Review

GATA-3 – Not Just for Th2 Cells Anymore

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GATA-3 was first cloned as a T cell specific transcription factor in 1991 and its importance in the transcriptional control of T helper type 2 cell (Th2) differentiation was established in the mid to late 90's. A role for GATA-3 during thymic development has long implied by its continuous and regulated expression through out T lineage development, but the absolute requirement for GATA-3 during early T lymphoid commitment/survival previously precluded definitive answers to this question. Several technical breakthroughs have fueled fruitful investigation in recent years and uncovered unexpected and critical roles for GATA-3 in CD4 thymocyte survival, invariant natural killer T cell generation and function, and also in beta selection. Not only does GATA-3 participate in nearly every stage of T cell development from common lymphoid progenitor to Th2, conditional knockout studies have indicated that the influence of GATA-3 also extends beyond the immune system. *Cellular & Molecular Immunology*. 2007; 4(1):15-29.

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Introduction

The development of pluripotent progenitors into differentiated effector cells relies on the ordered execution of distinct transcriptional programs. In the case of binary differentiation steps, lineage specific transcription factors, upregulated in response to external differentiation signals, evoke patterns of gene expression that drive cells into one lineage and suppress programs associated with the opposite lineage. Hematopoietic stem cells that mature into T lymphocytes must traverse several such binary branchpoints, including the T *vs* B, CD4 *vs* CD8, and T helper type 1 (Th1) *vs* T helper type 2 (Th2) decision steps. The last example, in which naïve precursor helper T cells differentiate into polarized T helper cell subsets with distinct cytokine production profiles and effector functions, has been the subject of intense study, resulting in a model in which the transcription factor T-bet drives Th1 differentiation, while the transcription factor GATA-3 drives Th2 differentiation (1, 2). In recent years the lineage specific transcription factor model has been extended by the identification of FoxP3 and RORγt as "master regulators" of naturally occurring T regulatory (Treg) and T helper type 17 (Th17) cells respectively (3, 4).

Our understanding of the Th2 master regulator GATA-3 has increased substantially, thanks to several technical breakthroughs, such as reaggregate fetal thymic organ culture, siRNA technology, retroviral transduction of primary T cells, and the generation of conditionally deficient mice. Unlike T-bet, whose tissue expression is limited to the immune system and which plays little role during early T progenitor or thymic development (5, 6), GATA-3 has emerged as having multiple roles in the earlier stages of T lymphoid and in non-immune cell development. Here we summarize the evidence that GATA-3 is not only a critical and essential regulator of Th2 cell development and function, but also of CD4 T cell survival, invariant natural killer T cell development and function, and T commitment. How a single transcription factor that is continuously expressed during T lymphoid development can perform different functions at each stage has yet to be understood. Our growing understanding of the interacting partners, post-translational modification and known direct targets of GATA-3 may elucidate the molecular mechanisms of transcriptional regulation in future years.

GATA-3 and the GATA family of transcription factors

GATA-3 is a member of the GATA family of transcription factors (7). There are at least 6 GATA proteins, GATA-1 to

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GATA-6, that have been identified in mammalian genome. All GATA proteins contain two highly conserved C2C2 type zinc fingers, which can bind to DNA containing the consensus WGATAR sequence (W = A or T and R = A or G), and are functionally interchangeable in some *in vitro* systems (8, 9). The GATA members can be further divided into two main groups based on their tissue distribution in adult animals. GATA-1, GATA-2, and GATA-3 are expressed mainly in hematopoietic cells, whereas GATA-4, GATA-5, and GATA-6 are found in endoderm-derived tissues/organs, such as heart and intestine. GATA-1, the founding member of the GATA family, is expressed mainly in erythroid/ megakaryocytic cells in adult animals, and was cloned based on its ability to bind to a functionally critical GATA sequence in regulatory regions of the α and β-globin gene clusters (10). The finding that several conserved and functionally important GATA binding sequences were also identified in the enhancers of the T cell receptor (TCR) genes suggested the presence of a GATA-1-like transcription factor that is expressed in T cells. By screening T cell cDNA libraries with the zinc finger region of GATA-1 under low stringency conditions, several groups independently cloned GATA-3 as the T cell-specific GATA member (11-13). Although GATA-3 is nearly exclusively expressed in T cells in adult animals, its expression can be detected in non-hematopoietic tissues/ organs, such as central nervous system, skin, inner ear, mammary glands, and kidney, during embryogenesis (14-18). Indeed the critical role of GATA-3 during embryogenesis is underscored by the fact that mice deficient in GATA-3 die at embryonic day 11.5 (19) and that haploinsufficiency of GATA-3 in human results in HDR syndrome, characterized by hypoparathyroidism, deafness, and renal dysplasia (20, 21).

