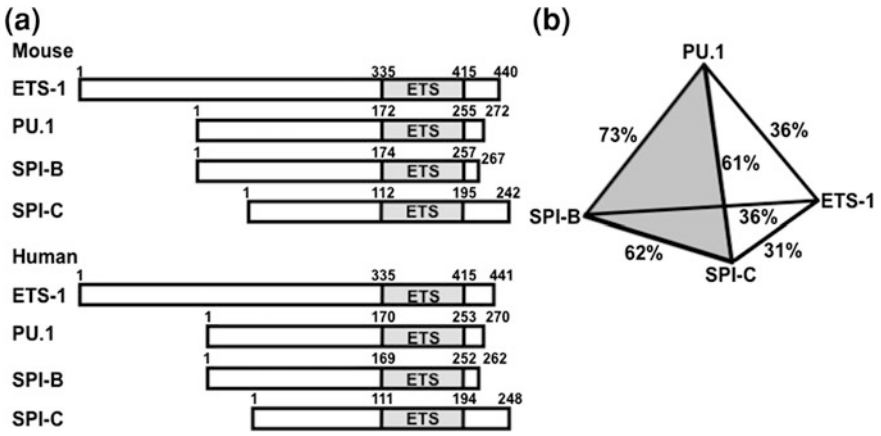

Fig. 3 *SPI-B-related subfamily* **a** Schematic representation of the overall molecular structure of ETS-1, PU.1, SPI-B, and SPI-C. The ETS domain is indicated by shaded boxes. Numbers represent positions of the amino acid residues. **b** Homology of the ETS domains of ETS-1, PU.1, SPI-B and SPI-C. The percentages of conserved amino acids in the ETS domains of indicated murine transcription factors are shown

These SPI-B subfamily members activate the promoters of target genes in cooperation with IRF family members. PU.1 can activate the target genes in cooperation with IRF-4 (Escalante et al. 2002). The target sequence is called as an ETS-IRF composite element (Brass et al. 1996). The elements were initially identified in the immunoglobulin light chain enhancers and found also in various kinds of genes. Crystal structures of the molecular complex consisting of PU.1, IRF-4 and the target DNA show that the DNA bends to juxtapose PU.1 and IRF-4 close together for selective electrostatic and hydrophobic interactions across the central minor groove (Escalante et al. 2002). This should be the basis for the synergistic effects of PU.1 with IRFs. In addition to the high expression in pDCs, this potential of SPI-B subfamily members to synergize with IRF family members highly motivated us to investigate whether or how SPI-B is involved in type I IFN gene induction in pDCs.

The activities of SPI-B on type I IFN promoters with or without IRFs are summarized in Table 1 (Sasaki et al. 2012). The *Ifna* promoter is not activated by SPI-B alone. Meanwhile, IRF-7 can activate the *Ifna* promoter and this IRF-7-induced activation is synergistically augmented when SPI-B is coexpressed. SPI-B does not show such synergistic activity when coexpressed with other IRFs. While IRF-1 can also activate the *Ifna* promoter, coexpression of SPI-B decreases the activity. The *Ifnb* promoter is activated by SPI-B, but not by IRF-7. However, as in the *Ifna* promoter, SPI-B can synergistically augment the transactivating activity with IRF-7. The synergy is also observed with IRF-4 or IRF-8, but at lesser levels than with IRF-7 (Sasaki et al. 2012). IRF-1, which can activate the *Ifnb* promoter, shows additive transactivating ability when coexpressed with SPI-B. Thus, SPI-B can transactivate the type I IFN promoters in the best synergy with IRF-7 among

Table 1 Type I IFN promoter activation 293T cells were transfected with *Ifna* or *Ifnb* promoter-driven luciferase reporter plasmids together with a combination of expression vectors for SPI-B and IRF family members

	IFN-α		IFN-ß	
SPI-B	–	+	–	+
No IRF	–	→	–	↑
IRF-1	↑	→[a]	↑	↑↑[b]
IRF-3	→	→	→	↑
IRF-4	→	→	→	↑↑↑[c]
IRF-5	→	→	→	↑
IRF-7	↑	↑↑↑[c]	→	↑↑↑[c]
IRF-8	→	→	→	↑↑↑[c]

Promoter activation is increased (↑), unchanged (→), or decreased (↓). The numbers of arrows represent the degree of activation

[a] IRF-1-induced activation was decreased by coexpression of SPI-B

[b] IRF-1-induced activation was increased additively by coexpression of SPI-B

[c] SPI-B showed synergistic activation with indicated IRFs

IRF family members, although the underlying molecular mechanisms for the synergy seem different between *Ifna* and *Ifnb* promoters. Although detailed mechanisms are still unclear, this synergy should result from the most intimate association of SPI-B with IRF-7 among IRF family members (Sasaki et al. 2012). Furthermore, analysis on SPI-B-deficient mice clarified that SPI-B is critical for TLR7/9-mediated type I IFN induction (Sasaki et al. 2012). In SPI-B-deficient mice, pDCs show defective production of type I IFNs in response to TLR7 or TLR9 stimuli. SPI-B-deficient mice also show decrease of serum type I IFN levels after injection of TLR7 or TLR9 agonists. Meanwhile, injection of double-stranded RNAs, which can induce type I IFNs through the cytosolic sensors mainly from fibroblasts, but not from pDCs, increase serum type I IFN levels similarly in both wildtype and SPI-B-deficient mice.

SPI-B-deficient pDCs also show defects in induction of TLR7/9-induced IL-12p40 and TNF-α production (Sasaki et al. 2012). This cannot be ascribed to the synergy of SPI-B with IRF-7, because the induction does not require IRF-7 but NF-κB. The NF-κB p65 subunit can activate the promoters of these proinflammatory cytokines. Although SPI-B expression alone fails to activate it, SPI-B can augment promoters of *Il12b* and *Tnf* in synergy with p65. It is unclear at present whether this synergy depends on the association of SPI-B with NF-κB, because the association could not be detected (our unpublished results). The results, however, indicate that SPI-B is also involved in TLR7/9-induced proinflammatory cytokine production in synergy with p65.

In response to TLR7/9 signaling, cDCs fail to produce IFN-α, but some, albeit not so large as in pDCs, amounts of IFN-ß and proinflammatory cytokines. Induction of IFN-ß and proinflammatory cytokines is not impaired in SPI-B-deficient cDCs. Thus SPI-B is dispensable for TLR7/9-induced cytokine production in cDCs in which SPI-B expression is low.

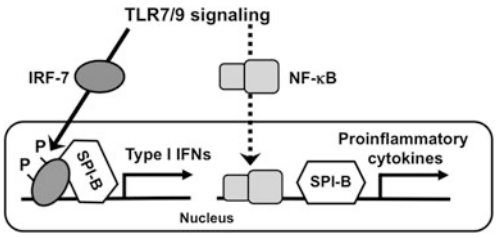

Fig. 4 *The model for the roles of SPI-B in activation of target genes* TLR7/9 signaling leads to activation and nuclear translocation of IRF-7 and NF-κB. In the nucleus, SPI-B functions in synergy with these activated transcription factors

Spib expression is constitutively high in unstimulated pDCs, but is not enhanced, rather suppressed upon TLR7/9 signaling. Although not formally tested, SPI-B is considered to be present in the nucleus. We therefore speculate that SPI-B makes the chromatins or target DNAs accessible for various transcription factors and activates the target genes in synergy with those transcription factors (Fig. 4).

4 Transcriptional Programs for pDC Generation

Knock-down of human SPI-B expression inhibits pDCs generation from $CD34^+$ precursor cells (Schotte et al. 2004). Meanwhile, SPI-B-deficient mice retain pDCs in both BM and spleen (Sasaki et al. 2012). Detailed analysis on SPI-B-deficient mice revealed novel regulatory points for pDC development. Before going into the phenotype of SPI-B-deficient mice, we describe how pDC development is regulated by transcriptional factors.

Generation of pDC depends on a pDC-specific transcription factor, E2-2 (encoded by *Tcf4*) (Fig. 1). E2-2 belongs to an E protein transcription factor family that includes basic helix-loop-helix (bHLH) transcription factors homologous to a drosophila protein, DAUGHTERLESS (Lazorchak et al. 2005). E proteins include E12, E47 (E12 and E47 are encoded by a single gene, *Tcf3*), HEB, and E2-2, which form homodimers or heterodimers with other E protein family members. E protein dimers bind E box sequences (CANNTG) with an apparent preference for C or G in the middle position. The activity of E protein is antagonized by the ID proteins (ID1-4), which sequester them into nonfunctional heterodimers. E2-2-deficient mice die at birth. So BM chimeric mice were generated and analyzed by transferring E2-2-deficient fetal liver cells into irradiated mice (Cisse et al. 2008). In E2-2-deficient chimeric mice, pDCs, defined by expression of CD11c, B220, and BST-2, are completely absent in the BM and all lymphoid tissues. Induced deletion of E2-2 in mature pDCs converts them to cDCs, indicating the roles of E2-2 in keeping the differentiation toward pDCs and against cDCs. ID2, expression of which is high in cDCs, but scarce in pDCs, can inhibit expression of pDC-specific genes and pDC

development (Spits et al. 2000). Upon deletion of E2-2, ID2 expression is induced and it drives differentiation into the cDCs. Thus, differentiation of pDCs and cDCs seems to be determined by the expression balance of E2-2 and its antagonist, ID2. Plasmactyoid DCs are derived from CDPs. Common DC precursors are heterogenous in terms of M-CSFR expression and M-CSFR$^-$ CDPs have a more skewed potential to generate pDCs than M-CSFR$^+$ CDPs (Fig. 1) (Onai et al. 2013). E2-2 is highly expressed in these M-CSFR$^-$ CDPs. The impairment of pDC function and development is also observed in heterozygous E2-2 mutant mice, indicating the haploinsufficiency of E2-2. Furthermore, a human disease, Pitt-Hopkins syndrome, which is caused by monoallelic loss of E2-2, also shows impairment in generation and type I IFN producing ability of pDCs (Amiel et al. 2007). Thus, E2-2 is a master gene required for pDC generation and expression of pDC signature genes in both mice and humans.

Another transcription factor, IRF-8, is highly expressed in CD103$^+$CD11b$^-$ cDCs and pDCs (Aliberti et al. 2003; Tsujimura et al. 2003). IRF-8-deficient mice show ablation of these two DC subsets, indicating that IRF-8 is critical for generation of them (Fig. 1) (Schiavoni et al. 2002).

Transcription factors expressed more broadly also play important roles in pDC development. IKAROS is a zinc finger type of transcription factors expressed in almost all hematopoietic cells, can associate with itself and the other related members, AIOLOS and HELIOS and essential for multilineage hematopoietic cell development (Georgopoulos et al. 1994). The mice expressing a hypomorphic IKAROS mutant show selective loss of pDCs (Allman et al. 2006). So pDC generation requires higher expression levels of IKAROS than the other hematopoietic cells (Fig. 1).

A transcription factor, RUNX2, is required for pDC-specific homing to the peripheral tissues (Fig. 1). The RUNX family of transcription factors consists of RUNX1, RUNX2, and RUNX3, which are orthologous to the RUNT protein of Drosophila melanogaster (Collins et al. 2009). They form heterodimers with CBFß and regulate the expression of target genes in various cells including T lymphocytes. RUNX2 is highly expressed in pDCs among DC subsets. In RUNX2-deficient mice, peripheral pDCs in the blood and lymphoid tissues are decreased, while BM pDCs are slightly increased (Sawai et al. 2013). This defect is caused by decreased expression of a set of chemokine receptors such as CCR5 in RUNX2-deficient pDCs. Thus, RUNX2 is involved in the migration of pDCs into the peripheral lymphoid tissues. Similar homing defects of pDCs are observed also in DOCK2-deficient mice (Gotoh et al. 2008). DOCK2 is a guanine nucleotide exchange factor that regulates the actin cytoskeleton at the upstream of a small GTPase. In DOCK2-deficient mice, pDCs normally develop in the BM, but peripheral pDCs are severely decreased. DOCK2 is required for the functions of chemokine receptors for pDC homing to the peripheral tissues.

5 Roles of SPI-B in pDC Generation

In SPI-B-deficient mice, pDCs are decreased in the BM, but increased in peripheral lymphoid tissues such as spleen and lymph nodes (Sasaki et al. 2012). Plasmactyoid DCs in the circulating blood are also increased. SPI-B-deficient pDCs show defects in expression of a set of surface molecules, including a type II C-type lectin, LY49Q, BST-2, SIGLEC-H, and B220, which are expressed more abundantly in pDCs than in cDCs. The defective expression of these markers is detected in both BM and splenic pDCs, but the defects are more severe in BM than splenic pDCs. BM chimeric mice analysis indicates that the impairment is caused by intrinsic deficiency of SPI-B in pDCs.

The findings that BM pDCs are decreased, while peripheral pDCs are increased is in contrast to the phenotype of chemokine signaling defective mice and unique for SPI-B-deficient mice (Table 2). In the BM pDC development proceeds from $CCR9^-$ to $CCR9^+$ stage (Fig. 1) (Schlitzer et al. 2011). Plasmactyoid DC-specific genes are enriched in $CCR9^+$ pDCs. In the SPI-B-deficient BM, $CCR9^-$ pDCs are increased, while $CCR9^+$ pDCs are decreased. This indicates the partial developmental block at this stage in SPI-B-deficient BM pDCs. However, this deterred development cannot account for the increase of peripheral pDCs and there should be an additional defect in the SPI-B-deficient mice. In wildtype mice, BM pDCs consist of proliferating and non-proliferating populations, whereas splenic pDCs are mainly non-proliferating (Fig. 5). We can assume that non-proliferating pDCs are more mature than proliferating pDCs. Therefore, the BM contain immature and mature pDCs and the peripheral tissues such as the spleen or lymph nodes mainly contain mature pDCs. In SPI-B-deficient mice, non-proliferating mature BM pDCs are severely decreased, although proliferating immature BM pDCs are comparable with wildtype mice (WT) (Fig. 5) (Sasaki et al. 2012). This should indicate that certain steps for retaining pDCs in the BM are defective in the SPI-B-deficient mice. It can be assumed that SPI-B-deficient BM pDCs cannot be fully retained in the BM and exit from the BM in a precocious state. Such immature pDCs can somehow mature, but the maturation is not completed in the periphery. Thus, precocious exit of the BM pDCs should contribute to the phenotype of the SPI-B-deficient mice. However, the molecular basis for keeping pDCs in the BM until full maturation is unclear at present.

