Dynamic balance between master transcription factors determines the fates and functions of CD4 T cell and innate lymphoid cell subsets

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CD4 T cells, including T regulatory cells (Treg cells) and effector T helper cells (Th cells), and recently identified innate lymphoid cells (ILCs) play important roles in host defense and inflammation. Both CD4 T cells and ILCs can be classified into distinct lineages based on their functions and the expression of lineage-specific genes, including those encoding effector cytokines, cell surface markers, and key transcription factors. It was first recognized that each lineage expresses a specific master transcription factor and the expression of these factors is mutually exclusive because of cross-regulation among these factors. However, recent studies indicate that the master regulators are often coexpressed. Furthermore, the expression of master regulators can be dynamic and quantitative. In this review, we will first discuss similarities and differences between the development and functions of CD4 T cell and ILC subsets and then summarize recent literature on quantitative, dynamic, and cell type–specific balance between the master transcription factors in determining heterogeneity and plasticity of these subsets.

The Journal of Experimental Medicine

Introduction

The differentiation of CD4 T helper cells (Th cells) is a central process during adaptive immune responses (Zhu et al., 2010). Upon activation through their TCR, naive CD4 T cells can differentiate into three major distinct Th subsets, type 1 Th (Th1), type 2 Th (Th2), and IL-17–producing Th (Th17) cells that produce unique sets of cytokines (IFN-γ for Th1; IL-4, IL-5, and IL-13 for Th2; and IL-17A, IL-17F, and IL-22 for Th17). These cells are critical for protective immune responses against a variety of pathogens. Inappropriate differentiation of Th cells can result in not only chronic infections but also various forms of inflammatory allergic and autoimmune diseases. The differentiation and functions of Th cell subsets depend on the induction of lineage-specific transcription factors, including the so-called master regulators: T-bet for Th1, GATA3 for Th2, and RORγt for Th17. Naive CD4 T cells can also develop into follicular T cells (Tfh cells) that express the master regulator Bcl6; Tfh cells are important for helping B cells in Ig class switching and considered as a separate Th lineage (Crotty, 2011). The master regulators cross-inhibit each other either at the transcriptional level or posttranscriptional level through protein–protein interactions. Therefore, their expression is usually mutually exclusive.

Some T regulatory cells (Treg cells), expressing Foxp3 as their master regulator, can derive from naive CD4 T cells in the periphery (Chen et al., 2003; Abbas et al., 2013). These cells are termed peripherally induced Treg cells (pTreg cells). Together with thymus-derived regulatory T cells (tTreg cells), they are important for regulating immune responses in addition to maintaining immune tolerance. Surprisingly, some Treg cells also express T-bet, GATA3, RORγt, or Bcl6, albeit at lower levels than that found in T effector cells.

Innate lymphoid cells (ILCs), particularly IL-7Rα–expressing ILCs, are a class of innate lymphocytes that display a cytokine-producing profile similar to Th cells (Diefenbach et al., 2014; McKenzie et al., 2014; Artis and Spits, 2015; Klose and Artis, 2016). Therefore, they can also be divided into group 1 ILC (ILC1), group 2 ILC (ILC2), and group 3 ILC (ILC3) subsets based on their signature cytokine production (IFN-γ for ILC1, IL-5 and IL-13 for ILC2, and IL-17A, IL-17F, and IL-22 for ILC3). Interestingly, just as Th subsets, ILC subsets also depend on T-bet, GATA3, and RORγt for their development and functions.

However, "one factor, one cell fate" is oversimplified and does not fully explain the functional heterogeneity of Th and ILC subsets. First of all, GATA3 is expressed at various levels by all CD4 T cells and ILCs. Different levels of GATA3 expression are associated with its unique functions in different cell types. Second, some Th cell and ILC subsets can coexpress two or more master regulators. Furthermore, the expression of these transcription factors in some subsets is often dynamic and quantitative. Lastly, the functions of a particular transcrip-

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Abbreviations used: DP, double positive; ILC, innate lymphoid cell; LTi, lymphoid tissue inducer; *Mtb*, *Mycobacterium tuberculosis*; Tfh cell, follicular T cell; tTreg cell, thymus-derived regulatory T cell.

The Rockefeller University Press $30.00
J. Exp. Med. 2017 Vol. 214 No. 7 1861–1876
https://doi.org/10.1084/jem.20170494

tion factor are cell type or stage specific, indicating that other lineage-specific transcription factors also participate in cell fate determination and functional regulation. In this review, we will discuss each of these topics mentioned above.

Similarities between Th cells and ILCs and their shared functions

As introduced above, effector Th cells can be classified into three major groups: Th1, Th2, and Th17 cells that produce IFN-γ, IL-4/5/13, and IL-17/22, respectively (Fig. 1 A). T-bet, GATA3, and RORγt are the master transcription factors in regulating the differentiation and functions of Th cell subsets (Zhu et al., 2010). Among these master regulators, GATA3 was first shown to be necessary and sufficient for Th2 cell differentiation (Zheng and Flavell, 1997). Conditional knockout of GATA3 indicates that GATA3 is required not only for inducing Th2 cell differentiation but also for suppressing Th1 cell differentiation through multiple mechanisms (Zhu et al., 2004; Yagi et al., 2011). T-bet is important for Th1 cell differentiation (Szabo et al., 2000), and it suppresses GATA3-dependent endogenous Th2 program by inhibiting GATA3 expression and function (Hwang et al., 2005; Zhu et al., 2012). T-bet and GATA3 can physically interact to modulate each other's functions; T-bet binds to the *Gata3* locus (Zhu et al., 2012), and GATA3 binds to the *Tbx21* (gene encoding T-bet) locus (Wei et al., 2011). Therefore, T-bet and GATA3 cross-regulate each other. RORγt is the master regulator for Th17 cells (Ivanov et al., 2006). It has also been reported that T-bet directly suppresses RORγt expression (Lazarevic et al., 2011). Because of cross-regulation among the master regulators, T-bet, GATA3, and RORγt expression is usually mutual exclusive, leading to the one factor–one fate hypothesis that T-bet, GATA3, and RORγt, as the master regulators for Th subsets, are preferentially expressed by Th1, Th2, and Th17 cells, respectively.

Similar to the Th cell subsets, IL-7Rα–expressing ILCs can be also divided into three major groups based on their unique cytokine-producing capacity. Interestingly, each ILC subset requires the same master regulator of the corresponding Th cell subset for their development and functions (Fig. 1 A). For example, GATA3, the master regulator of Th2 cells, is also critical for the development and maintenance of ILC2s (Hoyler et al., 2012; Mjösberg et al., 2012; Furusawa et al., 2013; Klein Wolterink et al., 2013; Yang et al., 2013; Yagi et al., 2014). T-bet is critical for ILC1 development, whereas no ILC3s are found in the absence of RORγt. Some investigators consider NK cells as a member of group 1 ILCs (Spits et al., 2016). However, conventional NK cells usually do not express IL-7Rα, and their development depends on transcription factor Eomes, which is also a critical transcription factor for CD8 T cell activation and function. On the other hand, like Th1 cells, ILC1s do not express Eomes. Thus, NK cells should be considered as the innate counterpart of CD8 T cells (Fig. 1 B).