GATA-3 and control of Th differentiation

The first described function of GATA-3 in the immune system is as a Th2 specific transcription factor, and the transcriptional regulation of Th subset differentiation has been the subject of a number of excellent reviews (1-4). This section will focus on recent *in vivo* confirmation of the role of GATA-3 as master regulator of Th2 differentiation and function.

Introduction to Th subsets

The differentiation of naïve CD4 T cells into Th1 and Th2 cells was a phenomenon described more than two decades ago (22, 23). Each subset has a set of signature cytokines and effector functions. Th1 cells produce mainly IFN-γ and are responsible for eradicating intracellular microorganisms, whereas Th2 cells express IL-4, IL-5, and IL-13, and orchestrate immunity against multicellular parasites. Unregulated Th1 function is pathogenic in many autoimmune diseases, such as type 1 diabetes mellitus, rheumatoid arthritis, or inflammatory bowel disease, whereas unbalanced Th2 function causes allergic inflammation. Two new functional subsets, Th17 and Treg cells, are also derived from CD4 single positive (SP) thymocytes (24-28). The signature cytokines of Th17 cells are IL-17, IL-17F, and IL-22. IL-17 is critical for eliminating extracellular bacteria (29). Th1, Th2, and Th17 cells can initiate or augment immune responses and are therefore generally referred to as effector Th cells. In contrast, Treg cells produce high level of anti-inflammatory cytokines, such as IL-10 and/or TGF-β, and transdominantly inhibit the proliferation and function of effector Th cells.

In the case of Th1 and Th2 cells, after initial upregulation of both Th1 and Th2 specific factors, certain costimulatory molecules, types of antigen presenting cells (APC), or doses of antigen, and most importantly, the cytokine milieu conspire to promote the dominant expression of either T-bet or GATA-3 and suppression of the factor responsible for the alternate fate, exemplifying the binary lineage decision process. Thus triggering IL-4/Stat6 signaling promotes and is required for Th2 development whereas IFN-γ/Stat1 and IL-12/Stat4 signaling are critical for the differentiation of Th1 cells. The cytokine milieu is critical also for the differentiation of Th17 cells, which requires IL-6 and TGF-β, while TGF-β alone promotes the differentiation of Treg cells. Whereas previously, the subsets of Th cells were primarily identified by their functional cytokine production profile, each subset can now be linked with a key lineage-specific transcription factor (T-bet, GATA-3, RORγt, FoxP3), that is both necessary and sufficient to drive development, and serves as a marker of subset identity (Th1, Th2, Th17, Treg).

Conditional knockout studies confirm GATA-3 as a "master regulator" of Th2 differentiation

GATA-3 was suspected to be important in Th2 differentiation based first on the discovery that GATA-3 binds cognate sites in the IL-5 promoter (30) and then its differential expression in Th2 cells compared to Th1 cells (31, 32). Upregulation of GATA-3 from the low levels normally expressed in naïve Th cells requires both TCR signals and exposure to IL-4, the most potent Th2-polarizing cytokine, whose main action is activation of Stat6. Remarkably, forced expression of GATA-3 is sufficient to induce the production of type 2 cytokines in cells lacking Stat6 and in the presence of IFN-γ and depletion of IL-4, a cytokine milieu that ordinarily drives Th1 differentiation (33-35). Taken together, these results indicate that GATA-3 is a downstream target gene of IL-4/Stat6 but, once induced, is sufficient to drive the expression of Th2 cytokines in an IL-4/Stat6-independent manner.

Studies of GATA-3 transgenic mice further support the notion that GATA-3 is sufficient to promote the differentiation and function of Th2 cells. Th cells obtained from GATA-3 transgenic mice already express a high level of T1/ST2, an IL-1R family member that is preferentially expressed on the surface of murine Th2 cells, and continue to produce type 2 cytokines even after cultivation under Th1 polarizing conditions (36). In several *in vivo* models, such as airway allergic inflammation, worm infection, or delayed type hypersensitivity, GATA-3 transgenic mice exhibit augmented Th2 immune responses and reciprocally display

attenuated Th1 responses (37-40).