E2-2 directly binds to the promoters of a group of pDC-specific genes including *Irf7, Irf8* and *Spib*. Expression of these genes is decreased in heterozygous E2-2 mutant pDCs. In SPI-B-deficient pDCs, expression of *Tcf4* is not decreased. The results indicate that *Spib* is a target gene of E2-2 and that SPI-B functions downstream of E2-2. However, comparison between E2-2- and SPI-B-dependent genes revealed a group of SPI-B-dependent and E2-2-independent genes (Sasaki et al. 2012). Thus, SPI-B also has its own functions independently of E2-2 and should also contribute to pDC developmental programs in an SPI-B-specific manner.

Table 2 pDC phenotype of gene targeting mice

	RUNX2	DOCK2	SPI-B
BM	↑	→	↓
Spleen	↓	↓	↑
Lymph node	↓	↓	↑
Blood	↓	ND	↑

pDC frequencies are increased (↑), unchanged (→), or decreased (↓) in the deficient mice
ND Not done

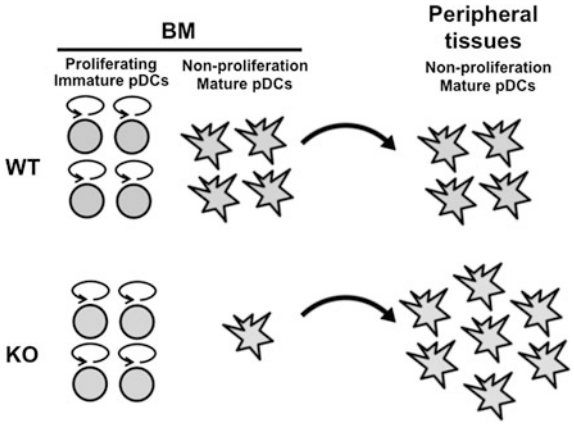

Fig. 5 *Precocious exit of SPI-B-deficient pDCs from the BM* In the WT, BM pDCs consist of both proliferating immature and nonproliferating mature pDCs, while peripheral pDCs consist of mainly nonproliferating mature pDCs. In the SPI-B-deficient mice (KO), immature pDCs are not so affected, but mature pDCs are severely decreased in the BM. Meanwhile, mature pDCs are increased in the peripheral tissues. These mature pDCs still have some functional and phenotypical defects. We assume that SPI-B deficiency should lead to precocious exit of pDCs from the BM and that SPI-B is critical for retaining the pDCs in the BM to complete their differentiation

6 Roles of SPI-B in Non-DCs

SPI-B is also expressed in B cells and the SPI-B-deficient mice were first analyzed for their B cell phenotype (Su et al. 1997). The SPI-B-deficient mice are normal in terms of basal levels of all Ig isotypes and T-independent B cell responses. However, the mutant mice show impairment in T-dependent secondary antigenic responses and produce lower levels of antigen-specific IgG1, IgG2a and IgG2b Abs, although they produce higher levels of antigen-specific IgM Abs after immunization. The SPI-B-deficient mice also show defective generation of germinal centers. SPI-B-deficient splenic B cells show poor proliferative responses against anti-IgM Abs. However, the responses against anti-CD40 Ab, IL-4 and various TLR agonists including TLR4, TLR7 and TLR9 agonists are not impaired (Su et al. 1997; Sasaki et al. 2012). Thus, SPI-B is critical for B cell receptor signaling.

SPI-B is expressed also in a subset of intestinal epithelial cells, called microfold cells (M cells). M cells are found in the follicle-associated epithelium (FAE) which covers the lymphoid follicles of gut-associated lymphoid tissues including Peyer's patches (PPs) (Neutra et al. 1996). M cells have a capacity for phagocytosis and transcytosis, thereby mediating the transport of antigens to underlying lymphoid tissues, initiating mucosal responses and leading to the T and B cell activation and generation of IgA-producing plasma cells. M cells are severely decreased in the mutant mice lacking receptor activator of nuclear factor κB ligand (RANKL), a cytokine of the TNF superfamily (Knoop et al. 2009). RANKL is produced by the mesenchymal cells in the subepithelial dome of PPs and exogenous administration of RANKL into RANKL-deficient mice rescues M cell differentiation, which is detected by sequential expression of various M cell markers. The kinetics and localization of the expression of those markers in M cells induced by RANKL are similar to those in M cells developing naturally around the birth. Shortly (1–2 h) after the treatment with RANKL, *Spib* expression is induced in a subset of FAE cells positive for *Ulex europaeus* agglutinin-1, a M cell-specific lectin. In SPI-B-deficient mice, mature M cells expressing the late stage markers, such as glycoprotein 2 or CCL9, are missing, although early stage M cell markers such as annexin V are normally expressed (Kanaya et al. 2012). SPI-B-deficient mice also show defects in the transcytotic activity across the intestinal epithelial barrier and, in the PPs, lack cells with irregular and sparse microvilli and a pocket-like invagination of the basolateral plasma membrane, which are morphological characteristics of M cells. Furthermore, SPI-B-deficient mice show impaired T cell responses against oral infection of *Salmonella typhimurium*. Thus, SPI-B is critical for generation of morphologically and functionally mature M cells (de Lau et al. 2012) (Sato et al. 2013).

Thus, not only in pDCs but also in B and M cells, SPI-B plays critical roles in the late stage development and acquisition of the functional properties of SPI-B-expressing cells rather than their early development. In the T cell development, SPI-B expression increases at the early stage, but decreases at the stage when pre-TCR signaling functions. SPI-B-deficient mice show no obvious defects in thymocyte differentiation. However, the decrease of SPI-B expression during the T cell development is critical for the cell lineage determination, because enforced expression of SPI-B arrests T cell development and instead accelerates DC development (Lefebvre et al. 2005).

Thus, SPI-B plays important roles in various cell lineages. However, the molecular mechanisms how SPI-B functions in those cells largely remain unclear.

7 Roles of PU.1 in DC and T Cell Generation

PU.1 shows 43 and 73 % identity with SPI-B at the amino acid level in the whole region and the ETS domain, respectively (Fig. 3b). PU.1 is not expressed in pDCs, but in all cDC subsets and their progenitors including MDPs and CDPs.

PU.1-deficient mice show loss of all lymphoid and myeloid progenitors and long-term multilineage repopulating HSC activity. Thus, PU.1 is critical for normal HSC activity and commitment of hematopoietic progenitors (Fig. 1) (Anderson et al. 2000). These severe defects in hematopoiesis made it difficult to clarify the roles of PU.1 in DC lineages. However, inducible deletion of PU.1 in the adult mice clarified the critical involvement of PU.1 in generation of cDCs and pDCs from CDPs (Fig. 1) (Carotta et al. 2010a). This phenotype can be ascribed to reduced expression of FLT3. PU.1 directly regulates FLT3 gene expression in a dose-dependent manner. PU.1 is also necessary for expression of other cytokine receptors including macrophage colony stimulating factor receptor (M-CSFR), granulocyte-macrophage colony stimulating factor receptor (GM-CSFR), and the interleukine 7 receptor (IL-7R) or molecules involved in DC functions. PU.1 is considered to function in synergy with IRFs. For example, PU.1 functionally interacts with IRF-8 to activate the expression of various target genes in myeloid cells (Rehli et al. 2000; Marecki et al. 2001). PU.1 also activates the cytostatin gene expression in synergy with IRF-4 in mature DCs (Xu et al. 2011). The roles of PU.1 in DCs and their progenitors are quite divergent and complex and remain largely unclear.

PU.1 is expressed in the early stage of T cell development and required for generation of T cell precursors (Carotta et al. 2010b). Multiple genes are regulated by PU.1 in early developmental stages of T cells (Zhang et al. 2012). Furthermore, decrease of PU.1 expression in T lineage cells corresponds to the commitment to T cells and loss of differentiating ability to DCs (Anderson et al. 2002). Thus, expression level of PU.1 is critical for determining the development to DCs or T cells.

8 Roles of SPI-C in Macrophages

SPI-C shows 34 and 62 % identity with SPI-B at the amino acid level in the whole region and the ETS domain, respectively, (Fig. 3b). SPI-C expression is scarce in pDCs and cDCs, but abundant in a macrophage subset, splenic red pulp macrophages. In SPI-C-deficient mice, splenic red pulp macrophages are missing (Kohyama et al. 2009). As a result, red blood cells are not efficiently phagocytosed and iron is accumulated in the red pulp, leading to splenomegaly. Vascular cell adhesion molecule (VCAM1) expression is decreased in the spleen of SPI-C-deficient mice and *Vcam1* was found to be a target gene of SPI-C. It, however, remains unclear how SPI-C is involved in generation of splenic red pulp macrophages. SPI-C is also expressed in B cells, albeit at lower levels, than in the red pulp macrophages. However, obvious B cell defect is not observed in SPI-C-deficient mice.

9 Transcriptional Programs for cDC Generation

Development of $CD103^+CD11b^-$ and $CD103^-CD11b^+$ cDCs is also regulated in a subset-specific transcriptional mechanisms.

9.1 Function and Generation of $CD103^+CD11b^-$ cDCs

$CD103^+CD11b^-$ cDCs are featured by high ability to incorporate dying cells and crosspresent Ags to generate $CD8^+$ T cell responses (Shortman and Heath 2010). Principally all DC subsets can exhibit crosspresenting activity, but $CD103^+CD11b^-$ cDCs highly express a set of molecules required for Ag presentation through MHC Class I. Crosspresentation includes both crosspriming and crosstolerance, which can lead to immune stimulation and immune suppression, respectively. DCs exhibit crosspriming by ingesting tumor or virally infected cells and contribute to anti-viral or anti-tumor defense. Crosstolerance can be achieved by DCs that incorporate host cells or tissues and should function for maintaining the homeostasis. Furthermore, $CD103^+CD11b^-$ cDCs are also featured by high ability to produce proinflammatory cytokines. In response to TLR3, TLR4 or TLR9, which recognize double-stranded-RNA, lipopolysaccharide (LPS) or CpG DNA, respectively, $CD103^+CD11b^-$ cDCs can produce the highest amounts of the cytokines such as IL-6 or IL-12p40 among all DC subsets.

As described above, IRF-8 is required for generation of pDCs and $CD103^+CD11b^-$ cDCs (Fig. 1). BXH2 mice are spontaneous mutant mice harboring a point mutation which leads to the change of arginine into cysteine in the position 294 of IRF-8 within the association domain required for the interaction with partner proteins (Schiavoni et al. 2002; Tailor et al. 2008). The mice show ablation of $CD103^+CD11b^-$ cDCs, but not of pDCs. This indicates that IRF-8 is involved in development of pDCs and $CD103^+CD11b^-$ cDCs through the differential protein interaction.

Basic leucine zipper transcription factor, ATF-like 3 (BATF3), which is also known as JUN-dimerization protein p21SNFT, belongs to a BATF family and can repress the functions of AP-1 family members, because it lacks a transcription activating domain (Dorsey et al. 1995). Although BATF3 is expressed in both $CD103^+CD11b^-$ and $CD103^-CD11b^+$ cDCs, BATF3-deficient mice show selective ablation of $CD103^+CD11b^-$ cDCs (Hildner et al. 2008). BATF3 is the first transcription factor shown to be critical for generation of $CD103^+CD11b^-$ cDCs (Fig. 1), although detailed analysis revealed the presence of $CD103^+CD11b^-$ cDCs in the peripheral lymph nodes of C57BL/6 BATF3-deficient mice (Edelson et al. 2011). This is due to the compensatory effects of closely related molecules, BATF and BATF2 (Tussiwand et al. 2012). However, BATF3-deficient mice have been used for a number of experiments, which clearly show the critical roles of $CD103^+CD11b^-$ cDCs in the crosspriming immune responses against virus,

bacteria, parasites, and tumors (Murphy et al. 2013). Critical roles of $CD103^+CD11b^-$ DCs in crosspriming were also shown by the analysis on the mutant mice in which $CD103^+CD11b^-$ DCs can be inducibly ablated upon injection of diphtheria toxin (DT). The mice were generated by knocking the gene for a fusion protein of DT receptor (DTR) and a fluorescence protein into the gene locus of a chemokine receptor, XCR1, which is specifically expressed in $CD103^+CD11b^-$ DCs (Yamazaki et al. 2013).

E4 promoter-binding protein 4 (E4BP4) is a basic leucine zipper transcription factor that is required for NK cell development. E4BP4 expression is not restricted to DCs and widely expressed in various tissues including macrophages. E4BP4-deficient mice also show selective loss of $CD103^+CD11b^-$ cDCs among DC subsets, indicating the critical involvement of E4BP4 in generation of $CD103^+CD11b^-$ cDCs (Fig. 1) (Kashiwada et al. 2011). BATF3 expression is decreased in E4BP4-deficient CDPs and defect in $CD103^+CD11b^-$ cDCs generation is rescued by enforced expression of BATF3, indicating that E4BP4 acts upstream of BATF3. As described above, ID2 expression is abundant in all cDC subsets, although low in pDCs. However, ID2 deletion leads to selective deletion of $CD103^+CD11b^-$ cDCs, indicating the involvement of ID2 in generation of $CD103^+CD11b^-$ cDCs (Fig. 1) (Ginhoux et al. 2009; Jackson et al. 2011). Thus, several transcription factors, although their expression is not specific to $CD103^+CD11b^-$ DCs, are required for generation of $CD103^+CD11b^-$ DCs.