Because Th cell and ILC subsets can produce similar sets of effector cytokines, they play similar roles in protective immune responses and in inflammation (Yagi et al., 2014; Artis and Spits, 2015; Koues et al., 2016; Shih et al., 2016). ILC1s and Th1 cells are involved in protective immune responses against intracellular pathogens such as bacteria, viruses and protozoa (Klose et al., 2014), whereas, ILC2s and Th2 cells mediate immune responses to helminthes (Fallon et al., 2006; Moro et al., 2010; Neill et al., 2010; Price et al., 2010). ILC3s and Th17 cells are critical for fighting against infections with extracellular bacteria and fungi (Qiu et al., 2013; Sano et al., 2015). Similar to the involvement of Th1 and Th17 cells in autoimmunity, ILC1s and ILC3s may also contribute to certain types of autoimmunity (Shikhagaie et al., 2017). Like Th2 cells, ILC2s can mediate allergic skin or lung inflammation and asthma (Chang et al., 2011; Monticelli et al., 2011; Halim et al., 2012; Roediger et al., 2013; Kim et al., 2014). Although the functions of ILCs may be redundant to Th cells in infections (Song et al., 2015; Rankin et al., 2016; Vély et al., 2016), activation of ILCs is sufficient to induce inflammation, even in the absence of T cells (Bando and Colonna, 2016). Furthermore, enforced activation of ILC2s by IL-25 is sufficient to expel worms in the absence of T cells (Fallon et al., 2006).

An important research topic in the field is to compare the similarities and differences between Th cell and ILC subsets. Two recent genome-wide studies show that not only the transcriptomes but also the epigenomes of an ILC subset are remarkably similar to those of the Th cell subset of the same type (Koues et al., 2016; Shih et al., 2016). Strikingly, the transcriptomes of ILC2s and Th2 cells harvested from the lungs of helminth-infected mice are so similar to each other that only 167 genes are differentially expressed (Shih et al., 2016). This difference is even smaller than the difference between activated ILC2s after helminth infection and ILC2s at the steady state. Both Th2 cells and ILC2s rely on GATA3 for their development; even after ILC2s are fully developed, GATA3 continues to be critical for their maintenance and functions, just like GATA3's critical function in mature Th2 cells. Indeed, GATA3 regulates a shared set of genes in both Th2 cells and ILC2s (Yagi et al., 2014). These genes, many of which are already known as critical genes for type 2 immune responses, such as *Il5*, *Il13*, *Areg*, *Il1rl1*, and *Ccr8*, represent the core elements that determine the shared functionalities between Th2 cells and ILC2s. There are also shared functional molecules and enhancers between Th1 cells and ILC1s or between Th17 cells and ILC3s (Koues et al., 2016).

Similarities between the development of Th cells and ILCs

Th cell differentiation starts at the activation of naive CD4 T cells, which are developed in the thymus. GATA3 is essential for CD4, but not CD8, T cell development (Ho et al., 2009). We have recently reported that, on the innate side, GATA3 is not required for NK cell development but indispensable for the development of all IL-7Rα–expressing ILCs (Yagi et al., 2014). This finding further confirms that ILC1s are developmentally