GATA-3 is not only sufficient to direct the differentiation of Th cells into the Th2 pathway, but earlier studies indicated that GATA-3 is also essential for the differentiation and function of Th2 cells. Anti-sense GATA-3 RNA has been shown to suppress the expression of IL-4 and/or IL-5 in Th2 cells (31, 41). In addition, nasal administration of anti-sense GATA-3 oligonucleotides suppressed the expression of GATA-3 and significantly attenuated allergic airway inflammation in an animal model of asthma (42). A "dominant negative" GATA-3 mutant has also been used to suppress the function of GATA-3 in transgenic models (43, 44). These KRR GATA-3 transgenic mice are more resistant to Th2 cell-mediated allergic airway inflammation and Th cells derived from these mice produce less type 2 cytokines than wild type cells. However, the KRR GATA-3 is not a true dominant negative mutant and also affects the proliferation and homing of the transgenic Th cells (45). Some of the features are probably caused by artifacts of overexpressing the KRR mutant instead of suppressing the function of endogenous wild type GATA-3.

Because germline deletion of GATA-3 results in embryonic lethality (19), confirmation of the essential role of GATA-3 in the differentiation and function of Th2 cells in whole animals required the generation of mice conditionally deficient in GATA-3 using the Cre-lox system. Two groups generated such mice independently. In one model, henceforth called G3-lckCre mice, GATA-3 was deleted using Cre recombinase driven by the proximal lck promoter. These Th cells exhibit a defect in differentiating into Th2 cells (46). In addition, the GATA-3 deficient Th cells, differentiated under Th2 polarizing conditions, often produce more IFN-γ than wild type Th1 cells. The inability of the GATA-3-deficient Th cells to produce type 2 cytokines does not originate from defects during thymic development because similar results were obtained when the GATA-3 gene was deleted in Th cells *in vitro* with a retroviral Cre recombinase. These results were confirmed in a second model using Cre recombinase driven by OX40 promoter, which is expressed only after activation of mature CD4 T cells, bypassing thymic development (47). In addition to impaired cytokine production, GATA-3-deficient Th2 cells also proliferated less robustly than wild type Th2 cells (47).

The effect of GATA-3 deficiency is also observed in both mice and humans *in vivo*. When GATA-3 deficient mice were immunized with an antigen mixed with alum, Th cells derived from draining lymph nodes produced very little IL-4 but an abnormally high level of IFN-γ in response to rechallenge with the antigen (46). This was in sharp contrast to likewise immunized wild type mice, which mounted a Th2 immune response. When GATA-3 deficient mice were infected with *N. brasilienesis*, which usually induces a dominant Th2 immune response, the mice had a reduced level of serum IgE, and the blasting Th cells harvested from these mice displayed a cytokine profile of Th1 cells instead of Th2 cells (47). Furthermore, human individuals, who carry only one functional GATA-3 allele and suffer from HDR syndrome, have a reduced serum level of Th2-dependent immunoglobulins, such as IgG4 and IgE, whereas the level of Th1-dependent IgG1 is reciprocally elevated (48). These results firmly establish the essential role of GATA-3 in regulating the differentiation of Th2 cells.

Transcriptional targets of GATA-3 in Th cells

It stands to reason that GATA-3 would have characteristic Th2 cytokines among its direct transcriptional targets, such as the IL-5 and IL-13 promoters, whose activity is critically dependent on GATA-3 binding (49-51). In the case of IL-4, regulation by GATA-3 is not likely to be *via* direct transactivation despite the identification of GATA-3 binding sites around the IL-4 gene (52, 53). Deletion of GATA-3 in well-differentiated Th2 cells results in a significant reduction in the production of IL-5 and IL-13, whereas the expression of IL-4 is unaffected (47), supporting this notion. Likewise GATA-3 probably also serves as a chromatin remodeling factor rather than a direct transactivator of the IL-10 gene (54), another Th2 cytokine. GATA-3 is sufficient to induce permissive epigenetic modifications in the IL-10 locus in an IL-4-independent manner but dose not directly transactivate the IL-10 promoter. Similar to IL-4, the expression of IL-10 is independent of GATA-3 in well-differentiated Th2 cells (S.Y.P. and I.C.H., unpublished data).

The epigenetic modification of the IL-4, IL-5, and IL-13 loci, present within a 200 kb Th2 locus and controlled by a highly conserved locus control region (LCR) within the Rad50 gene located between the IL-5 and IL-13 genes (55), on chromosome 11 has been extensively reviewed (56, 57). Various epigenetic modifications, including methylation, acetylation, and phosphorylation of histones and methylation/demethylation of CpG islands of DNA, impose "permissive" or "repressive" changes on the Th2 locus *via* several cis-acting regulatory regions that are conserved in sequence through evolution. Permissive modifications permit, whereas repressive modifications deny, the access of transcriptional machinery to the promoters of the Th2 cytokine genes. The critical role of epigenetic modifications in shaping the phenotype of Th cells has more recently been elucidated by studies using Th cells rendered deficient in either Dnmt1, a DNA methyltrasferase, or Mbd2, a methyl-CpG-binding protein (58, 59). Both Dnmt1 and Mbd2 enhance methylation of DNA and facilitate the formation of a repressive conformation. Th cells deficient in either Dnmt1 or Mbd2 aberrantly produce a high level of IL-4 even under Th1 polarizing conditions.