9.2 Function and Generation of $CD103^-CD11b^+$ cDCs

$CD103^-CD11b^+$ cDCs are less characterized than the other subsets, but are mainly involved in $CD4^+$ T cell responses (Liu and Nussenzweig 2010). An NF-κB family member, RELB, is expressed highly in $CD103^-CD11b^+$ cDCs and is the first transcription factor identified to be critical for generation of $CD103^-CD11b^+$ cDCs (Fig. 1) (Burkly et al. 1995; Wu et al. 1998). NF-κB family members include p50, p52, p65, CREL, and RELB. RELB tends to dimerize with p52, while p65 and CREL tend to dimerize with p50. At present, however, the molecular mechanisms by which RELB is involved in generation of $CD103^-CD11b^+$ cDCs remain unclear.

IRF-2-deficient mice also show defective generation of $CD103^-CD11b^+$ cDCs in the spleen. This phenotype is rescued by type I IFN receptor deficiency. IRF-2 seems to function in preventing $CD103^-CD11b^+$ cDCs from the suppressive function of type I IFNs (Fig. 1) (Ichikawa et al. 2004; Honda et al. 2004).

IRF-4 is broadly expressed in a variety of hematopoietic cells and play important roles in B and T cells (De Silva et al. 2012). In B lineage cells, IRF-4 is critical for Ig class switch recombination and plasma cell differentiation. In T lineage cells, IRF-4 is required for Treg cells to regulate Th2 responses and for differentiation of Th2 and Th17 cells. Among splenic DCs, IRF-4 expression is high in $CD103^-CD11b^+$ cDCs. IRF-4 deficiency leads to selective loss of

$CD103^{-}CD11b^{+}$ cDCs and slight reduction of pDCs in the spleen (Suzuki et al. 2004; Tamura et al. 2005). The findings reveal the differential involvement of IRF-4 and IRF-8 in development of DC subsets (Fig. 1).

Further analysis on IRF-4-deficient mice revealed tissue- and subset-specific roles of IRF-4 in DCs. In the skin lymph nodes, one DC subset, which express CD301b, also known as macrophage galactose-type C-type lectin 2 (MGL2), was found (Kumamoto et al. 2013). This DC subset is migratory and also expresses programmed death ligand-2 (PDL2), CD11b, and CCR7, but not CD103. Thus, $CD301b^{+}$ DC is included in the $CD103^{-}CD11b^{+}$ fraction of the skin lymph nodes. A DTR gene was knocked into the *Mgl2* locus to generate the mutant mice in which $CD301b^{+}$ DC can be inducibly depleted. IRF-4 deficiency in CD11c-expressing cells, i.e. DCs, also leads to selective ablation of $CD301b^{+}$ DCs (Gao et al. 2013). These mutant mice show defective Th2 responses induced by alum, papain, or helminths. Furthermore, $CD301b^{+}$ DC show high ability to support in vitro Th2 cell differentiation. Thus, IRF-4 is critical for a skin lymph node DC subset, which is required for Th2 responses.

$CD103^{+}CD11b^{+}$ DCs can be found in the small intestine lamina propria and mesenteric lymph nodes, but not in the spleen. The intestinal $CD103^{+}CD11b^{+}$ DCs and splenic $CD103^{-}CD11b^{+}$ DCs are developmentally related, because these two DC subsets show similar expression profiles (Persson et al. 2013; Schlitzer et al. 2013). These DC subsets show high IRF-4 and low IRF-8 expression, which is found also in splenic $CD103^{-}CD11b^{+}$ DCs. IRF-4 deficiency in DCs leads to severe reduction of $CD103^{+}CD11b^{+}$ DCs in the lamina propria and mesenteric lymph nodes. IRF-4 is required for survival of $CD103^{+}CD11b^{+}$ DCs, although it is unclear whether IRF-4 is also involved in the developmental process. Mice with IRF-4-deficient DCs show defective Th17 responses, indicating that the $CD103^{+}CD11b^{+}$ DCs are critical for Th17 responses. $CD103^{+}CD11b^{+}$ DCs show high ability to produce IL-6, thereby contributing to Th17 responses. $CD103^{+}CD11b^{+}$ DCs are also found in the lung and the mutant mice with IRF-4 deficiency in DCs exhibit impaired Th17 responses against *Aspergillus fumigatus* challenge in the lung (Schlitzer et al. 2013). Thus, analysis on the IRF-4-deficient mice clarified that $CD103^{+}CD11b^{+}$ DCs are critical for Th17 responses in the intestine and lung. Involvement of $CD103^{+}CD11b^{+}$ DCs in mucosal Th17 responses was also shown by the analysis on the gene-manipulated mice in which $CD103^{+}CD11b^{+}$ DCs are constitutively absent due to transgenic expression of DT A subunit (Welty et al. 2013).

10 Transcription Programs for Human DC Generation

Much progress has been achieved in the molecular and cellular mechanisms for function and development of murine DCs. It should be demanded how this knowledge can be translated to human systems (Collin et al. 2011). It largely remains unknown whether there exist committed DC progenitors in humans.

However, some mature DC subsets seem preserved. Human peripheral blood contains myeloid DCs and pDCs among the cells which show high expression of MHC Class II and are negative for several cell lineage markers. Plasmactyoid DCs can be defined as blood dendritic cell antigen 4 $(BDCA4)^+$ cells. Human pDCs can produce vast amounts of type I IFNs in response to TLR7/9 signaling. In myeloid DCs, $BDCA3^+$ DCs exist as a small but significant population. The gene expression profile of $BDCA3^+$ DCs closely resembles to that of murine $CD103^+CD11b^-$ cells, indicating that they are corresponding subsets. Furthermore, human blood and lung $CD1c^+$ DCs are similar to murine $CD103^+CD11b^+$ DCs in terms of expression patterns of genes including IRF-4 and the ability to support Th17 cell responses (Schlitzer et al. 2013). Thus, accumulating evidences show that some, although not all, human DC subsets correspond to murine DC subsets.

The study of primary immunodeficiency should provide us with critical information on the molecular mechanisms for human DC generation. Pitt-Hopkins syndrome is a rare genetic disorder caused by monoallelic loss of functions or deletions of E2-2 (Cisse et al. 2008). The patients show decrease and impaired type I IFN producing capacity of pDCs. Heterozygous mutation, including autosomal dominant or de novo mutation, of a transcription factor, GATA-binding factor 2 (GATA2), is the main cause for DC, monocyte, B and NK lymphoid (DCML) deficiency, which manifests combined mononuclear cell deficiency including complete absence of blood and interstitial tissue DCs (Dickinson et al. 2011). GATA2 is a zinc finger transcription factor involved in the homeostasis of hematopoietic stem cells. Three cases of impaired DC development likely due to mutations of IRF-8 were also reported (Hambleton et al. 2011). Similar to the patients with DCML deficiency, they show susceptibility to poor virulent mycobacteria, including Bacillus Calmette-Guérin. One case carries an autosomal recessive mutation (K108E) and two cases carry an autosomal dominant sporadic mutation (T80A) in the DNA-binding domain of IRF-8. IRF-8 with K108E or T80A mutation shows defective transcriptional activity due to impaired DNA-binding ability. The IRF-8 K108E patient manifests complete loss of peripheral blood myeloid DC, pDCs, and monocytes, while the IRF-8 T80A patients have normal numbers of myeloid DCs including $BDCA3^+$ DCs, pDCs, and monocytes, but lack $CD1c^+$ DCs. The phenotypes are different from the phenotype of IRF-8-deficient mice, which show a specific loss of $CD103^+CD11b^-$ DCs, a murine homologue of $BDCA3^+$ DCs. In spite of discordant phenotype, IRF-8 is critical for keeping the DC homeostasis in both human and mice.

11 Conclusions

Accumulating evidences have unveiled subset-specific transcriptional programs of DC development. If the deletion of one transcription factor led to selective ablation of one DC subset, then it should contribute a lot to understanding not only the

molecular mechanisms on the development of the subset but also the critical roles of the subset. However, we should carefully consider about the difference between a transcription factor-deficient mice and DC subset-ablated mice. For example, BATF3 is expressed in $CD103^{-}CD11b^{+}$ cDCs, which are not deleted in BATF3-deficient mice. Therefore, the possibility that phenotype of the BATF3-deficient mice is caused by the loss of BATF3 in $CD103^{-}CD11b^{+}$ cDCs should be excluded. It is also possible that certain cells unexpectedly increase due to activation of alternative differentiation pathway following the deletion of one transcription factor. Taking these possibilities into consideration should be necessary in interpreting the phenotype of the transcription factor-deficient mice.

DCs function not only in the host defense but also in the pathogenesis of immune disorders. Activating or depleting specific DC subsets should lead to enhancement of the host defense or amelioration of immune disorders. Therefore, clarifying the molecular mechanisms for DC subsets development should reveal the target mechanisms or molecules for developing novel types of immunoregulatory reagents. Although there are some differences in the human and murine systems, it is still necessary and quite useful to elucidate the molecular mechanisms for DC development in mice and to analyze the human system according to the findings on mice.

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Helper T Cell Plasticity: Impact of Extrinsic and Intrinsic Signals on Transcriptomes and Epigenomes

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Abstract $CD4^+$ helper T cells are crucial for autoimmune and infectious diseases; however, the recognition of the many, diverse fates available continues unabated. Precisely what controls specification of helper T cells and preserves phenotypic commitment is currently intensively investigated. In this review, we will discuss the major factors that impact helper T cell fate choice, ranging from cytokines and the microbiome to metabolic control and epigenetic regulation. We will also discuss the technological advances along with the attendant challenges presented by "big data," which allow the understanding of these processes on comprehensive scales.

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1 Introduction

The generation of diverse types of effector CD4 T cells and the balance with production of various types of regulatory T cells is crucial for the protection against infections, but is also critical for a variety of autoimmunity diseases (Reiner et al. 2007). In fact, understanding how CD4 T cells differentiate into these diverse fates has already provided insights not only into immunopathogenesis, but also has facilitated the development of new therapies. CD4 T cell fate choice has been recognized since the late 1980s, but the remarkable complexity of options available to these cells continue to be elucidated. Aside from T helper 1 (Th1) cells and Th2 cells, subsets termed Th17, Th22, Th9, and follicular T helper (Tfh) cells (Zhou et al. 2009a) have been recognized. Equally relevant for the pathogenesis of autoimmune disease are the mechanisms that lead to different types of regulatory T cells, including those that express Foxp3 and those that do not (Rudensky 2011; Ohkura et al. 2013; Awasthi et al. 2007; Gregori et al. 2012). But even among these defined subsets, we also appreciate considerable heterogeneity and plasticity (Cannons et al. 2013; O'Shea and Paul 2010; Coomes et al. 2013; Yamane and Paul 2012; Dong 2011; Zhu and Paul 2010). Consequently, the previous 1:1:1 model of differentiation (one lineage/function, one signature cytokine and one master regulator transcription) has given way to a more nuanced view of specification (Crotty 2012), and the plasticity versus stability of these subsets, both effector and regulatory continues to be intensively investigated. Thus, more sophisticated understanding of helper T cell differentiation will surely continue to be useful for immunologists both in terms of understanding and treating disease.

In this review, we will discuss the current views of helper T cell diversity and evolving insights into the mechanisms that underlie their differentiation. The appreciation of the enormous range of T cells fates has occurred at a time when our basic understanding of the regulation of gene expression is changing and new techniques are being devised. The impact of the epigenome on cell fate determination is being re-examined as new technologies to measure these changes also emerge. Indeed, the more flexible view of cell fate has been a general lesson of cell

biology, well beyond immune cells. It is premature at this time to propose a unifying framework of how networks of transcription factors and epigenomic changes converge to drive helper T cell fate choice while maintaining opportunities for plasticity. Nor can we hope to be comprehensive in covering all of these topics in a single review. Rather, we will try to provide a few illustrative examples of molecular mechanisms that can promote flexibility in the context of cellular differentiation. We will try to explain how new technologies have modified our views of the CD4 T cells biology and their capacity for plasticity in response to a constantly changing environment.

2 Old and New Players in Lineage Specification of Helper T Cells

Based on their function and cytokine expression, activated $CD4^+$ T helper (Th) cells were initially classified into two subsets (Mosmann and Coffman 1989): Th1 cells that produce Interferon-γ (IFN-γ) and Th2 cells that produce interleukin (IL)-4, IL-5, and IL-13 as their respective signature effector cytokines. In this way, CD4 T cells orchestrate the type of immune response that ensues upon encounter of diverse microbial pathogens. Regulated cytokine production is required for the proper elimination of microbial pathogens: Th1 cells for intracellular microbes and Th2 cell for helminthes (Abbas et al. 1996). Extrinsic factors, especially cytokines, are also critical in that they activate transcription factors especially members of the signal transducer and activator of transcription (STAT) family, which in turn control helper cell differentiation. IFN-γ and IL-12 activate STAT1 and STAT4, whereas IL-4 activates STAT6. Th2 cells lose their sensitivity to the Th1 cell-inducing cytokine IL-12 via downregulation of IL-12 receptor ß2 (IL-12R ß2) and STAT4 expression (Szabo et al. 1997; Usui et al. 2003). In addition to STAT4 and STAT6, STAT5 plays a critical role for both Th1 and Th2 cell differentiation, transmitting IL-2-dependent signals (Liao et al. 2011). Differentiation leads to high expression of transcription factors termed master regulators, which when over-expressed are sufficient to induce signature cytokines. For instance, T cell receptor (TCR) and STAT signals induce the T-box-containing protein, T-bet (encoded by *Tbx21*) (Szabo et al. 2000). Conversely, GATA-binding protein-3 (GATA-3) is critical for the development of the Th2 cell lineage (Zhang et al. 1997) and overexpression is sufficient to drive Th2 differentiation. Unlike T-bet though, GATA-3 also has other functions in thymic development.