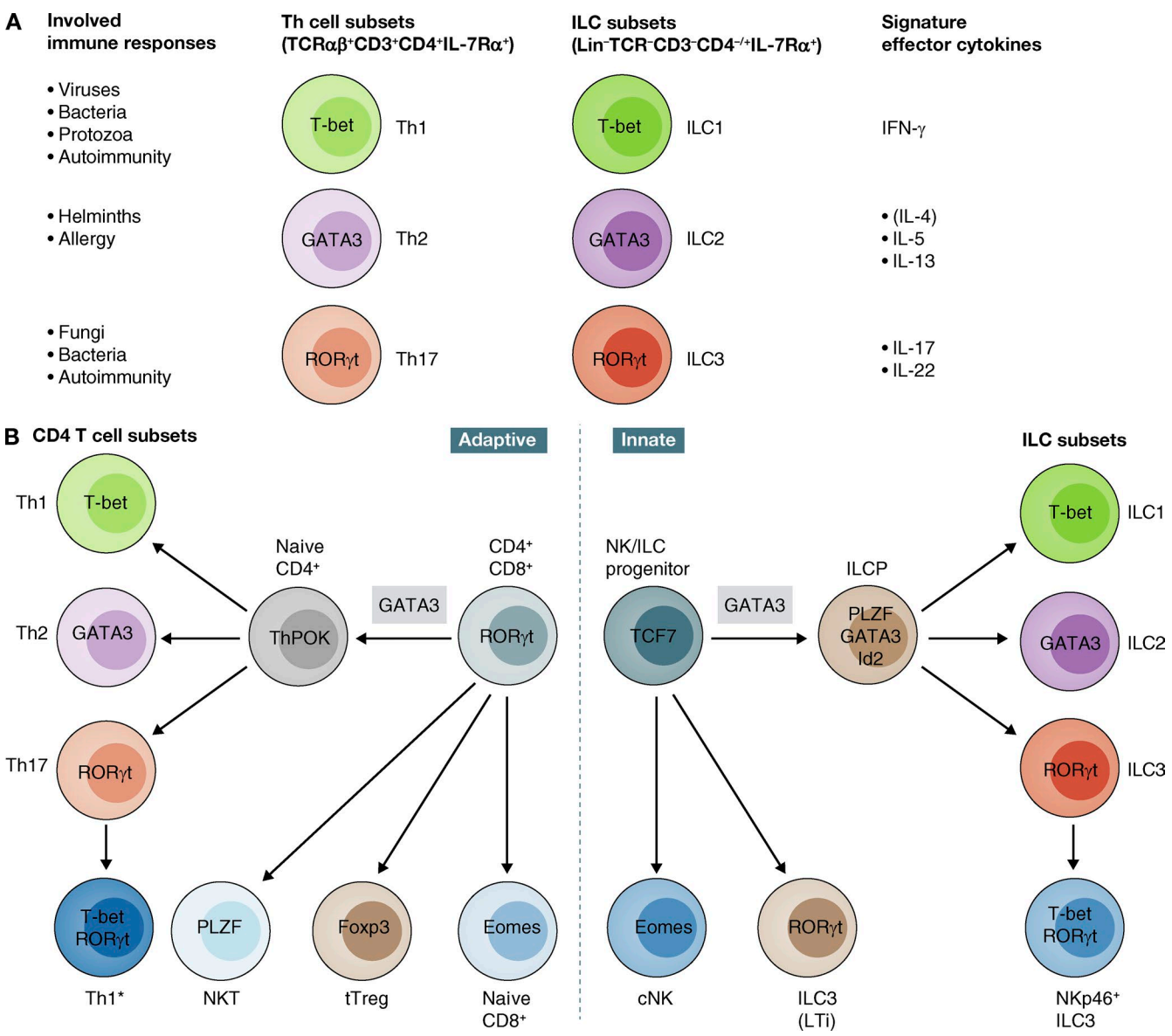

Figure 1. **Similarities and differences between the functions and development of T helper (Th) cell and innate lymphoid cell (ILC) subsets.** (A) Th cell and ILC subsets express identical sets of effector cytokines and share similar master transcription factors for their development and functions. Th1 cells and ILC1s are important for protective immunity against viruses, intracellular bacteria, and protozoa. Th2 cells and ILC2s are important for clearing helminths. Th17 cells and ILC3s are critical for protective immune responses against fungi and extracellular bacteria infection. Th2 and ILC2s are involved in allergic inflammation, and type 1 and type 3 Th cells and ILCs contribute to autoimmunity. (B) In the thymus, RORγt is essential for $CD4^+CD8^+$ double-positive (DP) cell survival and TCRα rearrangement. $CD4^+CD8^+$ DP thymocytes can develop into naive $CD4^+$ T cells, naive $CD8^+$ T cells, NKT cells, and tTreg cells through the induction of ThPOK, Eomes, PLZF, and Foxp3, respectively. GATA3 up-regulates ThPOK expression and thus is critical for CD4, but not CD8, T cell development. Upon T cell activation, naive $CD4^+$ T cells can further develop into T-bet–expressing Th1, GATA3-expressing Th2, and RORγt-expressing Th17 cells. On the innate side, transcription factor TCF7-expressing NK/ILC progenitor cells can develop into Eomes-expressing conventional NK cells, RORγt-expressing LTi or LTi-like cells, and $PLZF^+GATA3^{hi}Id2^{hi}$ ILC progenitors. GATA3 is critical for the generation of $PLZF^+GATA3^{hi}Id2^{hi}$ ILC progenitors but not NK cells. $PLZF^+GATA3^{hi}Id2^{hi}$ ILC progenitors can further develop into T-bet–expressing ILC1s, $GATA3^{hi}$ ILC2s and RORγt-expressing ILC3s. RORγt-expressing Th17 cells or ILC3s may further express T-bet to become RORγt/T-bet dual expressing Th1* cells or $NKp46^+$ ILC3s. ILCP, ILC progenitor.

distinct from NK cells. The development of T cells and ILCs also depends on several common transcription factors such as TCF7 and Tox, in addition to GATA3 (Yagi et al., 2014; Seehus et al., 2015; Yang et al., 2015; Zhong and Zhu, 2017). This demonstrates that there is an overall symmetry between the development of innate and adaptive lymphocytes (Fig. 1 B).

Although ILCs are currently classified into three major groups, there are two distinct ILC3 subsets, one of which ex-

presses CCR6 in mice (Zook and Kee, 2016). CCR6$^+$ ILC3s in mice represent lymphoid tissue inducer (LTi) or LTi-like cells that are required for the development of lymphoid tissues such as lymph nodes. The CCR6$^-$ ILC3s, ILC1s, and ILC2s share a common progenitor that expresses PLZF (Constantinides et al., 2014) and PD-1 (Yu et al., 2016), whereas CCR6$^+$ ILC3s do not develop from PLZF-expressing progenitors according to the fate-mapping study (Fig. 1 B). Our recent unpublished work indicates that CCR6$^+$ LTi or LTi-like cells exist in the GATA3-deficient mice (Vav-Cre-mediated *Gata3* deletion starting from the hematopoietic stem cell stage), whereas ILC1s, ILC2s, and CCR6$^-$ ILC3s are absent in these mice. This is consistent with a complete loss of the PLZF/PD-1–expressing ILC progenitors in the bone marrow of GATA3-deficient mice. Based on these findings, ILCs that are derived from PLZF/PD-1–expressing ILC progenitors should be considered as the bona-fide innate equivalent of Th effector cells, and PLZF/PD-1–expressing ILC progenitors are the innate counterpart of naive CD4 T cells.

CD4$^+$CD8$^+$ DP thymocytes not only develop into naive CD4 and CD8 T cells, but also give rise to other CD4 T cells, such as NKT cells and tTreg cells (Fig. 1 B). Whether LTi or LTi-like cells resemble either the NKT cell or tTreg cell counterpart on the innate side requires further investigation. Nevertheless, some LTi-like ILCs may express some molecules that are identified as the Treg cell signature genes (Zhong et al., 2016), and MHC-II–expressing ILC3s within the CCR6$^+$ LTi or LTi-like compartment have some regulatory functions (Hepworth et al., 2013, 2015). However, Foxp3-expressing ILCs have not been found. Because NKT cells express PLZF particularly at the immature stage (Constantinides and Bendelac, 2013) and NKT cells can further develop into NKT1, NKT2, and NKT17 subsets (Lee et al., 2013), it is also possible that ILC development mimics the development of the NKT branch of the adaptive lymphocytes.

Differences between the development and functions of Th cells and ILCs

Although both Th cell and ILC subsets can produce similar sets of effector cytokines, they differ from each other in several aspects. First of all, Th cells are antigen specific, but ILCs lack antigen receptors. Differentiated Th cells can respond to antigen restimulation to produce effector cytokines. However, ILCs mainly respond to cytokine stimulation (Artis and Spits, 2015). For example, IL-12 and IL-18 can stimulate ILC1s to produce IFN-γ; IL-25 or IL-33 induces IL-5/IL-13 production by ILC2s; and IL-23 is a potent inducer of IL-22 production by ILC3s. Interestingly, in mature Th cells, IL-1 family receptors (IL-18R, IL-33R, and IL-1R) are preferentially expressed by Th1, Th2, and Th17 cells, respectively (Guo et al., 2009, 2012). As a result, Th cell subsets can also respond to cytokines to produce their effector cytokines in an antigen-independent manner (Guo et al., 2015). Strikingly, Th2 cells found in the lung require stimulation by IL-25, IL-33, and/or thymic stromal lymphopoietin (TSLP) to become functional Th2 effector cells (Van Dyken et al., 2016).

ILC development does not depend on pathogen insults and/or antigen stimulation. Thus, ILCs preexist even before an immune response occurs, whereas Th cells that differentiate from naive CD4 T cells require TCR-mediated signaling triggered by antigen stimulation. Cytokine-mediated activation of STAT family proteins, including STAT3, STAT4, and STAT6, is also essential for the differentiation of CD4 T cells (Shih et al., 2014). On the other hand, the involvement of STATs in ILC development and function may be quite different. At least for STAT3, it has been shown that this transcription factor is not necessary for the development of ILC3 subsets, even though IL-22 production by ILC3s requires STAT3 (Guo et al., 2014).

Although the development of T cells and ILCs depends on several common transcription factors, Id2, which only has a modest effect on T cell development, plays a critical role during ILC development (Yokota et al., 1999; Klose et al., 2014; Zook and Kee, 2016; Zhong and Zhu, 2017). In contrast, whereas Bcl11b is the critical factor for T cell development, it only regulates the development of ILC2s (Li et al., 2010; Califano et al., 2015; Walker et al., 2015; Yu et al., 2015b; Zhong and Zhu, 2015, 2017; Zook and Kee, 2016).

ILCs, but not Th cells, mainly reside in tissue (Sojka et al., 2014; Gasteiger et al., 2015). ILC3s are the major ILC population found in the gut (Qiu et al., 2013; Yagi et al., 2014). Although ILC2s are also enriched in the gut, they are also found in lung, skin, and adipose tissues (Moro et al., 2010; Halim et al., 2012; Artis and Spits, 2015). ILC1s are highly enriched in the liver; they are also referred to as tissue-resident NK cells (Sojka et al., 2014). Parabiosis experiments indicate that Th cells are in circulation but ILCs are tissue resident (Sojka et al., 2014; Gasteiger et al., 2015). Because of their predevelopment and tissue residency, ILCs can provide first line of host defense before adaptive immune responses are activated.

Although several genes are similarly regulated by GATA3 in Th2 cells and ILC2s as mentioned above, many other genes are regulated by GATA3 in only one of these two cell types (Yagi et al., 2014). These gene products may exert Th2 cell– or ILC2-specific functions. It will be also interesting to carry out parallel studies to compare the function of T-bet in Th1 cells and ILC1s or RORγt in Th17 cells and ILC3s, respectively. Revealing the differences and similarities in gene expression between Th cells and ILCs will help us better understand the biology of these two evolutionarily related populations. Some gene products may serve as drug targets specific in ILC or Th cell subsets or in both cell populations of the same type.