It is believed that GATA-3 plays a critical role in the establishment of a full permissive conformation of the Th2 locus (35, 51, 60). But how GATA-3 accomplishes this is poorly understood. The C-terminal finger of GATA-1 can interact with CREB-binding protein, CBP, which contains histone acetyltransferase activity (61). A recent study further suggests that GATA-3, T-bet, CBP/p300, HDAC-3, and HDAC-5 may form a high order protein complex (62). But how the formation of this high order complex, which contains nuclear proteins that have opposite effect on the chromatin conformation, influences the epigenetic modification of the Th2 locus in Th cells is unclear. Finally, several recent

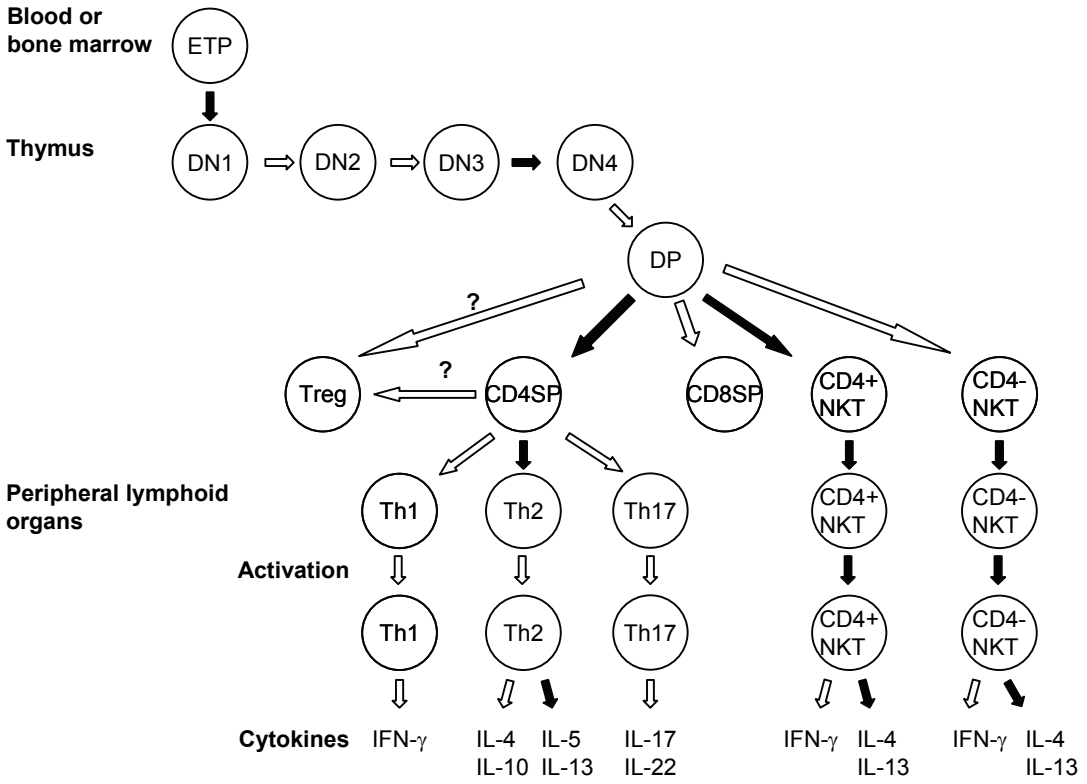

Figure 1. Schematic diagram of the development of T cell lineage. ETP stands for early thymic progenitor. The dark arrows indicate GATA-3-dependent steps.