While the appreciation of diverse CD4 helper cell fates was a breakthrough in elucidation of immunoregulatory mechanisms, it also became clear that a dichotomous view of Th1 and Th2 cells failed to explain many aspects of immune responses and especially autoimmunity. The discovery of another subset of cells, Th17 cells, subsequently provided a number of insights (Yang et al. 2008b; Stockinger et al. 2007; Korn et al. 2009; Weaver et al. 2007; Steinman 2007). Th17 cells are characterized by the production of IL-17A, and IL-17F, but can also

produce IL-21 and IL-22. Th17 cells are an important component of the adaptive immune response to certain microbes, particularly extracellular bacteria and fungi (McGeachy and Cua 2008). A number of transcription factors including retinoic acid-related orphan nuclear hormone receptor ROR$\gamma\tau$, STAT3, Batf, and IRF4 are involved in the differentiation of Th17 cells (Acosta-Rodriguez et al. 2007b; Huber et al. 2008; Yang et al. 2011; Lohoff et al. 2002; Brustle et al. 2007; Biswas et al. 2010). A variety of cytokines also contribute to the differentiation of Th17 cells including transforming growth factor (TGF)-β, IL-1, IL-6, IL-23 (Mangan et al. 2006), and IL-21 (Korn et al. 2007; Zhou et al. 2007; Mangan et al. 2006). However, it is becoming increasingly clear that "Th17" cells represent a heterogeneous collection of cells, which may or may not be pathogenic (Ghoreschi et al. 2010; Zielinski et al. 2012; Wu et al. 2013; Yosef et al. 2013; Lee et al. 2012). IL-17 and IL-22 production is also influenced by environmental factors such as aryl hydrocarbon receptor (AHR) ligands (Veldhoen et al. 2008, 2009), which include environmental pollutants. Dietary components can also activate AHR and IL-17 is even influenced by dietary salt (Yosef et al. 2013; Wu et al. 2013; Lee et al. 2012; Kleinewietfeld et al. 2013). In this context, it needs to be emphasized however, that $CD4^+$ Th17 cells are by no means the only source of IL-17 and IL-22 cytokines.

In addition to Th1, Th2, and Th17 cells, there are subsets of CD4 T cells that preferentially produce IL-9 and IL-22 (Veldhoen et al. 2009; Dardalhon et al. 2008; Duhen et al. 2009; Trifari et al. 2009). These are referred to as Th9 and Th22 cells; however, there are circumstances in which these cytokines are not produced exclusively, but are produced with other effector cytokines. IL-4 together with TGF-β induce a population of IL-9^+IL-10^+ T cells, which do not express Foxp3, but induce colitis and peripheral neuritis after transfer into RAG-1-deficient mice (Dardalhon et al. 2008). TGF-β can reprogram committed Th2 cells to switch to IL-9 secretion and therefore drives the differentiation of Th9 cells (b).

Adding to the complexity of $CD4^+$ T cells are follicular helper T cells, which provide help to B cells and are found within B-cell follicles or germinal centers. They express CXCR5, which contributes to their localization, PD-1 and IL-21; however, PD-1 and IL-21 are not exclusively expressed by Tfh. Moreover, in addition to IL-21, Tfh cells can produce IL-4, IL-17, and IL-10 (Crotty 2011; Reinhardt et al. 2009). The "master regulator" for Tfh is the transcription factor Bcl-6 (Crotty 2011; Johnston et al. 2009; Yu et al. 2009; Nurieva et al. 2009). The factors responsible for Tfh differentiation remain perplexing and the search for unique inducers has been frustrating; rather, it appears that multiple factors can contribute to Tfh differentiation. That is, STAT1, STAT3, and STAT4 all contribute to Tfh cell development, whereas STAT5 supresses formation of this subset (Choi et al. 2014; Johnston et al. 2012; Yang et al. 2008a; Nurieva et al. 2012; Ma et al. 2012; Nakayamada et al. 2011). Cytokines that activate these STATs, including IL-6, IL-21, IL-27, and IL-12, have all been implicated in driving Tfh cell differentiation, whereas IL-2 has a negative effect (Eto et al. 2011; Schmitt et al. 2013; Poholek et al. 2010; Harker et al. 2011; Batten et al. 2006; Ballesteros-Tato et al. 2012). A summary of diverse T cell subsets can be found in Fig. 1.

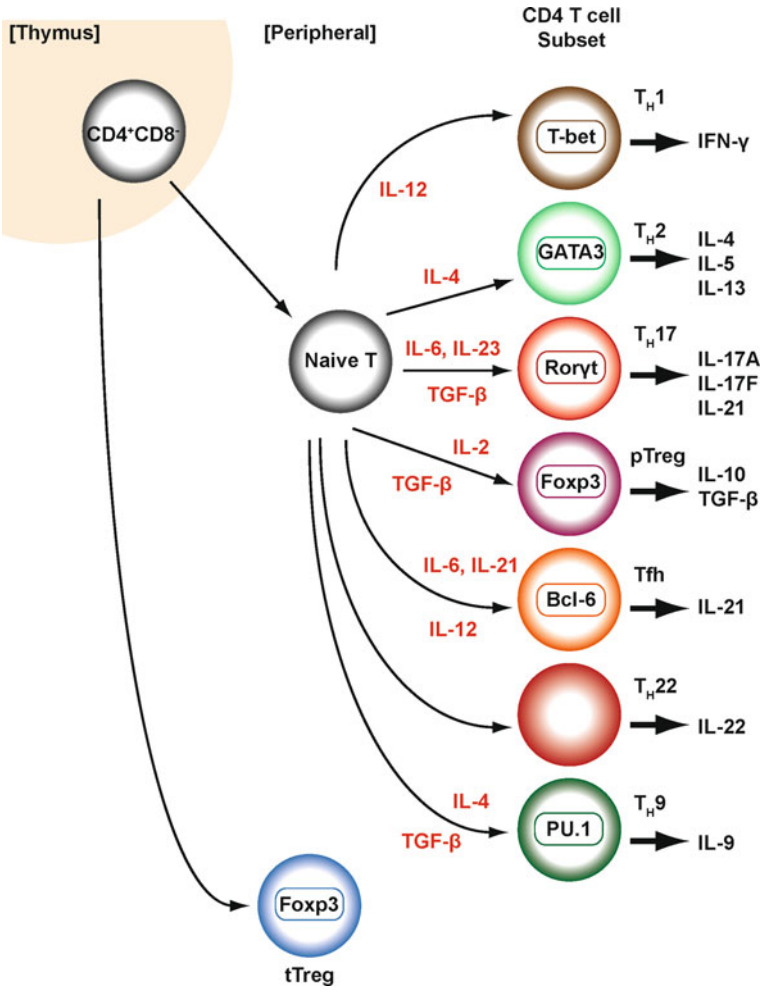

Fig. 1 Classical view of helper T cell differentiation. Helper T cells can differentiate from naïve T cells into diverse helper T cells, including Th1, Th2, Th17, T follicular helper (Tfh) cells, Th9 and Th22 cells and peripheral derived regulatory T cells (pTreg) and in contrast to thymically derived regulatory T cells (tpTreg) cells that develop in the thymus

Traditionally, helper T cell differentiation has been viewed as a largely irreversible process akin to terminal differentiation of other specialized cells. In this way, naive CD4 T cells can be regarded as a pluripotent cell, specification of which is initiated by environmental triggers to generate distinctive "lineages" in which different master regulator transcription factors are induced that in turn supervise unique transcriptional programs. However, as the recognition of the number of different "lineages" and fate choices available for CD4 T cells continue to expand, the extent to which this view is apt has been reexamined. Moreover, considerable evidence

continues to mount that argues for a view in which differentiated $CD4^+$ T cells retain the capacity to redirect their functional programs in response to changing milieus. The complexity of helper cell fate determination is made further complicated by the appreciation of overlap between "effector" and "regulatory" functions.

2.1 The Diversity of Regulatory T Cells

In addition to orchestrating immune responses by production of key effector cytokines, diverse types of regulatory $CD4^+$ T (Treg) cells are essential for maintenance of immune self-tolerance and homeostasis by suppressing excessive immune responses (Sakaguchi et al. 2008). A subset of Treg cells is defined by their expression of the transcription factor forkhead boxp3 (FoxP3), which is necessary for the development and maintenance of these key cells (Zheng and Rudensky 2007). Mutations of *FOXP3* underlie the disorder termed immunodysregulation polyendocrinopathy eneropathy X-linked (IPEX) syndrome (Bennett et al. 2001). Likewise, mice that carry a mutation or deletion of *FoxP3* develop a fatal systemic autoimmune disease (Brunkow et al. 2001; Fontenot et al. 2003). Overexpression of FoxP3 confers suppressive activity to conventional T cells, leading to the conclusion that FoxP3 serves as the master regulator and a lineage-specifying transcription factor for Treg cells (Hori et al. 2003). Equally though, it is also recognized that an array of other transcription factors contribute to the phenotype of Treg cells; indeed, FoxP3-dependent and FoxP3-independent factors are important for the phenotype of regulatory cells (Fu et al. 2012).

Treg cells that are derived from the neonatal thymus in response to self-antigens have been termed thymic derived regulatory (tTreg) T cells (Shevach 2000). Their program of specification is critically dependent upon TCR signals that lead to induction of FoxP3 (Ohkura et al. 2012). Peripheral derived (p)Treg cells, by contrast, develop in the periphery in response to environmental self-antigens (Chen et al. 2003; Horwitz et al. 2008; Abbas et al. 2013). Induced Treg (iTreg) cells can be generated from naïve precursor cells in vitro through activation and cytokine receptor engagement (Fig. 1). More specific markers, like the Ikaros family transcription factor Helios, or neuropilin-1, which are thought to distinguish tTreg and pTreg cells, have been described recently (Thornton et al. 2010; Yadav et al. 2012; Hansen et al. 2012; Delgoffe et al. 2013). IL-2 signals through STAT5, which in turn binds directly to the *FoxP3* promoter. Continued stable expression of Foxp3 is influenced by IL-2 receptor expression, which presumably invokes STAT5 in maintenance of FoxP3 expression (Miyao et al. 2012). Conversely STAT3-dependent signals can destabilize Foxp3 expression (Laurence et al. 2012). Stable Foxp3 expression depends on DNA methylation of a selected 5'intronic region, called Treg-specific determining regions (TSDR) of the Foxp3 gene (Huehn et al. 2009). All *trans* Retinoid acid (RA) has been shown to augment TGF-beta and TCR induced effects (Coombes et al. 2007; Mucida et al. 2007). RA facilitates the binding of pSMAD3 to the enhancer of Foxp3 (Xu et al. 2010).

FoxP3-expressing Treg cells, however, are not the only regulatory T cells. On the contrary, multiple types of regulatory T cells exist and go by a variety of names, such as Th3, Tr1, or Tr35 cells. These cells produce critical anti-inflammatory cytokines like TGF-β, IL-10, and IL-35 (Weiner et al. 2011; Battaglia et al. 2006; Collison et al. 2007; Gagliani et al. 2013; Awasthi et al. 2007). Absence of TGF-β or its receptors results in lethal autoimmunity. Many cells produce TGF-β, but T cell expression of TGF-β and its receptors is critical (Li and Flavell 2008; Oh and Li 2013; Li et al. 2006, 2007a). Similarly, IL-10 is another key immunoregulatory cytokine. Mutations and polymorphisms of *IL-10* or its receptor are associated with inflammatory bowel disease (Glocker et al. 2009; Engelhardt et al. 2013). IL-22 is yet another critical cytokine; in certain circumstances, it too has essential anti-inflammatory properties (Nakagome et al. 2011). Finally, IL-35 is a regulatory cytokine that is able to generate a population of cells with suppressive activity called Tr35 cells (Collison et al. 2007). Added to this is recognition that cytotoxic effector cells, including NK cells that express perforin, can have regulatory functions by limiting T and myeloid cell function (Magnani et al. 2011; Crome et al. 2013).

Thus, it should be clear that what comprises the constellation of regulatory T cells and how they exert their immunosuppressive effects is anything but simple.

3 Blurring the Lines of "Lineages"

Despite views of different T cell subsets as stable terminally differentiated lineages, many lines of evidences argue for a more fluid view of regulation (Zhou et al. 2009a; O'Shea and Paul 2010). There are many examples at this point, so just a few illustrative examples will be provided: For instance, extended culture of Th17 cells leads to IFN-γ production (Mukasa et al. 2010) and the IFN-γ^+ cells that arise also under Th17-inducing conditions can have properties distinct from conventional Th1 cells (Boniface et al. 2010). IL17/IFN-γ double positive T cells are found under normal and inflammatory conditions in mouse and man (Annunziato et al. 2007; McGeachy et al. 2007; Acosta-Rodriguez et al. 2007a; Chen et al. 2007; Lee et al. 2009; Bending et al. 2009). Recent data show that co-expression of T-bet and Runx1 or Runx3 is required for the generation of pathogenic IFN-γ producing Th17 cells. At this point, the notion that Th17 cells are heterogeneous collection of cells is no longer a subject of debate.

Many cells also produce IL-10, not just Tr1 cells. In fact, IL-10 was first noted in Th2 cells and Th17 cells also make IL-10. In *S. aureus*-primed cultures, Th17 cells produce IL-17 and IL-10 (Zielinski et al. 2012). Again, it needs to be remembered that cells other than T cells clearly produce IL-10; this cytokine is widely produced by myeloid, B, and NK cells in addition to T cells.