Quantitative GATA3 expression and its functions in different lymphocytes

GATA3 is the master regulator for Th2 cells and ILC2s (Zhu et al., 2004; Yagi et al., 2014). Beyond that, GATA3 has many other important functions in different cell subsets, partly because all CD4 T cells and ILCs express GATA3 at different levels (Ho et al., 2009; Tindemans et al., 2014; Zhong et al.,

2016). In fact, GATA3 promotes the expression of IL-7Rα in all T cells and ILCs, indicating a common mechanism for how GATA3 regulates lymphocyte homeostasis (Wang et al., 2013; Zhong et al., 2016). Low levels of GATA3 expression in CD8 T cells or ILC3s are sufficient to regulate IL-7Rα expression in these cells. Binding of GATA3 to the *Il7r* gene in ILC3s is similar to that in ILC2s, indicating that this binding site may be a high-affinity one for GATA3 (Zhong et al., 2016).

Mature ILC1s express low levels of GATA3 (Klose et al., 2014; Zhong et al., 2016). Interestingly, the maintenance of mature ILC1s requires GATA3 (Klose et al., 2014). Whether this is because of GATA3's effect on IL-7Rα expression in ILC1s is unknown. Furthermore, despite of its low expression levels in mature ILC3s, GATA3 affects the expression of several important genes, including *Il22*, in addition to *Il7r* (Zhong et al., 2016). GATA3 regulates IL-22 expression in both $CCR6^+$ LTi-like cells and $CCR6^-$ ILC3s. Mice with GATA3 deficiency only in ILC3s (RORγt-Cre mediated GATA3 deletion) all died of *Citrobacter rodentium* infection, which correlated with a dramatic reduction in IL-22 production; however, these mice have the lymph node structures indicating the LTi cells in these mice are functional (Zhong et al., 2016).

The physiological importance of low GATA3 expression in Th1 and Th17 cells is still unclear. However, GATA3 expression in Treg cells seems to modulate Treg cell functions to a certain extent, which will be discussed later. Because quantitative expression of GATA3 may translate to qualitative effects, the combination of titratable GATA3 transgene expression and endogenous GATA3 deficiency may be necessary to distinguish the effects of high and low GATA3 expression at different developmental stages for the generation of mature CD4 T cells and ILCs.

Coexpression of multiple master transcription factors in Treg cells

Although the master regulators are often preferentially expressed in a cell type–specific fashion, coexpression of these factors has also been noticed. For example, RORγt and Foxp3 can be coexpressed (Fig. 2), presumably because of the shared program between Th17 and Treg cell differentiation, both of which require TGF-β. The RORγt/Foxp3-coexpressing cells were initially considered as an intermediate stage before lineage commitment to either Th17 or Treg cell fate (Yang et al., 2008; Zhou et al., 2008; Komatsu et al., 2014). Two recent studies indicated that RORγt/Foxp3-coexpressing cells, highly enriched in the large intestine, may represent a subset of Treg cells (Ohnmacht et al., 2015; Sefik et al., 2015). Interestingly, deletion of RORγt specifically in Foxp3-expressing cells results in Th2-related gut inflammation (Ohnmacht et al., 2015).

Besides RORγt, T-bet and GATA3 are also expressed by the Treg cell subsets (Yu et al., 2015a). However, the expression level of T-bet and GATA3 in Treg cells is much lower than that found in T effector cells. Possibly because of their low expression, T-bet and GATA3 fail to induce effector cytokine production in these Treg cells. Nevertheless, they are responsible for inducing chemokine receptor expression in Treg cells. For example, CXCR3 expression on Treg cells depends on T-bet (Koch et al., 2009; Yu et al., 2015a). It has been proposed that Treg cell subsets may utilize the master regulators of Th effector cells to specifically regulate the expression of chemokine receptors that are associated with T effector cell migration; these Treg cell subsets then may colocalize with the same type of Th effectors to precisely control their activation during this particular type of immune response (Chaudhry et al., 2009; Zheng et al., 2009; Campbell and Koch, 2011). Therefore, it has been predicted that T-bet expression in Treg cells is necessary for suppressing Th1 responses (Koch et al., 2009). However, T-bet deletion specifically in Treg cells do not result in Th1-related autoimmune diseases, indicating that T-bet–deficient Treg cells are still able to control autoreactive Th1 cells at steady state (Yu et al., 2015a). However, it remains to be tested whether T-bet expression in Treg cells contributes to their suppressive ability in limiting ongoing Th1-associated immune responses (Oldenhove et al., 2009; Yamaguchi et al., 2011).

GATA3 is also expressed by Treg cells. Although all T cells, including all Treg cells, express GATA3 at a basal level, some Treg cells may express high levels of GATA3, particularly upon activation (Wang et al., 2011; Wohlfert et al., 2011; Rudra et al., 2012; Yu et al., 2015a). GATA3 regulates the expression of several "Th2-specific" genes, including *Il1rl1* and *Ccr8*, in Treg cells (Wei et al., 2011). GATA3 deletion specifically in Treg cells results in mild to severe Th2-like autoimmunity; the difference in disease severity is possibly because of variations in mouse strains and/or animal facilities (Wang et al., 2011; Wohlfert et al., 2011; Rudra et al., 2012; Yu et al., 2015a). Because GATA3 is expressed by all Treg cells at a basal level, and because GATA3 also regulates IL-7Rα expression in Treg cells, whether partial loss of Treg cell function is caused by the absence of "Th2-like" Treg cell subset or removal of GATA3 basal expression in all Treg cells remains further investigation.

The master regulator for Tfh cells is Bcl6 (Nurieva et al., 2008, 2009; Johnston et al., 2009; Yu et al., 2009). Interestingly, some Treg cells are also found in the B cell follicle, and these cells, designated follicular T regulatory cells, express both Bcl6 and Foxp3 (Chung et al., 2011; Linterman et al., 2011). Follicular T regulatory cells may suppress Tfh cells and thus limit germinal center reaction in the B cell follicle.

Coexpression of multiple master transcription factors in Th effectors

Within the Th effector compartment, some subsets also express multiple master regulators. For example, T-bet/RORγt–coexpressing cells have been found, especially in the gut environment (Fig. 1 B). These cells are also called Th1* cells and are capable of producing both IL-17 and IFN-γ (Ivanov et al., 2006; Lee et al., 2009; Ghoreschi et al., 2010; Lexberg