studies have revealed that GATA-3 and Stat6 binding are associated with complex long-range intrachromosomal interactions that generate loops of DNA in the Th2 locus and physically juxtapose the promoters of the cytokine genes (63, 64). The formation of densely packed loops of DNA in activated Th2 cells requires the induction of a "genomic organizer" SATB1 (special AT-rich sequence binding protein 1), which binds to nine base unpairing regions (BURs) within the Th2 locus. Four of the nine BURs are located in the LCR. SATB1 then folds the locus into densely packed loops by anchoring the locus to scaffold proteins. Other proteins including c-maf, also a Th2 cell-specific transcription factor that is a potent transactivator of the IL-4 gene (65, 66), RNA polymerase II and Brg1, a chromatin-remodeling ATPase subunit of BAF complex also are recruited to the looped Th2 locus in activated Th cells. These intriguing data portray the temporal sequence of the molecular events leading to stable and coordinated expression of Th2 cytokines. But the role of GATA-3 in maintaining the permissive conformation of the Th2 locus once established is probably limited. "Quasi-Th2" cells were generated by introducing a "deletable" GATA-3 gene into differentiating Th1 cells (67). These Th2 cells expressed type 2 cytokines and had undergone permissive epigenetic modifications in the Th2 locus. Deletion of the exogenous GATA-3 gene in these Th2 cells, however, only had a subtle impact on the epigenetic modifications of the Th2 locus.

GATA-3 and control of thymocyte differentiation

GATA-3 is not only preferentially expressed in the Th2 lineage and critical for Th2 differentiation and function, but also exhibits regulated expression throughout thymic development. The following section reviews critical studies using knock-in, transgenic, RNAi and conditional knockout approaches demonstrating that GATA-3 is required at both the β-selection step and CD4/CD8 differentiation step of thymic development.

Normal expression pattern and the effects of overexpression

Thymic development is traditionally described by the pattern of cell surface markers expressed at each stage (Figure 1). T progenitors that seed the thymus initially express neither CD4 nor CD8 and are termed double-negative (DN), which in turn are divided into DN1, DN2, DN3, and DN4 stages based on their expression of CD44 and CD25. After expression and triggering of the pre-TCR, composed of the invariant pre-T α chain and the TCRβ chain, DN3 thymocytes undergo so-called β-selection, a checkpoint that ensures that only those cells which have rearranged the TCRβ gene successfully are allowed to divide and differentiate. DN4 cells then undergo proliferation and differentiate into cells expressing both CD4 and CD8, termed double positive (DP). DP cells rearrange the TCRα gene, and expression of mature

α/β TCR leads to the process of positive and negative selection, ensuring a diverse repertoire of T cells with appropriate avidity and specificity. $CD4^{hi}CD8^{hi}$DP thymocytes that have undergone positive selection upregulate markers such as CD69 and downregulate CD8 slightly (Figure 2). These $CD4^{hi}CD8^{int}CD69^{hi}$ positively selected cells contain committed CD4 and CD8 progenitors (68, 69). These committed progenitors then undergo a binary lineage decision step and differentiate into CD4 single positive (CD4SP) and CD8 single positive (CD8SP) cells, which have fully downregulated the opposite co-receptor and then are exported to the periphery.

The expression of GATA-3 during the development of thymocytes has been characterized using a lacZ gene inserted into the GATA-3 locus as a reporter (70). A low to moderate level of expression of lacZ can be detected in every stage of thymic development and its expression is further induced during the transition from the DN3 to the DN4 stage and from the DP to CD4SP stage. These two waves of induction correspond nicely with β-selection and positive selection, when engagement of antigen receptors with selective antigens takes place. Indeed, expression of GATA-3 is induced in DP thymocytes by signals derived from TCR (71). CD4SP thymocytes and peripheral $CD4^+$ T cells maintain a high level of GATA-3, whereas the expression of GATA-3 is quickly downregulated once DP cells mature into CD8SP thymocytes and peripheral $CD8^+$ T cells.

Several strains of mice expressing transgenic GATA-3 specifically in T cells during thymic development have been generated. One transgenic line, in which a GATA-3 transgene is driven by a CD2 promoter, displays impaired maturation of CD8SP cells and consequently has a reduced number of peripheral $CD8^+$ T cells (72). Surprisingly, approximately 50% of these mice spontaneously develop thymomas composed of $CD4^+CD8^{lo}CD3^+$ cells. These results, although interesting, have not been reproduced in other GATA-3 transgenic lines, which have otherwise unremarkable thymic development (31, 38). The number and subset distribution of thymocytes in these lines are very comparable to those of wild type mice despite the apparent overexpression of GATA-3. The overexpression approach thus suggests that endogenous GATA-3 is probably already expressed at a saturating level in thymocytes of later stages.

GATA-3 is indispensable for the development of CD4SP thymocytes

The molecular mechanism of how the CD4/CD8 lineage decision is made has been recently reviewed (73-75). Over the past few years, the landscape of our understanding of the transcriptional regulation of CD4/CD8 development has changed from that of a blank slate thanks to definitive studies demonstrating the indispensable roles of Th-POK (also known as c-krox) and GATA-3 in CD4SP development.