Th2 cells have a certain degree of plasticity too. Infection with lymphocytic choriomeningitis virus can reprogram Th2 cells into GATA-3$^+$T-bet$^+$ cells, which gain the capability to produce IL-4 and IFN-γ (Hegazy et al. 2010). This reprogramming is

mediated by IFN-γ and STAT1. Hybrid Th1/Th2 cells can develop in response to IFN-γ, IL-12 and IL-4 (Peine et al. 2013).

Tfh cells are one of the hardest cells to pigeonhole as a "lineage," as they do not have a unique pattern of signature cytokine secretion and have the ability to produce cytokines of other lineages. IL-21-producing Tfh-like cells generated in vitro can be re-differentiated upon re-culture with the appropriate cytokines to make IFN-γ, IL-4, or IL-17 (Lu et al. 2011). In addition, Tfh features are present early in Th1 differentiation, but as differentiation proceeds, Tfh characteristics are repressed and Th1 features dominate (Nakayamada et al. 2011). This flexibility is not limited to in vitro differentiation. In vivo, Tfh cells express the cytokines that are signatures of a given infection. During helminth infection, for example, all the IL-4-producing cells in the lymph nodes are located in germinal centers, suggesting they are in fact Tfh cells (King and Mohrs 2009; Glatman Zaretsky et al. 2009; Reinhardt et al. 2009). Similarly, during a Th1-type bacterial infection, Tfh cells express IFN-γ Reinhardt et al. 2009). Given this flexibility, it is hard to view Tfh cells as a classic lineage analogous to Th1 or Th2 cells. An alternative model that would incorporate inherent flexibility would be to differentiate between cells that are retained in the lymph nodes versus those that exit: only the former would be bonafide Tfh cells and those that migrate to the tissues would be viewed as Th1 or Th2 cells. In this way, the "lineage" would be defined by localization and not production of signature cytokines. T cells retained in the lymph node would likely receive additional signals from B cells, which would further enforce or stabilize the Tfh features of the T cell, while permitting acquisition of some features of Th1 or Th2 cell (Crotty 2011; Deenick et al. 2010; Goenka et al. 2011; Cannons et al. 2013). Until the specific signals to drive Tfh cells are worked out, this will likely remain an area of active investigation.

While it is accepted that iTreg cells are prone to Foxp3 instability and can produce effector cytokines (Yang et al. 2008a), the debate to what extent tTreg cells are plastic or not is still ongoing (Bailey-Bucktrout and Bluestone 2011; Hori 2011). Using Foxp3 lineage reporter mice, two groups reported that tTreg cells were stable (Rubtsov et al. 2010; Miyao et al. 2012). Using a different model, it has been reported that Treg cells can lose Foxp3 expression, gain effector attributes, and become drivers of inflammation (Zhou et al. 2009b; Bailey-Bucktrout et al. 2013; Komatsu et al. 2014).

3.1 Responding to More than One Master

The recognition of substantial plasticity of differentiated helper T cells begs the question as to what mechanisms drive stability and flexibility of phenotype. In the following sections, we will review these mechanisms. In broad, nonmutually exclusive terms can be influenced by flexible expression of "master regulators" and other transcription factors, which themselves are regulated by post-translational regulation of transcription factors and exert their effect in conjunction with metabolic, dietary, and microbial factors. Along with well-known factors like cytokines,

all these signals are integrated to modify diverse genomic switches in developing CD4 T cells and techniques are rapidly being devised to measure the impact of transcription factors and epigenomic changes on global, comprehensive scales. We will start the discussion with insights into flexible and complex expression of master regulator transcription factors.

One important insight that has emerged recently is recognition of the limitations of viewing helper T cells from the perspective of a single "master regulator" effecting lineage commitment. Perhaps such a model is reasonably apt for some sessile tissues, like muscle, in which MyoD can appropriately be visualized as a true master regulator. Though the classical view of helper T cells as a homogeneous population that expressed a signature cytokine in response to a single master regulator transcription factor was a useful construct, the limitations of this "fallacy" has also become increasingly apparent (Crotty 2012). A major limitation to a monolithic view of CD4 lineages is the proliferation of observations that helper cells can express multiple "master regulators." T-bet, for example is now appreciated to be expressed in FoxP3$^+$-Treg, Bcl6$^+$-Tfh, Rorγt$^+$-Th17, and even GATA3$^+$-Th2 cells (Koch et al. 2009; Oldenhove et al. 2009; Ghoreschi et al. 2010; Cosmi et al. 2008, 2011; Nistala et al. 2010).

In some cases, key transcription factors work in opposition, titering functionality. On one hand, T-bet can recruit the transcriptional repressor Bcl-6 (Oestreich et al. 2011); at low cellular concentrations of Bcl-6 the formation of a T-bet-BCL6 complex can block Bcl6 from repressing its target genes (Oestreich et al. 2012). Analogously, FoxP3 represses ROR$\gamma\tau$ and both transcription factors can be co-expressed (Zhou et al. 2008; Yang et al. 2008a; Lochner et al. 2008; Voo et al. 2009). In some circumstances, Treg cells can produce effector cytokines (Stock et al. 2004; Sawitzki et al. 2005; Wei et al. 2009), and in humans some IL17-producing cells arise from cells that expressed FoxP3 (Beriou et al. 2009). This is important to keep in mind insofar as FOXP3 is generally more fluid in its expression in human cells, and is broadly induced in activated T cells, and is not necessarily associated with suppressor cell functionality (Wang et al. 2007; Roncador et al. 2005).

Another view is that expression of more than one "master regulator" can help provide specialized functions. Like other helper cell subsets, Treg cells are also heterogeneous. Co-expression of FoxP3 with T-bet, GATA3, Bcl6, or STAT3 has been argued to produce specialized subsets of Treg cells that control Th1, Th2, Tfh, or Th17 responses (Oldenhove et al. 2009; Koch et al. 2009, 2012; Wang et al. 2011d; Chung et al. 2011; Wohlfert et al. 2011; Chaudhry et al. 2009). Similarly, Treg cell flexibility can also be controlled by transcription factors such as Eos (IKZF4), a known corepressor for FoxP3 (Sharma et al. 2013). The marked heterogeneity of Treg cells is further illustrated by the identification of populations of Treg cells in nonlymphoid tissues that can be characterized by differential chemokine receptor expression (Burzyn et al. 2013a, b). In adipose Treg cells, PPAR-γ collaborates with Foxp3 to impose on naïve T cells the distinctive transcriptional profile (Cipolletta et al. 2012).

Adding to this complexity is the increasing recognition that master regulators work in concert with an array of other transcription factors. Even though overexpression

of a factor might be associated with a phenotype, the array of other factors present in the cell is certainly not irrelevant. Indeed, in Treg cells much of the transcriptional signature is not ascribable to FoxP3, but rather to other factors and other signaling pathways (Fu et al. 2012). Another good example of a critical transcription factor that preserves immune tolerance is Bach2, which pervasively limits effector cell differentiation. Bach2 is important for homeostasis of both Treg and conventional T cells. In reality, a cadre of factors are important for helper T cells including Runx1, Runx3, Bcl6, c-Maf, Blimp1 (encoded by Prdm1), IRF4, Batf, NFκb, IKZF2, IKZF3, and many others. Exactly how these factors all work together is very poorly appreciated. What is clear is that along with GATA3 and Rorγt, which have functions at multiple points in development, networks of transcription factors may not neatly track with a single lineage. IRF4 is an obvious example—it is important for Th2, Th17, Th9, iTreg, and TFH cells (Staudt et al. 2010; Ahyi et al. 2009; Brustle et al. 2007; Kwon et al. 2009).

A technology that is revolutionizing how we think about transcription factor action is chromatin immunoprecipitation and massive parallel sequencing. This technique, developed in 2007, allows the enumeration of genome-wide binding sites for DNA-binding proteins (Rivera and Ren 2013). This information is rapidly accumulating thanks to efforts of Encode and Roadmap (http://www.encode-roadmap.org); however, our understanding of the functional consequence of the binding of all these different transcription factors in distinct states of T cell activation is in its infancy. Even more challenging though is trying to make sense of this onslaught of data. As will be discussed below, we have some sense of what different transcription factors are doing with respect to organizing the epigenome and the results are surprising. Despite the daunting prospect of this task, it is now feasible to rigorously map binding of the major factors and try to impute their functions.

3.2 Transcription Factors are Regulated Post-Translationally

Another mechanism that needs to be borne in mind regarding transcription factors, including the so-called master regulators is that they can be regulated post-transcriptionally. In other words, mere presence of transcription factor may not be sufficient for action of a transcription factor; covalent modifications of diverse types can positively and negatively regulate function. For instance, master regulators like T-bet are regulated by phosphorylation (Hwang et al. 2005a, b; Jang et al. 2013). Linking all the relevant pathways that might impinge upon this modification is important, but this area is surprisingly understudied and the number of potential modifications beyond phosphorylation is immense.

Also, like other proteins, transcription factors can be actively degraded and this offers another molecular mechanism for regulating phenotypic stability. This appears to be the case for FoxP3; the levels of this key protein can be controlled in a number of ways. HIF-1 attenuates Treg cell development by binding FoxP3 and

targeting it for proteasomal degradation. Importantly, this regulation occurs under both normoxic and hypoxic conditions, and HIF-1α-deficient T cells have reduced pathogenic potential (Dang et al. 2011). FoxP3 can be also regulated by poly-ubiquination of multiple lysine residues (van Loosdregt et al. 2013). Stress signals, like proinflammatory cytokines or lipopolysaccherids can also lead to the degradation of FoxP3 through the ligase Stub1 (Chen et al. 2013). Degradation of key factors like FoxP3 and other "master regulators" obviously can have profound effects on helper cell function and stability of phenotype.

3.3 Control of Helper Cell Fate by Metabolic Cues

We have discussed the importance of exposure to cytokines in the determination of how T helper cells differentiate after exposure to cognate peptide. In addition to cues from co-receptors and cytokines, the availability of nutrients, oxygen, and even the balance of salts in their local environment influence T cell differentiation.

A resting naïve T cell requires little energy, and what little metabolism occurs does so aerobically. By contrast, an activated T lymphoblast switches on metabolic pathways, similar to cancer cells, catabolizing glucose anaerobically and using the metabolites to generate building blocks of protein, lipid, and DNA synthesis (Fox et al. 2005). The serine kinase mTOR (mammalian Target of Rapamycin) acts as a nutrient sensor: it is inhibited by a deficiency in the availability of amino acids particularly leucine and glutamine (Christie et al. 2002; Nicklin et al. 2009) or by a decrease in the ATP:AMP ratio (Gwinn et al. 2008). By contrast, mTOR is activated by T cell receptor, CD28 co-receptor stimulation, and cytokines known to support T cell proliferation via their ability to activate Phospho-inositol phosphatidyl 3′ kinase (PI3 K) (Brennan et al. 1997; Lafont et al. 2000; Kane et al. 2001). mTOR in the centerpiece of two signaling complexes: mTOR complex 1 (mTORC1) and complex 2 (mTORC2). mTORC1 is critical for the translation of many proteins including both metabolic enzymes and the transporters required for cells to take up local nutrients (Gordan et al. 2007; Wang et al. 2011c). mTORC2 has a specialized role in activating members of the AGC serine kinase family including protein kinases B and C (PKB/Akt and PKC) and the serum and glucocorticoid inducible kinase-1 (SGK-1) (Guertin et al. 2006; Garcia-Martinez and Alessi 2008). Complete inhibition of mTOR by the immunosuppressive rapamycin, blocks T effector cell differentiation and facilitates the development of $FoxP3^+$ Treg cells (Delgoffe et al. 2009) in part through its regulation of expression of HIF1a (Quinlan and Hall 2010).

Using genetic models it is possible to see the contributions of the individual mTOR complexes; loss of mTORC1 prevents Th1 and peripheral Th17 differentiation (Delgoffe et al. 2011), whereas loss of mTORC2 prevents Th2 differentiation and Th17 differentiation within the Thymus (Delgoffe et al. 2011; Kim et al. 2013). Recent work has linked SGK-1 with IL-23 dependent Th17 differentiation, a process that is influenced by the sodium concentration of the environment (Kleinewietfeld et al. 2013; Wu et al. 2013).

3.4 Control of Helper Cell Differentiation by the Microbiome

In thinking about factors that drive helper cell differentiation, it cannot be ignored that all barrier sites are inhabited by a community of commensal organisms, which greatly out-numbers the host in number of cells and genes (Grice et al. 2009; Ley et al. 2006; Beck et al. 2012). Our knowledge of composition of the microbiota has grown exponentially over the past decade as sequencing technology has allowed us to capture a better picture of the diversity among various commensal communities. What has also emerged is that it is not just pathogens that drive helper T cells specification—commensals are also critical factors. The commensal microbiota of the GI tract in particular contributes to host health via the provision of key metabolites and nutrients derived from food that is otherwise indigestible by the host. However, since commensals are microbes, they have the potential to induce inflammatory immune responses. Therefore, the mammalian immune system has evolved complex mechanisms to prevent untoward activation at barrier sites (Hooper and Macpherson 2010). For instance, the immune system has evolved to receive homeostatic cues from the microbiota, as it has long been noted that the immune system does not properly develop in germ-free animal models (Smith et al. 2007; Bauer et al. 1963). Multiple studies have revealed that some, but not all commensal strains can compliment certain immune defects associated with germ-free development, implying that the immune system interacts with specific commensals and their products (Mazmanian et al. 2005; Umesaki et al. 1999; Hall et al. 2008). Perhaps the best example of this phenomenon is the effect of segmented filamentous bacteria (SFB) on the development of Th17 T cells in the GI tract. The direct role for SFB in T cell differentiation came from studies of genetically identical animals from two different vendors that either lacked or possessed Th17 T cells in their GI tissue and the presence of SFB was shown to be sufficient for the differentiation of Th17 cells (Ivanov et al. 2008, 2009; Gaboriau-Routhiau et al. 2009). In contrast, bacteria of the *Clostridia* class have been shown to be associated with the differentiation of Treg cells in the colon (Atarashi et al. 2011, 2013). Interestingly, these bacteria are particularly adept at the production of Short-Chain Fatty Acids (SCFA) and three recent studies have shown that the presence of SCFA powerfully supports the production and maintenance of Tregs in the GI tract (Smith et al. 2013; Furusawa et al. 2013; Arpaia et al. 2013; Pryde et al. 2002). Since the production of SCFA from dietary fiber requires multiple bacterial strains, perhaps SCFA is a key metabolite via which the immune system can ascertain the health of the microbiota. It should be noted that while SFB and *Clostridia* are sufficient for the production of Th17 cells and Treg cells, respectively, they are not strictly necessary as Treg cells are present in reduced numbers in the total absence of the microbiota (Atarashi et al. 2011). As well, some strains of *Bacteroides fragilis* are potent inducers of Treg cells in the GI tract (Round and Mazmanian 2010). Finally, Th17 T cells can be induced in some gnotobiotic mice that lack SFB entirely and Th17 cells at other barrier sites, such as the skin, rely on

local bacterial populations, and are independent of the GI microbiota (Geuking et al. 2011; Naik et al. 2012).