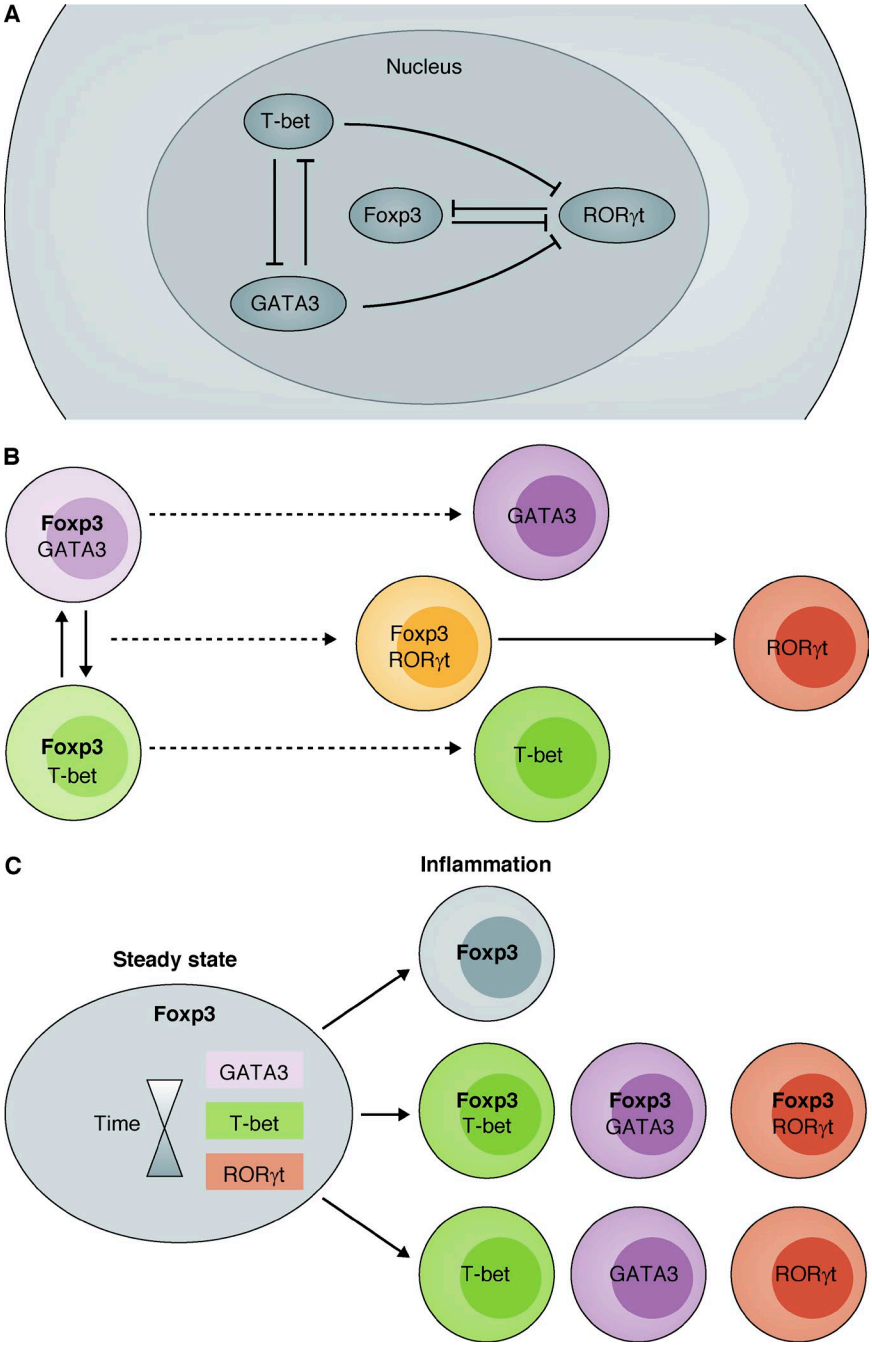

Figure 2. **The plasticity of Treg cells.** (A) In Treg cells, Foxp3 and RORγt antagonize each other's function, T-bet and GATA3 cross-regulate each other in Foxp3-expressing Treg cells, and T-bet and GATA3 also repress RORγt in Treg cells in a redundant manner. (B) Depending on the cytokine environment, T-bet or GATA3 can be induced in Foxp3-expressing Treg cells. Dynamic and low expression of T-bet or GATA3 in Treg cells is essential for preventing Treg cells from acquiring the Th1, Th2, or Th17 phenotype. (C) In steady state, T-bet, GATA3, or RORγt can be transiently expressed by Foxp3-expressing Treg cells. During immune responses, Treg cells may have three different fates: (1) Treg cells only expressing Foxp3; (2) specialized Treg cells expressing both Foxp3 and T-bet, GATA3, or RORγt; and (3) T-bet–, GATA3-, or RORγt-expressing Th effectors derived from Treg cells.

et al., 2010; Hirota et al., 2011). Importantly, T-bet/RORγt (IFN-γ/IL-17) double-producing CD4 T cells are abundant in several inflammatory settings such as Crohn's disease. It has been proposed that these cells are the most potent cells in inducing inflammation. T-bet/RORγt dual expressor, with their TCR specificity for *Candida albicans* or *Mycobacterium tuberculosis* (*Mtb*), are also found in human patients (Zielinski et al., 2012; Okada et al., 2015). In addition, GATA3/T-bet and GATA3/RORγt dual expressors are found in mice or humans during type 2 immune responses such as helminth infection and asthmatic inflammation (Cosmi et al., 2010; Wang et al., 2010; Peine et al., 2013). However, it is still elusive what the unique functions of these mixed phenotype cells are and whether two master regulators have a synergistic and/or antagonistic effect on the expression of a set of lineage-specific genes.

Coexpression of multiple master transcription factors in ILCs

GATA3 is highly expressed by ILC2s. So far, it has been shown that T-bet– and/or RORγt-expressing ILCs only express low

levels of GATA3. On the other hand, coexpression of T-bet and RORγt are found in some CCR6$^-$ ILC3s (Fig. 1 B), some of which express NKp46 (Sciumé et al., 2012; Klose et al., 2013; Zhong et al., 2016). Both T-bet and RORγt are indispensable for the development of NKp46$^+$ ILC3s. CCR6$^+$ ILC3s, which do not express T-bet, and NKp46$^+$ ILC3s are functionally redundant in protective immune responses to *Citrobacter rodentium* infection, although NKp46$^+$ ILC3s may have some unique functions during inflammation, such as anti-CD40–induced colitis, and gut homeostasis (Song et al., 2015; Rankin et al., 2016). However, the precise function of T-bet in NKp46$^+$ ILC3s is still unclear. It is also unclear how T-bet and RORγt can be stably coexpressed in these cells, and whether a unique program is activated only when both master regulators are present. Bcl6 but not Foxp3 mRNA is also detected in ILC population. However, it is not known whether Bcl6 can be expressed by a subset within the ILC1, ILC2, or ILC3 populations.

Dynamic expression of master transcription factors in regulating cell plasticity and development

The expression of some master regulators could be dynamic, which may contribute to cell plasticity. Th17 and Treg cells are more flexible in changing their phenotypes compared with Th1 and Th2 cells (Zhou et al., 2009a). Both Treg cells and Th17 cells can acquire IFN-γ–producing capacity through T-bet induction in these cells (Mathur et al., 2006; Xu et al., 2007; Bending et al., 2009; Komatsu et al., 2009, 2014; Lee et al., 2009; Oldenhove et al., 2009; Zhou et al., 2009b; Noval Rivas et al., 2015). Treg cells may also acquire a Th2 or Th17 phenotype after losing Foxp3 expression (Xu et al., 2007; Komatsu et al., 2009, 2014; Oldenhove et al., 2009; Zhou et al., 2009b; Noval Rivas et al., 2015). In contrast, some studies have shown that Treg cells remain stable during inflammation (Rubtsov et al., 2010; Sakaguchi et al., 2013). Interestingly, most of the IFN-γ–producing cells found in the central nervous system during an autoimmune disease induction are derived from "Th17" cells that have expressed IL-17, based on an IL-17 fate-mapping marker (Hirota et al., 2011). In addition, Th17 cells can transdifferentiate into Treg cells after inflammation is resolved (Gagliani et al., 2015). These lineage switches and/or transdifferentiation is accompanied by an alteration in the expression of master regulators.