The transcriptional control of CD4/CD8 lineage determination was previously elusive, though there was reason to suspect that the processes of positive selection, commitment to the CD4 lineage and post-commitment CD4SP survival were separable. Temporally, the testing of randomly generated α/β TCR for the appropriate range of specificity and avidity through positive and negative selection occurs simultaneously with the downregulation of one coreceptor, thereby matching of CD4 expression with MHC class II (MHC-II) restriction and CD8 expression with MHC class I (MHC-I) restriction. That the helper-deficient or so-called HD mutant mouse (76) exhibited a selective lack of CD4SP thymocytes while generating positively selected CD8SP thymocytes demonstrated that positive selection and lineage determination were functionally separable. Indeed, when these mice were crossed to the MHC-II restricted AND TCR transgenic mice, in which all positive selecting thymocytes are expected to become CD4SP cells, transgenic T cells were nevertheless driven into the CD8SP lineage, indicating that the HD gene was required for CD4SP lineage determination (77). Positional cloning of the HD gene revealed a spontaneous A to G change at nucleotide position 1165 resulting in Arg to Gly substitution within the second zinc finger of a transcription factor which the group named T-helper-inducing POZ/Kruppel-like factor or Th-POK (78). This gene was previously known as c-Krox, is induced in positively selected thymocytes and is preferentially expressed in CD4SP but not in CD8SP thymocytes (78, 79). Forced expression of Th-POK in thymocytes destined to become CD8SP cells was sufficient to drive thymocytes into the CD4SP lineage (78, 79). Thus, Th-POK is essential and sufficient for the development of CD4SP thymocytes and is the only factor shown to control lineage determination, independently of MHC restriction.

The role of GATA-3 in CD4SP development, demonstrated by gene deletion and RNA interference approaches, was unexpected, and GATA-3 appears to control not lineage commitment but post-commitment CD4SP survival. Conditional GATA-3-deficient mice were generated using the Cre-lox system to further study the role of GATA-3 during intrathymic T cell development. Mice deficient in GATA-3 at the DN4 stage, henceforth called G3-CD4Cre mice, were created by deleting the GATA-3 gene with a Cre recombinase driven by the CD4 promoter. These mice had a drastic and selective reduction in the number of CD4SP subset (80). In contrast, total thymocyte numbers and the number and maturation of DP and CD8SP thymocytes were apparently normal even though the GATA-3 gene was also deleted in these cells. This result is similar but not identical to what was
observed when the expression of GATA-3 was suppressed using RNA interference in reaggregate fetal thymic organ culture (71). In this *in vitro* system, the development of CD4SP thymocytes was also attenuated but there was a reciprocal increase in the percentage and number of CD8SP subset. This discrepancy most likely originates from differences between *in vitro* fetal thymic organ culture and *in vivo* thymocyte development in adult animals.

Although G3-CD4Cre mice have almost no mature CD4SP thymocytes, a substantial number of mature CD4SP thymocytes can actually be found in G3-lckCre mice, and as described above, exhibit defective Th2 differentiation. This discrepancy is not due to incomplete deletion of GATA-3 by