One of the crucial unanswered questions in mucosal immunology is how any given T cell is directed to differentiate to a specific state, given the multitude of coincident signals associated with a diverse commensal microbiota. During infection, T cells specific to commensal antigens have been shown to differentiate according to the dominant inflammatory milieu, but at homeostasis where multiple signals may compete for prominence, how this works is less clear (Hand et al. 2012). Perhaps as a result of multiple signals, the barrier surfaces are home to many T cells that co-express transcriptional modules associated with different states. For example, Treg cells that co-express the transcription factor Rorγt, T-bet, and GATA-3 are commonly found at barrier sites (Wohlfert et al. 2011; Zhou et al. 2008; Koch et al. 2009; Wang et al. 2011d). As well, under inflammatory conditions "pathogenic" Th17 cells that co-express IL-17A and IFN-γ are commonly found in the GI tract (Ahern et al. 2010). While it is clear that commensal microbiota along with pathogenic microbes have a major impact on helper T cell differentiation and plasticity, where are really only just beginning to understand the rules. Suffice it to say that in considering all the environmental factors from cytokines to nutrients and pollutants that provide signals to helper T cells, the enormous diversity of microbial ecosystem is undoubtedly a major factor.

4 Gene Expression, Genomes and Chromatin Organization

It should be very evident at this point that the selective pattern of gene expression that occurs in differentiating helper T cells is the result of numerous extrinsic and intrinsic cues. Precisely how the cell interprets these cues to induce and maintain characteristic gene expression is obviously the critical question. However, the issues of selective gene expression that accompanies lineage commitment and terminal differentiation along with the capacity of differentiated cells to retain plasticity obviously go far beyond T cells. This is a fundamental issue in developmental biology. Accordingly, lessons learned from many systems have implications for understanding these processes in T cells. Reconsidering T cell plasticity is timely insofar as completion of the human and other genomes and advances in sequencing technologies are dramatically changing how fundamental biological problems are viewed (Rivera and Ren 2013).

A surprising finding resulting from the elucidation of human and other genomes was the paucity of "genes" within the genome. Of the roughly three billion of nucleotides in the genome, there are only on the order of 2×10^5 genes in mammals. Despite the paucity of classical genes, as defined by the central dogma of molecular biology (DNA transcribed to RNA translated to protein), it is now recognized that consideration of selective gene expression needs is controlled by a vast number of distal "switches" and that the majority of the genome is transcribed. How accessibility of genes is controlled both locally and by large-scale changes in genomic

architecture is currently the focus of intense investigation. In this context, it is worth reviewing some of the newer insights and how these might relate to helper T cell diversity. Like other specialized cells, for the most part (*TCR* locus excluded), changes in DNA sequence (genome) is not responsible for differential functions; rather it is the "epigenome" that changes dramatically. The epigenome refers to a variety of alterations in chromatin including histone tail modifications, DNA methylation, nucleosome compaction, and long-range chromatin interactions. Production of long noncoding RNAs (lncRNAs) and microRNAs (miRNAs) are additional mechanisms that to contribute to gene regulation, as do distal elements termed enhancers transcribed to produce eRNAs. Better understanding of the dynamic epigenomes of helper T cells have provided useful insights into our notions of plasticity, but this is certainly an area that will receive increasingly greater attention (Wilson et al. 2009; Ansel et al. 2003; Kanno et al. 2012; Zhu et al. 2010). We will start by briefly summarizing the most important epigenetic modifications (Fig. 2).

4.1 DNA Methylation

The classic modification associated with silencing of gene expression is DNA methylation (Fig. 2). DNA modification occurs at cytosine-guanine (CpG) dinucleotides, which are often clustered within promoters and are associated with chromatin-remodeling factors such as methyl-DNA-binding proteins that promote the condensation and inaccessibility of chromatin. In contrast, demethylation of CpG motifs leads to a relaxation of chromatin and an increased accessibility of target sequences, thereby allowing the binding of specific transcription factors. Therefore, CpG methylation at promoters is generally associated with transcriptional repression. CpG methylation is maintained by DNA methyltransferases (DNMTs), which use S-adenosylmethionine as the methyl group donor (Goll and Bestor 2005). Maintenance of a preexisting methylation pattern is catalyzed by Dnmt1; Dnmt1 recognizes a hemi-methylated DNA and introduces methyl-groups on the unmethylated strand (Bestor et al. 1988). In contrast, de novo DNA methylation is mediated by Dnmt3 methyltrasferases (Okano et al. 1998, 1999; Bestor et al. 1988). Both processes are important for stabilizing helper cell phenotypes (Okano et al. 1999; Young et al. 1994; Winders et al. 2004; Thomas et al. 2012; Lee et al. 2001; Makar et al. 2003).

As mentioned earlier, *FoxP3* expression and stability of Treg cells is also regulated by the DNA methylation status at several key genomic loci called the Treg-specific demethylation region (TSDR) (Floess et al. 2007; Baron et al. 2007; Lal et al. 2009; Janson et al. 2008; Polansky et al. 2008; Zheng et al. 2010; Huehn et al. 2009).

Furthermore, recent data show that methylation of tTreg cells is necessary for FoxP3 to acquire a genome-wide Treg cell type gene expression pattern, lineage stability, and suppressive activity. The Treg cell-specific hypomethylation pattern is dependent on T cell receptor stimulation and Foxp3 independent (Ohkura et al. 2012).

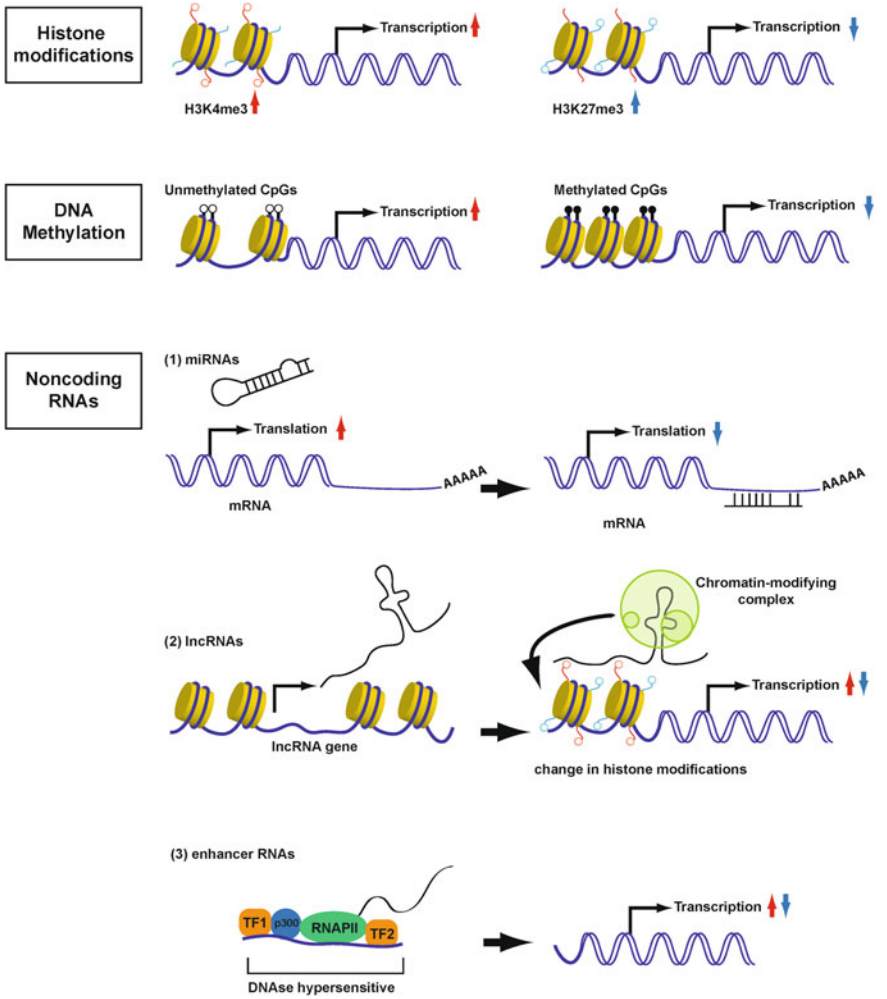

Fig. 2 Epigenetic mechanisms that modify gene transcription. Specific alterations in chromatin including histone tail modifications, DNA methylation, production of long noncoding RNAs (lncRNAs), microRNAs (miRNAs) and enhancer RNAs (eRNAs) are mechanisms that to contribute to gene regulation

While DNA methylation is important for the maintenance of T cell lineage stability, it is also true that massive demethylation occurs during T cell activation, and is selective with specification of subsets. For instance, the *Ifng* locus is demethylated during a Th1 differentiation, whereas differentiated Th2 or Th17 cells sustain their methylation status. Similarly, the *Il4, IL13,* and *IL17a* loci are methylated in naïve T cells and are demethylated during Th2 and Th17 differentiation, respectively (Santangelo et al. 2009; Schoenborn et al. 2007; Aune et al. 2013).

The ability of Th17 to express IL-17 and IFN-γ concomitantly is associated with hypomethylation of the *IFN-γ*, *IL17a,* and *Rorc* promoters (Cohen et al. 2011). DNA demethylation of *Rorc* under inflammatory conditions can lead to IL-17 production in Treg cells (Schmidl et al. 2011). Although this is a widely appreciated event, we have almost no understanding of the molecular basis of these changes. On one hand, the massive proliferation of T cells that occurs with activation is no doubt an important factor; what is less clear is how this process can be so selective.

Global dysregulation of DNA methylation has long been recognized as a potential contributor to autoimmune disease including RA and SLE (Richardson et al. 1990; Corvetta et al. 1991; Absher et al. 2013; Altorok et al. 2013). To some degree it is hard to separate cause and effect since these diseases are associated with T cell activation; however, the fact that drugs known to affect DNA methylation are associated with lupus suggest at least some degree of causality. DNA methylation inhibitors like 5-azacytidine can induce T-cell autoreactivity and lupus symptoms in mice (Quddus et al. 1993), and drug induced lupus is associated with reduced DNA methylation (Cornacchia et al. 1988).

Over the past decade, the global DNA methylation technologies have rapidly evolved; instead of measuring small, localized changes in DNA methylation, it is now possible to measure genome-wide changes. At present though, the quality as compared to the average cost has to be taken into account and the high costs have limited acquisition of data for complete analysis of methylomes. Techniques like endonuclease digestion-based DNA methylation assays (MRE-seq) or affinity-based enrichment assays (MeDIP-seq) for example have moderate costs; although the resolution is comparatively low compared to MethylC-seq or reduced representation bisulfite sequencing (RRBS). Newer techniques like oxidative bisulfite sequencing (oxBS-seq) and TET-assisted bisulfite sequencing (TAB-sequ) yield single base resolution methods, but these remain costly (Rivera and Ren 2013). Array-based methylation assays (such as the Illumina HM450 array), however, can detect DNA methylation at single nucleotide resolution with low cost by only targeting known CpG islands using locus specific probes. As the costs of sequencing continue to decline, the expectation is that DNA methylation datasets will become far more commonplace. This is welcome as this epigenetic modification is thought to be one of the more stable marks.

4.2 Enumerating Global Histone Modifications

Another important factor that dictates accessibility of genes and therefore transcription is that DNA is wrapped into nucleosomes that consist of histone octamers. Histones can be modified by many different post-translational modifications including acetylation, methylation, phosphorylation, ubiquination, sumoylation, etc., which all contribute to define the overall structure of chromatin (Rivera and Ren 2013; Kanno et al. 2012) (Fig. 2); in fact, more than 130 different histone modifications have been described. In the interest of brevity, we will only discuss a

few major modifications. Both active (euchromatin) and silenced (heterochromatin) configurations are associated with certain combination of histone modifications. For example, histone H3 lysine 4 tri-methylation (H3K4me3) is associated with active accessible promoters whereas H3K36 tri-methylation is associated with actively transcribed coding regions. Enhancers are marked by histone H3 lysine 4 mono- and di-methylation (H3K4me1 and H3K4me2). H3 lysine 27 acetylation (H3K27Ac) in the context of H3K4me1 is a mark of an active enhancer; without this mark, H3K4me1 alone indicates a "poised" enhancer. In contrast, histone H3 lysine 27 tri-methylation (H3K27me3) and histone 3 lysine 9 tri-methylation (H3K9me3) are present in broad domains that encompass inactive genes. H3K4me3 and H3K27me3 modifications can both be present at some genomic regions, and these bivalent modifications have been suggested to poise genes ready for either activation or repression during differentiation.