As discussed above, T-bet and RORγt are coexpressed by NKp46$^+$ ILC3s. Interestingly, these cells may become ILC1-like cells by turning off RORγt expression (Klose et al., 2013). These ex-ILC3s can be distinguished from "real" ILC1s by an RORγt-Cre–mediated fate-mapping tool in mice. A recent study has shown that ILC1s may become ILC3s when cells receive IL-1/IL-23 stimulation (Bernink et al., 2015). However, such conversion seems to be limited to the "ex-ILC3s" portion of ILC1s.

It is noteworthy that similar to GATA3, RORγt is also expressed during T cell development (Sun et al., 2000). RORγt expression in CD4$^+$CD8$^+$ DP cells is required to maintain DP cell survival and consequently promote TCRα rearrangement (Guo et al., 2002). Reduced TCR repertoire is found in RORγt-deficient mice; such alteration in T cell development may also result in defective immune responses to self-antigens and/or to infections (Guo et al., 2016). In RORγt-deficient patients, impaired immune responses against *Candida* infection and *Mtb* infections are noted (Okada et al., 2015). Susceptibility to *Candida* infection is consistent with critical role of RORγt in generating Th17 cells. The unexpected *Mtb* infection in these patients suggests either the generation of effective Th1 cells needs to go through a "Th17" stage or altered T cell development in these patients indirectly affects immune responses against *Mtb*.

Although all the T cells have expressed RORγt during their development, RORγt expression is limited to a small proportion of ILC progenitors. Fate-mapping experiments in mice show that ∼10% of mature ILC2s have expressed RORγt (unpublished data). Whether such ILC2s represent a unique ILC2 population is not known. It has been reported that the CD34$^+$ RORγt-expressing ILC progenitors in human second lymphoid tissues have already committed to the ILC3 lineage (Montaldo et al., 2014). However, another recent study identified an RORγt-expressing ILC progenitor population in human, which is capable of developing into all ILC subsets and NK cells (Scoville et al., 2016). Prior expression of RORγt in a subset of non-ILC3s may imprint the plasticity of these cells to become ILC3-like cells upon an appropriate stimulation.

Dynamic expression of T-bet and GATA3 in Treg cells

As discussed above, subsets of Treg cells may express T-bet and/or GATA3. Interestingly, T-bet and GATA3 expression in Treg cells is not stable (Yu et al., 2015a). When Treg cells become activated, they can turn on either T-bet or GATA3 depending on the cytokine environment (Fig. 2). When they are removed from cytokine stimulation, the expression of T-bet and GATA3 may return to a basal level. Such dynamic expression may be physiologically important to prevent Treg cells from acquiring either Th1 or Th2 effector phenotype. The dynamic changes in T-bet and GATA3 expression in Treg cells is also important for suppressing RORγt expression in Treg cells, thus preventing Treg cells from acquiring IL-17–producing capacity. Indeed, when both T-bet and GATA3 are absent, these Treg cells up-regulate RORγt expression and lose their suppressive activity. It is well known that Foxp3 and RORγt antagonize each other. This new study (Yu et al., 2015a) indicates that T-bet and GATA3 may assist Foxp3 in suppressing RORγt in a redundant manner to maintain immune tolerance (Fig. 2).

Dynamic expression of T-bet and GATA3 potentially regulates Tfh subsets

Tfh cells, through IL-21 production, play an important role in helping B cells in Ig production (Crotty, 2011). However, B cell Ig class switching to IgE or IgG2a/2c depends on IL-4

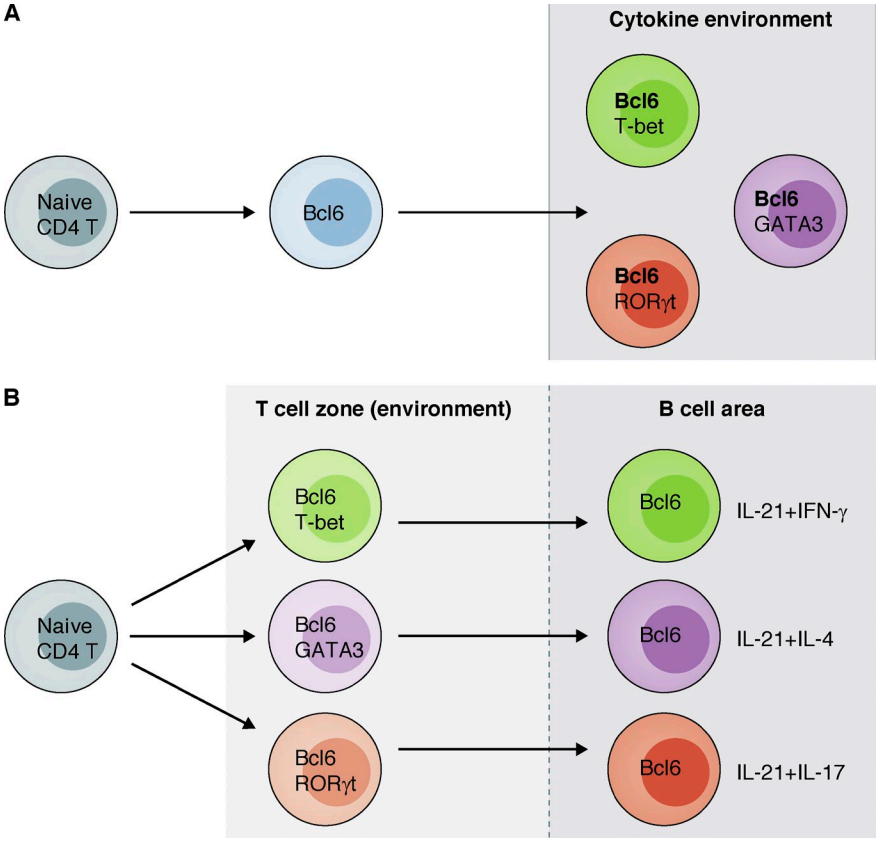

Figure 3. **Models for the development of effector cytokine–producing Tfh cells.** Bcl6 is the master transcription factor for Tfh cell development. Tfh cells express no or low levels of T-bet, GATA3, or RORγt. IL-21 is the signature effector cytokine of Tfh cells; however, IFN-γ and IL-4 can also be produced by Tfh cells. (A) Model 1. The capacity of Tfh cells to produce IFN-γ or IL-4 is acquired after their development. Low levels of T-bet, GATA3, or RORγt induced by cytokines in Tfh cells are responsible for IFN-γ, IL-4, or IL-17 production, respectively. (B) Model 2. At an early stage of Tfh cell development, transient expression of T-bet, GATA3, or RORγt precommits these Bcl6-expressing Tfh cells to become IFN-γ–, IL-4–, or IL-17–producing Tfh cells in the B cell follicles by leaving "epigenetic memory" marks at the cytokine loci.

or IFN-γ, respectively (Kopf et al., 1993; King and Mohrs, 2009; Reinhardt et al., 2009; Zaretsky et al., 2009). Therefore, besides producing signature effector cytokine IL-21, Tfh cells may also express IL-4 or IFN-γ. Interestingly, majority of the IL-4–producing Th cells in vivo indeed display a Tfh phenotype (King and Mohrs, 2009). Although conventional Th2 cells found in the lung mainly produce IL-13, IL-4–producing Tfh cells, mainly located in the secondary lymphoid organs such as lymph nodes, do not express IL-13 (Liang et al., 2012).