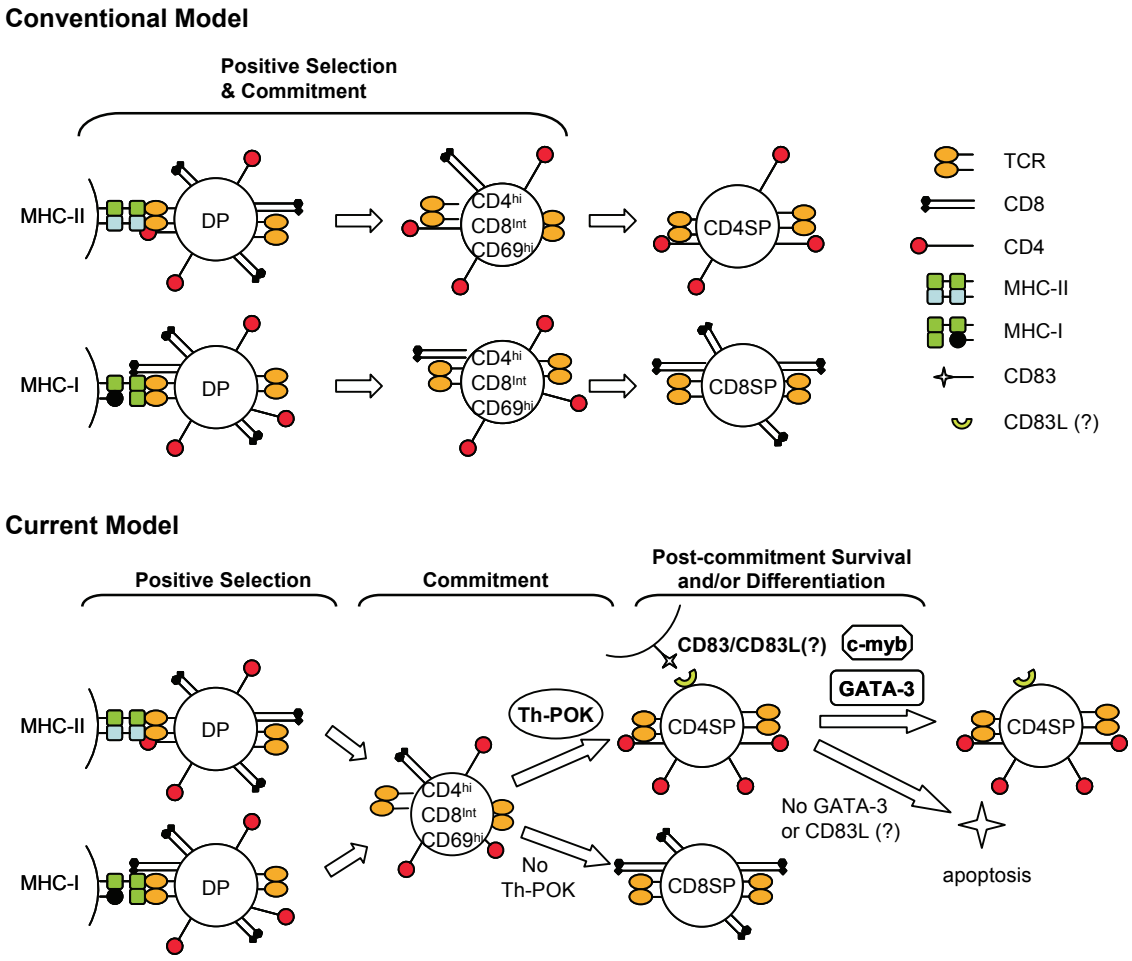

Figure 2. Two models of the generation of single positive thymocytes. CD83L stands for CD83 ligand.

the lckCre transgene because the mature CD4SP population found in the G3-lckCre mice have undergone Cre-mediated deletion of the GATA-3 gene, contain only residual GATA-3 protein, and express a slightly reduced level of TCR, a feature of GATA-3 deficient T cells. It is still unclear how the CD4SP thymocytes found in G3-lckCre mice can develop in the absence of GATA-3. One possible scenario is that thymocytes, once they survive β-selection in the absence of GATA-3, become less dependent on GATA-3 in subsequent developmental stages. Alternatively, the kinetics of GATA-3 gene deletion in the G3-lckCre mice and half-life of previously translated protein could allow GATA-3 to persist just long enough to allow CD4SP maturation to occur.

The virtual absence of CD4SP thymocytes could indicate that GATA-3 is essential for the commitment of DP to CD4SP lineage. According to this scenario, all positively selected DP cells, including those destined to become CD4SP cells, would be channeled into CD8SP cells in the absence of GATA-3, such as in the HD mouse. An alternative scenario is that GATA-3 is required for the survival or final differentiation of CD4SP cells after commitment. These two scenarios can be distinguished by introducing an MHC-II restricted TCR transgene into GATA-3-deficient mice. If GATA-3 is required for CD4 lineage commitment, transgene-expressing DP thymocytes will be converted into CD8SP cells. In contrast, a lack of transgene-expressing CD4SP and CD8SP cells will argue for a defect at a post-commitment stage. When the DO11.10 or AND TCR transgene (S.Y.P. and I.C.H., unpublished data) was introduced into GATA-3-deficient mice, there was still a marked reduction in the number of CD4SP cells and no conversion of transgene-expressing DP cells into CD8SP cells (80), a result in agreement with the latter scenario.

The mechanism by which GATA-3 controls CD4SP survival has not been characterized, though potential avenues of exploration are suggested by two other gene-deficient mouse models, CD83 deficient and c-myb deficient mice, which have similar phenotypes. CD83 is an immunoglobulin-like transmembrane protein expressed mainly in APC and thymic epithelium (81). CD83-deficient mice also have a striking reduction in CD4SP population but a normal number of CD8SP cells (82). Similar to what was observed in GATA-3-deficiency, the AND TCR transgene failed to rescue the differentiation of CD4SP cells or give rise to AND-

positive CD8SP cells. The defect appears to reside in thymic radio-resistant cells because CD83-deficient thymus was unable to support the differentiation of AND-bearing wild type CD4SP thymocytes in mixed chimeric hosts. It is proposed that thymocytes express an ill-identified ligand for CD83 and that the interaction between CD83 on thymic stromal cells and CD83L on differentiating thymocytes is indispensable for the differentiation of CD4SP cells. This intriguing result suggests that GATA-3 and CD83L pathway may intersect at the post-commitment stage.