H3K27 tri-methylation is mediated by polycomb proteins, which comprise two major repressive complexes: polycomb repressive complex 1 (PRC1) and PRC2. The polycomb proteins Enhancer of Zeste Homolog 1 (Ezh1) and Ezh2 form two closely related PRC2 complexes that can trimethylate H3K27 and are required for maintenance of cellular identity at several stages of development. Ezh2 constrains differentiation and plasticity of Th1 and Th2 cells, by controlling the expression of *Tbx21* and *Gata3* in developing Th1 and Th2 cells and inhibiting spontaneous generation of IFNγ-producing cells via repression of *Eomes* (Tumes et al. 2013). Interestingly, in Th9 differentiation, TGF-β acting via Smad2 and Smad4 serve to displace Ezh2 from the *Il9* locus (Wang et al. 2013).

The global enumeration of histone marks for helper T cells has been accomplished and the findings help to explain some of their apparent plasticity (Wei et al. 2009). One striking finding is genes that encode key regulatory transcription factors including *Tbx21*, *Gata3*, *Rorc*, *Prdm1*, and others. The presence of bivalent poised domains suggests a potential for phenotypic plasticity even in terminally differentiated cells. This helps explain the previously unanticipated breadth of expression of "lineage-defining master regulators." Assessment of histone modifications reveal that Bcl6 is accessible in all subsets, thus explaining the flexibility of Tfh cells (Nakayamada et al. 2011). Thus, the epigenetic landscape of genes encoding master regulators may allow flexibility in expression and thereby permit the blurring lineages, allow fine tuning or provide subspecialization.

It is important to note that histone modifications can be targeted therapeutically. HDAC inhibitors are the best studied epigenetic therapeutic agents and have been proven to reduce the levels of proinflammatory cytokines (Leoni et al. 2002, 2005). Beneficial effects of HDAC inhibitors have been initially described in animal models of arthritis (Nishida et al. 2004; Lin et al. 2007; Nasu et al. 2008; Saouaf et al. 2009; Joosten et al. 2011). In humans 18 HDACs have been described and further characterized (Wang et al. 2009b). In line with this, it has been reported that blocking histone deacetylase is efficient in treatment of juvenile idiopathic arthritis (Vojinovic et al. 2011). However, the data on T cells are very limited. First of all, some HDAC inhibitors are not very specific and can target other transcription factors like NF-κB or STAT3, which are also important in

inflammation. Second, most data come from other cell types like fibroblasts or macrophages. Studies from in vivo models, like CIA, point to a role of Treg cells, whose number and function was reported to be increased after treatment with valproic acid (Saouaf et al. 2009; Wang et al. 2009a).

An additional level of gene regulation derives from proteins that "read" histone and DNA modifications, such as bromodomain-containing proteins that bind acetylated histones including BRD2, BRD3, and BRD4. The BET family member, BRD4 has a unique C-terminal domain that binds to the positive transcription elongation factor b (P-TEFb; composed of the cyclin-dependent kinase CDK9 and its partner, cyclin T1) complex. BRD4 recruits P-TEFb to acetylated histones, promoting phosphorylation of paused RNA polymerase II (Pol II) and the repressive complexes DSIF and NELF by CDK9, thereby allowing productive mRNA elongation. Of note, BET inhibitors appear to have efficacy in preclinical models of autoimmune disease (Bandukwala et al. 2012). In addition BRD2 and BRD4 associate with the *Il17* locus and control the Th17 differentiation (Mele et al. 2013).

4.3 Defining Enhancers

Many aspects of human biology depend on the tissue-specific control of gene expression (Noonan and McCallion 2010). Regulatory information creating such complex gene expression patterns is accommodated across the noncoding part of genome in the form of cis-regulatory elements. The key point is that only a subset of these cis-regulatory elements is brought into play in every cell type in a temporal and spatial-dependent manner.

Among many types of regulatory elements, enhancers play essential roles in cell type-specific gene expression. Enhancers are noncoding DNA sequences that "enhance" transcription of cognate target genes, regardless of their location or orientation. The *Ifng* and *Il4* loci are good examples of the complicated architecture of finely regulated lineage-specific genes (Wilson et al. 2009; Ansel et al. 2006). For instance, a conserved noncoding sequence in the distal site of the *Il4* locus is critical for IL-4 expression in Tfh cells but not in Th2 cells (Harada et al. 2012). Likewise, a *FoxP3* enhancer (CNS1) is essential for peripheral Treg cells but dispensable for natural Treg cell generation; notably, this element is only active in placental mammals (Zheng et al. 2010; Samstein et al. 2012).

A major breakthrough for enhancer biology was the discovery of the chromatin signatures of enhancers, facilitated by next-generation sequencing technology. It is now believed that presence of a unique combination of histone modifications map the enhancer elements of each cell type. Pioneering work identified H3K4me1-high, H3K4me3-low as a chromatin signature of enhancers in human cells (Heintzman et al. 2009). Not all H3K4me1-high, H3K4me3-low regions are functionally active and these regions are representatives of "poised" enhancers. Additional marks such as the binding of acetyltransferase p300 or H3K27Ac have been related to "active enhancers" in many studies.

It has been argued that the prominent action of master regulator factors supervise the creation of the enhancer landscape (Natoli 2010). Among key transcription factors in the immune system, early studies characterized the role of PU.1, encoded by *Sfpi1,* as an organizer of enhancer elements (Ghisletti et al. 2010; Heinz et al. 2010). These studies suggested that master regulators of other cell types might have similar pervasive impacts on the enhancer cohorts. In the context of $CD4^+$ T cell subset differentiation, master regulators are defined as required and sufficient transcription factors for programming a T cell subset. Another aspect of this definition is that the master regulator has a restricted expression pattern, which means that it is only found in cells of a specific fate. As mentioned above FoxP3 has been argued to be necessary and sufficient for Treg cell development. However, FoxP3 has limited impacts on the enhancer landscape of Treg cells (Samstein et al. 2012). The major changes in the epigenetic landscape of tTregs are FoxP3-independent and are created by TCR-dependent signals. Likewise, the master regulators of Th1, Th2, and Th17 cells, have also limited impacts on key enhancer cohorts. Taken together, these studies provide compelling evidence that the master regulators involved in helper cell specification are not the factors that shape the enhancer landscape of $CD4^+$ T cell subsets. It remains a question then how the many T cell expressed transcription factors work in concert to create the genomic enhancer landscape of T cells.

A key aspect in the acquisition of distinct T helper cell phenotypes is the cytokine milieu. Upon encounter of diverse microbial pathogens, dendritic cells and other cells of the innate and adaptive immune system produce cytokines, which serve to instruct distinct T cell fates. The major specifying cytokines exert their effect through STAT family transcription factors. Strikingly, the majority of differentially active enhancers in Th1 and Th2 cells and Th17 cells are STAT-dependent and many are direct STAT targets of STATs (Hirahara et al. 2011). Importantly, reconstitution of STAT4- and STAT6-deficient cells with the master regulators T-bet and GATA3 failed to recover the active enhancer landscapes, again arguing for a primary role of environmental sensors in dictating global landscapes.

A new subset of enhancers termed “super” or “stretch” enhancers has recently been appreciated. Super enhancers regions have been suggested to have crucial functions in defining cell identity. In embryonic stem cells for instance, super enhancer domains are associated with genes that play essential roles in ESC biology. Transcription at super enhancer genes is also disproportionately sensitive to any perturbations. The super enhancer landscapes of helper T cells have not been cataloged, but this is obviously an area of considerable interest.

4.4 Transcription Abounds

A recent surprise that even though only 2 % of the genome represents standard, protein-coding genes, most of the genome is active and roughly 80 % of the genome transcribed despite the fact that no protein is produced. RNA-seq technology

and other technologies have permit this abundance of transcription, including the identification of the repertoire of noncoding RNAs that include microRNAs (miR), enhancer RNA (eRNA) and long noncoding RNA (lncRNA) (Birney et al. 2007; Kapranov et al. 2007; Kim et al. 2010). Work on eRNAs and lncRNAs is just beginning, although we already know that eRNAs are functionally important for promoting accessibility of canonical protein-coding genes (Mousavi et al. 2013; Hu et al. 2013a).

4.5 MicroRNA and Helper T Cells

MiRNAs are transcribed by RNA polymerase II as long precursor transcripts termed pri-miRNAs, which are then processed into premiRNAs by the enzyme complex including the RNase III type endonuclease Drosha and its partner DiGeorge syndrome critical region gene 8 (DGCR8). In the cytoplasm, premiRNAs are further cleaved by the RNase III endonuclease Dicer to generate a miRNA-induced silencing complex (miRISC), which destabilizes target mRNA and reduces their translation into proteins (Bartel 2009; Fabian et al. 2010). Loss of Drosha and Dicer can lead to instability of Th cells and cause autoimmunity (O'Connell et al. 2010b; Bartel 2004; Chong et al. 2008; Liston et al. 2008; Baumjohann and Ansel 2013). By targeting crucial T cell lineage commitment genes, miRNAs are involved in regulation of gene expression network that determines T cell identity, plasticity, and function (Fig. 2).

Individually, a variety of miRNAs have been shown to influence effector cell differentiation and stability. Once again, this is a fast-moving field and our goal is not to be comprehensive, but rather to give a few relevant examples of factors that influence gene expression. For instance, MiRNA-155 is a multifunctional miRNA that regulates a number of aspects of B and T cell functions. Its effects include the development of Th1, Th17 cells, and miR-155 deficient mice are also protected from EAE and CIA (Murugaiyan et al. 2011; Bluml et al. 2011; O'Connell et al. 2010a). MiR-155 is also expressed in Tregs and miR155 deficient mice have lower numbers of Treg cells (Lu et al. 2009) and develop enteric and lung inflammation (Rodriguez et al. 2007). Mir-155 is regulated by several transcription factors, such as STAT3 (Escobar et al. 2013) and FoxP3 (Zheng et al. 2007; Marson et al. 2007), which is compatible with miR-155 expression in Th1, Th17, and Treg cells.

One important target of MiR-155 is suppressor of cytokine signaling 1 (*Socs1*; Lu et al. 2009), which negatively regulates IL-2 signaling, and is important for the maintenance of Treg cells. MiR-155 also targets SMAD2 (Louafi et al. 2010), SMAD5 (Rai et al. 2010), and alters TGF-β signaling. In effector T cells, *Socs1* and SH2 domain-containing inositol polyphosphate 5≠ phosphatase 1 (*Ship1*), which is negative regulator of cytokine signaling in addition to Socs1, are important targets of miR-155 as well as *Ifngr1* (Huffaker et al. 2012; Banerjee et al. 2010). Recently, miR-155 directly targets Ets1, a negative regulator of Th17

differentiation, and subsequence down regulation of Th17-specific genes, such as IL-17F, IL-22, and IL-23R (Hu et al. 2013b).

Mir146a is another multifunctional MiRNA. Mir-146a-deficient T cells show increased IFN-γ production (Yang et al. 2012; Huffaker et al. 2012; Lu et al. 2010) because miR-146a regulates Th1 differentiation by targeting Stat1 (Lu et al. 2010). MiR-146a also targets IL-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6), and derepression of IRAK1 and TRAF6 increase activation of NFκB. This leads to TCR hyper-responsiveness and subsequent upregulation of IFN-γ expression from effector T cells (Yang et al. 2012). In addition, miR-146a seems to have an effect on Treg cell function (Lu et al. 2010).

Mir-181 also augments TCR signaling. Mir-181a targets several phosphatases involved in proximal TCR signaling, such as tyrosine phosphatases H2 domain-containing protein tyrosine phosphatase 2 (*Shp2*) and protein tyrosine phosphatase nonreceptor type 22 (PTPN22) and inactivator of MAP kinases dual-specificity protein phosphatase 5 (*Dusp5*) and *Dusp6* (Li et al. 2007b). Skewed TCR signaling by inhibition of miR-181a impairs sensitivity of double positive cells in thymus and obstruct positive and negative selection (Li et al. 2007b). Mir-181a is also important in the peripheral $CD4^+$ T cell response. Expression of miR-181a is high in human neonatal naïve $CD4^+$ T cells, and age-associated decrease of miR-181a increased DUSP6 expression, and diminished ERK phosphorylation via TCR stimulation (Palin et al. 2013). Reconstitution of miR-181a lowered DUSP6 expression, and restored $CD4^+$ T cell responses (Li et al. 2012).

The miR-17$\sim$92 cluster regulates several aspects of T cell activation but importantly, regulates Tfh differentiation, relevant targets being Rora (Baumjohann et al. 2013) and CXCR5 (Yu et al. 2009).

Mir-10a attenuates the conversion of iTregs into Tfh cells by targeting Bcl6; miR-10a can also limit Th17 differentiation (Takahashi et al. 2012).

MiRNAs regulate broad range of gene modification in various immune cells including Th cells. The role of miRNAs in different diseases has been studied extensively. Especially, the role of miR-155 and miR-146 has been best described as pro and anti-inflammatory genes in both in infectious and autoimmune diseases in human and mouse. Silencing of miR-155 reduced the susceptibility of LPS treatment in murine model (Androulidaki et al. 2009). Contrary, lack of miR-146a exhibited exaggerated inflammatory response to LPS (Boldin et al. 2011). MiR-155 and miR-326 are overexpressed in active multiple sclerosis (MS) brain lesions, and mice lacking or silencing of these miRNAs leads to ameliorate the symptom of EAE, the mouse model of MS (Junker 2011). In another autoimmune disease, systemic lupus erythematosus (SLE), underexpression of miR-146a, a negative regulator of interferon signaling, has a negative correlation with the disease activity (Tang et al. 2009). The involvement of MiR-155 and miR-146 is also reported in rheumatoid arthritis (Kurowska-Stolarska et al. 2011; Nakasa et al. 2008; Pauley et al. 2008), atopic dermatitis (Sonkoly et al. 2010), Sjoegren's syndrome, and IgA nephropathy (Wang et al. 2011b). Modulation of miRNAs might be an effective

therapeutic approach. Recent reports have shown experimental evidences of the efficacy about blocking miRNA. Antagonizing miR-126 reduce the Th2 response in asthma model (Collison et al. 2011).