Tfh cells, which need the transcription factor Bcl6 for their development, are distinct from conventional Th1, Th2, Th17, and Treg cells (Nurieva et al., 2008, 2009; Johnston et al., 2009; Yu et al., 2009). It has been reported that Tfh cells express no or very low levels of T-bet. However, high levels of T-bet are detected in Bcl6-expressing cells at an early stage of cell differentiation (Nakayamada et al., 2011). By using T-bet fate-mapping tools, we previously reported that T-bet expression by Treg cells is dynamic (Yu et al., 2015a). Follow-up analysis indicates that even in the T effector compartment, T-bet expression is also dynamic. Interestingly, some ex–T-bet cells actually display a Tfh cell phenotype. Thus, it is likely that transient expression of T-bet during early stage of Tfh cell differentiation may be sufficient to prepare the *Ifng* locus for its later expression. Once the *Ifng* locus is in an open conformation, T-bet may no longer be needed for IFN-γ production by Tfh cells (Fig. 3).

Even though Tfh cells express very low levels of GATA3, IgE class switching still depends on GATA3 (Zhu et al., 2004), indicating that IL-4–producing Tfh cells require GATA3 for their development. It is possible that IL-4 production by Tfh cells only needs low levels of GATA3 expression (Yusuf et al., 2010; Liang et al., 2012). Alternatively, GATA3 expression during the differentiation of IL-4–producing Tfh cells is also dynamically regulated. Although chromatin remodeling at the *Il4* locus may require transient induction of GATA3 expression, IL-4 production by mature Tfh cells may occur in a GATA3-independent manner. This is consistent with our early study showing that *Gata3* deletion in fully differentiated Th2 cells only has a modest effect on IL-4 production (Zhu et al., 2004).

The relationship between IFN-γ–producing Tfh cells and conventional Th1 cells, and between IL-4–producing Tfh cells and Th2 cells, is still unclear. In vitro, conventional Th1 or Th2 cells may develop into IFN-γ– or IL-4–producing Tfh-like cells, and Tfh-like cells may subsequently acquire IFN-γ– or IL-4–producing capacity (Lu et al., 2011). In vivo, IL-4–producing T cells can acquire a Tfh cell phenotype after their interaction with B cells (Zaretsky et al., 2009). Based on the importance of T-bet or GATA3 expression in regulating IFN-γ or IL-4 production, and the fact that T-bet or GATA3 is not highly expressed in Tfh cells, it is possible that transient expression of these master regulators during early Tfh cell dif-

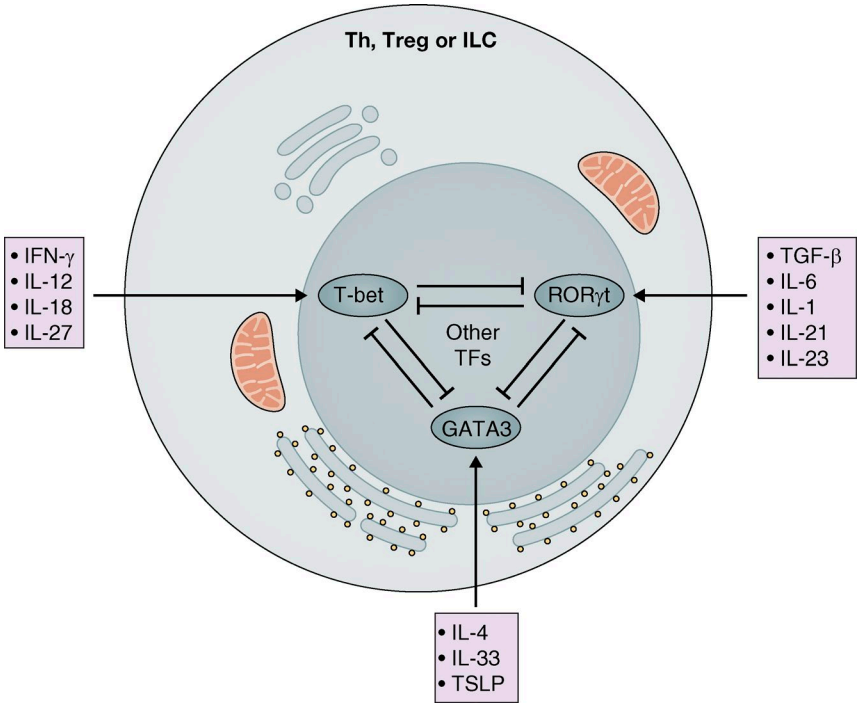

Figure 4. **Quantitative, dynamic, and cell type–specific balance between master transcription factors in determining the fates and functions of CD4 T cell and ILC subsets.** T-bet, GATA3, and RORγt can be expressed by Th effectors, Treg cells, and ILCs at the single-cell level. The balance between these factors is achieved through quantitative and dynamic expression of each molecule in a cell context–dependent manner. Extracellular stimuli such as cytokines may transiently influence the balance, resulting in cell heterogeneity and plasticity. The alteration of this balance may cause immune-related human diseases. On the other hand, fine-tuning the transcriptional regulatory network may be considered as an effective way to treat certain diseases. TFs, transcription factors.

ferentiation may ultimately determine the ability of Tfh cells to produce either IFN-γ or IL-4. Such a model should be tested with experiments using fate-mapping tools.

Molecular basis for dynamic expression of master transcription factors

The status of epigenetic modifications/changes often provides additional information on gene regulation. This is also true for T cell differentiation and ILC development (Kanno et al., 2012; Shih et al., 2014). The combination of multiple epigenetic modifications not only predicts whether a gene is being expressed or not but also indicates whether a gene is silenced or poised for future expression. A poised gene locus is usually linked to bivalent modifications with both H3K4me3 (active chromatin) and H3K27me3 (repressive chromatin) marks (Barski et al., 2007). Interestingly, such bivalent modifications are commonly found in the promoters of many master regulator genes, including *Tbx21*, *Gata3*, *Rorc*, and *Bcl6* in Th cell subsets that do not express these genes (Wei et al., 2009; Nakayamada et al., 2011). Bivalent modifications at the *Gata3* and *Rorc* loci in different T cell population may simply reflect their expression history during T cell development. For *Gata3*, it could also reflect its expression at low levels. However, T-bet is not expressed during T cell development. This is confirmed by the T-bet-Cre–mediated fate-mapping study showing that naive CD4 T cells are all T-bet fate-mapping negative (unpublished data). Nevertheless, the *Tbx21* locus in Th17 and Treg cells has bivalent modifications. It is likely that bivalent modifications also exist in the ILC populations at the master transcription factor loci. Such bivalent modifications may allow the induction of a master regulator of one cell fate in an alternative lineage, first leading to the appearance of mixed phenotype cells coexpressing multiple master regulators and then resulting in lineage switch. Master regulator–coexpressing cells can be generated when cells receive multiple cytokine signals either at the early stages of cell differentiation or after differentiation (Fig. 4). Because of the cross-regulation among the master regulators, even a transient extracellular stimulus may change the balance between these master regulators, resulting in cell heterogeneity and plasticity.

Quantitative balance between master transcription factors

The delicate balance between the master regulators is fine-tuned by quantitative expression of these factors. Multiple studies have indicated haploinsufficiency of the master regulators. Heterozygous deletion of T-bet results in derepression of Th2 cytokine expression (Szabo et al., 2002), reduction of GATA3 expression level by half in ILC2s causes a partial defect in ILC2 development and function (Klein Wolterink et al., 2013), and T-bet expression is up-regulated in RORγt heterozygous NKp46$^+$ ILC3s (Zhong et al., 2016). These results may be explained by the alteration of a quantitative balance among these three transcription factors. Indeed, as discussed above, the NKp46$^+$ ILC3s express all three factors (T-bet, GATA3, and RORγt). It is well known that T-bet and RORγt are required for the development of NKp46$^+$ ILC3s. Interestingly, our recent study shows that GATA3 is also required for the development of these cells through regulating the balance between T-bet and RORγt (Zhong et al., 2016). GATA3 suppresses RORγt expression in ILC3s. An approximately twofold increase in RORγt expression is found in GATA3-deficient ILC3s, correlated with a loss of NKp46$^+$

ILC3s. On the other hand, RORγt heterozygous mice in which one copy of *Rorc* was replaced by GFP have a dramatic increase in the proportion of NKp46$^+$ ILC3s. Strikingly, restoration of RORγt level by crossing GATA3-deficient mice with *Rorc* heterozygous mice completely rescues the development of NKp46$^+$ ILC3s. These findings indicate that GATA3 fine-tunes the balance between T-bet and RORγt during NKp46$^+$ ILC3 development and the development of NKp46$^+$ ILC3 requires cooperation among these three master regulators that were thought to be mutually exclusive in defining distinct lineages. Cross-regulation and balance between these factors has also been reported in Treg cells (Yu et al., 2015a). Therefore, it is likely that during Th cell differentiation at certain stages, the quantitative balance among these three master regulators may also play a critical role in determining Th effector lineage fates (Fig. 4).

Transcriptional regulatory network

Although various cell types at different developmental stages may express the same transcription factor, the functions of this transcription factor are usually cell type and/or stage specific. GATA3, when expressed at high levels, may regulate different sets of genes in ILC2s versus in Th2 cells (Yagi et al., 2014). Similarly, when it is expressed at low levels, GATA3 regulates different sets of genes in CCR6$^+$ ILC3s versus in NKp46$^+$ ILC3s (Zhong et al., 2016). Furthermore, genes that are regulated by GATA3 in developed ILC3s are very different from those that regulated by GATA3 in developing ILC3s, which may explain why early, but not late, GATA3 deletion affects LTi functions (Yagi et al., 2014; Zhong et al., 2016). GATA3 is also critical for the generation of PLZF$^+$ ILC progenitors in the bone marrow and CD4 single-positive cells in the thymus. It appears that GATA3 collaborates with Th-POK during CD4 T cell development (Wang et al., 2008) but may collaborate with Id2 during ILC development (Klose et al., 2014). Furthermore, even though PLZF$^+$ ILC progenitors express very high levels of GATA3 (equivalent to the GATA3 levels in ILC2s), it is not sufficient to induce type 2 cytokine production in these progenitors. Therefore, a given transcription factor, even when it is expressed at a fixed level, could have unique functions in different cells at various stages.

In addition to complex cross-regulation among the master regulators, many other transcription factors may act upstream or downstream of the master regulators, and some may serve as cofactors of the master regulators. To understand T cell differentiation, ILC development, and their functional regulation at a systems level, several genome-wide studies have been performed to compare the transcriptomes of Th cell subsets, ILC subsets, and ILC progenitors (Wei et al., 2011; Hu et al., 2013; Yosef et al., 2013; Yagi et al., 2014; Robinette et al., 2015; Koues et al., 2016; Shih et al., 2016; Yu et al., 2016; Zhong et al., 2016). Indeed, dozens of other transcription factors are also expressed in a cell type–specific manner (Wei et al., 2011; Hu et al., 2013). Among these factors, some may also be important for regulating cell fate determination and/or cytokine production just as the master regulators. For example, Runx3 is preferentially expressed by Th1 cells, and it plays an important role in regulating IFN-γ production while suppressing IL-4 expression (Djuretic et al., 2007; Naoe et al., 2007; Yagi et al., 2010). Runx3 also regulates the development of ILC1 and ILC3 subsets and the maintenance of ILC1s (Ebihara et al., 2015). Another important example is AHR, which is critical for the production of IL-22 in both Th17/22 subset and in ILC3s (Zhou, 2016).

Transcriptional regulatory network during Th17 cell differentiation has been studied in a great detail (Ciofani et al., 2012; Yosef et al., 2013). Besides the five transcription factors (RORγt, STAT3, IRF4, BATF, and Hif1α) that are previously known to be important for Th17 cell differentiation, a dozen other transcription factors (including Egr2, Etv6, Fosl2, IRF8, and Sp4) are also found to regulate IL-17 expression either positively or negatively. Therefore, although the master regulators play a major role, ultimately, the transcriptional regulatory networks consisting of many transcription factors determine cell differentiation and functions. Extracellular stimuli and cell-intrinsic changes in pathways that regulate the expression of any component of the network could influence the balance between the master regulators in a cell context–dependent manner (Fig. 4). Revealing the transcriptional regulatory network structures in different cell types at various stages with the input signals from extracellular stimuli is important to fully understand the development, activation, and functions of ILC and Th cells.

Conclusions

Different master regulators that determine distinct cell fates are usually expressed in a mutually exclusive manner. However, they can also be coexpressed in both Th cells and ILCs. Activated Treg cells can also express the effector cell–related master regulators, albeit at lower levels. Thus, coexpression and cross-regulation among the master regulators may exist in T effector cells, Treg cells, and ILCs (Fig. 4). The balance between the master regulators is quantitative, dynamic, and cell type specific. A precisely regulated balance would be important to ensure the appropriateness of immune responses. Furthermore, coexpression, cross-regulation, and dynamic expression of the master regulators could result in ILC and Th cell plasticity and may be involved in the generation of Tfh cell subsets. Alterations in signaling pathways and/or cis-regulatory elements that control the expression of these master factors as well as other transcription factors within the transcriptional regulatory network are likely to contribute to certain immunological diseases.

Many studies have been performed using in vitro–generated Th cells or ILC subsets in the steady state. In the future, research should be focused on gene regulation in in vivo–generated Th subsets and activated ILC subsets during infection, allergy, and autoimmunity given that gene regulation is highly cell type and stage dependent. With rapid advances in technology, including the application of CRISPR/

Cas9 in gene modification, some research may be performed directly on human cells (Shalem et al., 2014). Nevertheless, because of the limitations in studying human immune responses in vivo, some important future discoveries will continue to rely on animal models, particularly mouse models. For example, transcription factor reporter mouse strains have served as powerful tools in studying Th and ILC subsets (Zhu et al., 2012, 2016; Yu et al., 2015a; Zhong et al., 2016). Generation of multicolor reporter mice will facilitate the separation of Th and ILC subsets in vivo. New fate-mapping tools using transcription factor locus–driven Cre expression will help us gain further insights into cell stability, plasticity, and heterogeneity (Yu et al., 2015a). However, because of dynamic expression of some transcription factors during development, especially for RORγt and GATA3, cell fate mapping mediated by inducible Cre, such as Cre-ER^{T2}, under the control of these transcription factor loci will be necessary. Furthermore, single-cell RNA sequencing (Gaublomme et al., 2015; Björklund et al., 2016; Chea et al., 2016; Gury-BenAri et al., 2016; Ishizuka et al., 2016; Yu et al., 2016) and CyTOF mass cytometry (Gury-BenAri et al., 2016; Simoni et al., 2017) may allow us to discover novel molecules that regulate ILC and Th cell development and functions during immune responses or in inflammation. Future investigation of Th and ILC cell heterogeneity and plasticity in vivo using new tools and technologies may help explain and/or treat a variety of immune-related human disorders.

ACKNOWLEDGMENTS

Both authors are supported by the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health (1-ZIA-AI001169).

The authors declare no competing financial interests.

Submitted: 16 March 2017

Revised: 25 May 2017

Accepted: 2 June 2017

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