4.6 Long Noncoding RNAs

More recently, long noncoding RNAs (lncRNAs) have taken the center stage within the noncoding world as critical regulators of cellular identity. Dramatically, germline knockouts of several lncRNAs have proven to be embryonic or postnatal lethal, emphasizing that lncRNA are not simply transcriptional noise (Sauvageau et al. 2013; Marahrens et al. 1997). Work with ES and iPS cells have shown that lncRNAs are highly dynamic and play an integral role in both maintaining pluripotency and promoting differentiation, so it is attractive to envision that they would be essential players in the face of a dynamic $CD4^+$ immune response (Guttman et al. 2011; Sheik Mohamed et al. 2010; Loewer et al. 2010) (Fig. 2).

lncRNAs are a remarkably diverse class of transcripts that together are defined as being larger than 200 nucleotides and lacking a functional open reading frame. Like mRNA, lncRNAs may be transcribed by RNA polymerase II, 5′ capped, spliced, 3′ polyadenylated, and bound by ribosomes (Ingolia et al. 2011). Several large-scale studies have been conducted to identify lncRNAs resulting in an overwhelming number of transcripts: 9277 human lncRNA genes were identified through the ENCODE project (Derrien et al. 2012); using cDNA sequencing FANTOM found 34,030 lncRNA transcripts (Maeda et al. 2006; Carninci et al. 2005); 1,586 mouse and 1,833 human novel lncRNAs were identified using adjacent H3K4me3 and H3K36me3 chromatin signature signifying activated genes (Guttman et al. 2009; Khalil et al. 2009); and 4,662 lncRNAs were established using RNA-seq across 24 human tissues and cell types (Cabili et al. 2011). Two studies have examined the lncRNA population with the adaptive immune compartment. The first used custom microarrays to identify ~1,200 lncRNAs within human and mouse $CD8^+$ T cell with differential lncRNA expression between the naïve/memory and effector populations (Pang et al. 2009). Recently Keji Zhao's group through an impressive tour de force cataloged the both the poly-A and total lncRNA transcriptome from murine thymocytes and Th1, Th2, Th17, and iTreg differentiated $CD4^+$ T cells across a 2-week time course (Hu et al. 2013a). 1,524 lncRNA-expressing genomic regions were identified, the majority of which were polyadenylated, with 464 regions in the double-negative thymocytes, 515 in the double- and single-positive thymocytes, and 646 in the naïve /differentiated $CD4^+$ helper T cell subsets. The lncRNAs were dynamically regulated both temporally and across the various cell types; with the T helper cells subsets only 15.5 % of the lncRNAs were shared, in contrast to 78 % of the 13,934 mRNA transcripts.

lncRNAs have been shown to act through an assortment of differing mechanisms. A strong trend that has emerged is that lncRNAs plays a prominent role forming ribonucleoprotein complexes that mediate epigenetic regulation and

transcriptional expression of highly specified target genes (Mattick et al. 2009; Bernstein and Allis 2005; Rodriguez-Campos and Azorin 2007). Not only is RNA is an integral component of chromatin but many histone methyltransferases lack DNA-binding properties, instead possessing RNA-binding motifs, indicating that lncRNAs may play a much more pervasive role that currently recognized (Rodriguez-Campos and Azorin 2007; Khalil et al. 2009). One such example is TMEVPG1 (NeST; LincR-Ifng-3′AS), a lncRNA expressed in Th1 and $CD8^+$, but not NK cells (Vigneau et al. 2003; Collier et al. 2012; Gomez et al. 2013). This lncRNA protects against persistent Theiler's virus infection by regulating the epigenetics of Inf-γ through association with WDR5, a scaffolding protein for the SET1 family of methyltransferases.

In addition to acting as scaffolds for histone modifying proteins, lncRNAs have been reported to regulate multiprotein complexes responsible for transcription factor activity. NRON (ncRNA repressor of the nuclear factor of activated T cells), a lncRNA expressed in both mice and humans, acts to bring together NFAT, IQGAP1, and the NFAT kinases CK1, GSK3, and DYRK, thereby maintaining NFAT in a phosphorylated, inactive state (Willingham et al. 2005; Sharma et al. 2011). Depletion of NRON resulted in enhanced NFAT dephosphorylation, nuclear transport, and increased IL-2 production. None of the proteins within the NRON-NFAT complex contained an identified RNA-binding domain, suggesting that lncRNAs may play a wider role in transcription factor regulation than previous appreciated.

lncRNAs can also serve as direct regulators of transcription, as is with the case of the lncRNA T early alpha (TEA). The act of transcribing TEA regulates transcription across the Jα segments of the TCRα by activating the Jα58, Jα57, and Jα59 promoters while also opening the chromatin associated with the promoterless Jα61, Jα53, and Jα52 regions. Knocking out TEA, or inhibiting its transcription through the insertion of a transcription terminator cassette abrogates germline transcription across these segments (Abarrategui and Krangel 2006, 2007; Corcoran 2010).

lncRNAs can also regulate T cell function by acting as decoy targets for protein and miRNAs. GAS5 represses the glucocorticoid pathway by acting to inhibit glucocorticoid receptor (GR) transcriptional regulation of glucocorticoid-responsive genes (Kino et al. 2010). Repression is mediated through a hairpin structure on GAS5 that resembles the glucocorticoid response element (GRE) found within DNA. The GAS5 GRE-decoy binds to the GR (Kd ~30 nM, compared to ~60 nM for DNA-GRE binding to GR), thereby preventing GR binding to the DNA. The lncRNA contains a 5′ terminal oligopyrimidine tract (5′ TOP) that results in active degradation by nonsense-mediated decay in proliferating cells but confers transcript stability under conditions that result in growth arrest such as serum starvation, loss of growth factors, or pharmacological inhibition of the mTOR pathway (Schneider et al. 1988; Coccia et al. 1992). Depletion of GAS5 by siRNA in T cells prompted continued proliferation in otherwise exhausted cultures, while overexpression of GAS5 attenuated proliferation (Mourtada-Maarabouni et al. 2008). lncRNAs can also function as miRNA decoys (Poliseno et al. 2010; Salmena et al. 2011). Termed competing endogenouse RNAs (ceRNAs), they can

act as miRNA sponges to sequester miRNAs and protect protein-coding mRNAs from degradation. Recently, a new class of circularized miRNA sinks (cirRNAs) has been identified with increased stability and a higher capacity to sequester miRNAs than ceRNAs (Hansen et al. 2013; Memczak et al. 2013).

An exciting new area of RNA biology is the discovery that active enhancers produce non-polyadenylated RNA transcripts, called eRNA (enhancer RNA) (Djebali et al. 2012). eRNAs are bi-directionally transcribed from the enhancer and function to promote chromatin looping and formation of the RNAPII transcription machinery (Mousavi et al. 2013; Melo et al. 2013; Li et al. 2013). Notably, the presence of eRNAs appear to have a strong association with active enhancers, and may prove to be a better indicator of enhancer activity than currently identified epigenetic markers (Wang et al. 2011a) (Fig. 2).

Noncoding RNA is highly dynamic both in its expression and functionality. Given the central role that it plays in regulating epigenetic states and transcriptional networks, we predict that it will be increasingly found to play a pervasive role in the regulation of helper T cell plasticity.

4.7 Higher Order Chromatin Organization for Cell Identity

As mentioned previously, enhancers are the key to tune the cell type-specific gene expression profile. It is believed that enhancers act through physical contacts with target gene promoters. Therefore, higher order chromatin conformation that forms loops to facilitate enhancer–promoter interactions and to exclude intervening genes has become a critical aspect for studying gene regulation. Indeed, the genome-wide mapping of promoter–enhancer interactomes reveals that global gene expression is fine-tuned by tissue-specific enhancers, even for those genes that are not cell type-specific (Kieffer-Kwon et al. 2013). For instance, within near 5,000 promoter interactions shared by B cells and ES cells, up to 90 % use at least one cell type-specific enhancer, indicating genome-wide dynamics of enhancer landscape during development and differentiation (Kieffer-Kwon et al. 2013).

In nucleus, the genome is folded complexly in a hierarchical manner (Gibcus and Dekker 2013). Instead of randomly tangling with each other, chromosomes tend to occupy their own territories that contain euchromatin and heterochromatin compartments for aggregation of active and inactive genes, respectively (Lieberman-Aiden et al. 2009). Using 3C-based assays, megabase-scaled topologically associated domains (TADs) are found in both euchromatin and heterochromatin compartments. The boundaries of TADs are invariant between different cell types and are conserved between distinct species (Dixon et al. 2012; Nora et al. 2012). However, submegabase-sized interactions that reside within the TADs reveal cell type specificity (Phillips-Cremins et al. 2013).

The DNA looping within sub-TADs can be partially explained by the interplay between three well-recognized chromatin organizers, CCCTC-binding factor (CTCF), Mediator, and cohesin. It is appreciated that CTCF and cohesin colocalize

to form long-range structural loops that may support short-range enhancer–promoter interactions bridged by Mediator and cohesin (Phillips-Cremins et al. 2013). Master transcription factors and Polycomb proteins also play essential roles for the formation of cell type-specific chromatin architecture. For instance, in mouse pluripotent stem cells, lineage-specific master transcription factors, Nanog, Sox2, and Oct4, binding sites preferentially interact with each other and frequently colocalize with Polycomb proteins. Depletion of one master regulator or Polycomb subunit disrupts their associated DNA contacts, but not the overall chromosome topology (de Wit et al. 2013; Denholtz et al. 2013).

Studies have identified cell type-specific and stimulus-inducible chromatin architecture on immune-related genes; most of them focus on uni-gene regulation. For instance, Ifng locus forms a chromatin hub specifically in Th1 cells but not in Th2 or naïve $CD4^+$ T cells. This Th1-specific chromatin hub is CTCF/cohesin/T-bet-dependent and is conserved between human and mouse (Sekimata et al. 2009; Hadjur et al. 2009). Similarly, the contacts across Th2 cytokine (IL-4, Il-5, and Il-13) locus appear preferentially in T and NK cells but not in B cells or fibroblast (Spilianakis and Flavell 2004). Upon activation of Th2 cells, the Th2 cytokine locus transforms from a "poised" status to a more complicated "cage-like" configuration with multiple loops along with gene upregulation (Cai et al. 2006). Intriguingly, in primary human fibroblast cells, the enhancers of TNF-γ responding genes already interact with their target promoters before stimulation (Jin et al. 2013), suggesting the cell type-specific chromosome topology can also set the stage for inducible genes to be rapidly activated.

Interchromosomal interactions also play a role in hierarchical gene regulation and co-regulation. During $CD4^+$T cells differentiation, Ifn-γ locus on chromosome 10 and Th2 cytokine on chromosome 11 interact with each other specifically in naïve $CD4^+$ T cells while they are inactive and then dissociates during differentiation, suggesting a co-regulation or "poised" nuclear organization for lineage-specific genes (Spilianakis et al. 2005). To sum up, the cell type-specific networking of chromatin architecture may determine cell identity by framing cis-regulatory element activity for gene expression.

5 Conclusions

What should be obvious at this point is that there is no shortage of mechanisms that can promote flexibility of responses in CD4 T cells. Indeed, recent advances have shaken fundamental views of terminal differentiation and lineage commitment—indeed, thanks to the pioneering work of Yamanaka and colleagues, it is commonplace to reprogram fully differentiated cells with four transcription factors and/or a cocktail of drugs (Yamanaka and Blau 2010; Malik and Rao 2013; Obokata et al. 2014) and most recently by incubating cells in acidic medium. If lymphocytes turn into stem cells by brief exposures to relatively innocuous stressors such as low pH or pipetting, it is increasingly difficult to argue that helper T cell subsets are

immune from changing phenotypes. A few years ago, we would have said with certainty that CD4 and CD8 cells are unquestionably stable lineages; however, recent work points to the fact that even such cells can alter their transcriptional program (Mucida et al. 2013). In view of these developments along with much experimental data in CD4 T cells, it seems appropriate to recognize that terms like Th1, Th2, and Th17 cells, should really be thought of as a shorthand that approximates functionality. But we should not deceive ourselves: this convenient, but artificial shorthand should by no means be interpreted to suggest that populations of T cells are terminally differentiated and unable to acquire new functionalities.

In this review, we have tried to emphasize that helper T cells express a network of transcription factors that can work in concert, in opposition or to allow for specialized function. T cell master regulators, if they exist, work with a vast array of other factors. Transcription factors can be covalently modified to activate or inactivate their function and can be degraded. The epigenetic modifications of many of these key transcription factors permit flexible expression of master regulator transcription factors, even when modifications of effector cytokine genes appear "fixed." Formation of the epigenetic landscape that modulates the accessibility of transcription factors and other key genes is profoundly affected by signals emanating from the T cell receptor and cytokine receptors. Moreover, we now recognize that cell signaling is intimately linked to cellular metabolism and microbiome. Epigenomic landscapes integrate all these environmental signals and thereby can be considered as an extension of signal transduction.

The good news is that we can now measure it all: genomes, epigenomes, metabolomes, microbiomes, and transcriptomes. The bad news is that we can measure it all. How do we understand the complexity of multilayer networks built on top of each other? What tools do we need to develop to assist in providing models that explain the biology of CD4 T cells in health and disease? Moving ahead, all T cell biologists will need to adapt "systems biology" approach in some way. Although hypothesis-driven studies with a focus on a few select genes remain useful, one needs to bear in mind all the things that could, but are not being measured would influence the experimental outcome. As our annotation of the human genome becomes more complete and we develop more sophisticated understanding of the impact of human genetic variation, we are approaching a new era that can redefine T cell function in health and disease by employing "omic" views to comprehensively quantitate states of dynamic cellular activities.

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