The thymic phenotypes of GATA-3 deficiency also resemble those of c-myb-deficient mice. Germline deficiency of c-myb resulted in a complete and early block of thymocyte development (83). Descendants of c-myb-deficient ES cells could still be detected in the thymi of chimeric hosts in a RAG-2 complementation system, a result different from what was observed with GATA-3-deficient ES cells. These c-myb-deficient thymocytes are $CD44^{lo}CD25^{-}$, carry unrearranged TCRβ loci, and may represent very early thymic progenitors, a stage even more immature than the classical DN1 cells. When the c-myb gene was deleted at an early DN stage through the Cre-lox system, a profound block at β-selection was also observed, which was similar to G3-lckCre mice as detailed in a later section (84, 85). This block is most likely caused by a defect in Dβ-Jβ and Vβ-DβJβ recombination rather than in post-transcriptional expression of the TCRβ gene as seen in GATA-3-deficient DN3 cells. Interestingly, the c-myb-deficient mice also have a significant reduction in the number of CD4SP thymocytes but the number of CD8SP thymocytes is nearly normal. Introduction of an MHC-II-restricted TCR transgene not only failed to rescue the development of CD4SP cells but also did not convert the transgene-positive cells into the CD8SP subset, suggesting a post-commitment defect.

One emerging model is that GATA-3 and c-myb may act in concert to regulate the same molecular events, or act in sequential steps of the same pathways at several stages of thymic development. Such functional interaction between c-myb and GATA proteins has been demonstrated during the differentiation of erythrocytes (86, 87). At the post-commitment stage, GATA-3 (and c-myb) may regulate the expression of the elusive CD83L or alternatively CD83L signaling may be essential for maintaining the expression of GATA-3. In contrast, Th-POK and GATA-3 clearly act in two independent steps. Th-POK dictates the lineage determination of the CD4 lineage, which requires GATA-3 for subsequent differentiation or survival (Figure 2).

Critical roles of GATA-3 in invariant NKT cell development, maturation, and function

Invariant NKT (iNKT) cells are a unique subset of T cells expressing Vα14Jα18 (mouse) or Vα24Jα18 (human) T cell receptors, which are often coupled with Vβ8, Vβ7, or Vβ2. iNKT originate from thymic DP cells but, unlike conventional α/β T cells, iNKT cells are positively selected in the thymus by CD1d molecules expressed on the surface of DP thymocytes. iNKT cells that have successfully undergone positive selection further differentiate into either $CD4^{+}$ or $CD4^{-}CD8^{-}$ mature iNKT cells and emigrate to peripheral lymphoid organs. iNKT cells only make up approximately 5% of peripheral T cells but they are capable of rapidly, within hours, producing large amounts of various cytokines, including IFN-γ, IL-4, and IL-13, upon encountering antigens, a phenomenon called cytokine storm. The natural antigens for iNKT cells are still being discovered but thus far α-galactosylceramide (α-GC), a glycolipid derived from marine sponge, is the most potent agonist for iNKT cells. In addition to mounting cytokine storm, iNKT cells also express many NK receptors and are capable of killing tumor cells. A large body of evidence has demonstrated that iNKT cells play important immunomodulatory roles in infection, autoimmunity, allergy, and tumor immunity. Readers should refer to several recent reviews for details of iNKT cell biology (88, 89). Recent studies indicate that GATA-3 plays a crucial role in regulating the development, maturation, and function of this unique subset of T cells.

GATA-3 is also expressed in iNKT cells (90-92). Until recently, very little was known about the role of GATA-3 in regulating the development and function of iNKT cells. The generation of conditional GATA-3-deficient mice has made it possible to address this question. G3-CD4cre mice have a near normal number of thymic iNKT cells but, surprisingly, more than 90% of the iNKT cells are $CD4^{-}CD8^{-}$ iNKT (93). This is in sharp contrast to control mice, in which the ratio between $CD4^{+}$ and $CD4^{-}CD8^{-}$ subsets of iNKT cells is approximately 1:1. Most of the GATA-3-deficient iNKT cells undergo apoptosis during the transition from the thymus to peripheral lymphoid organs, resulting in a 6-fold reduction in the number of iNKT cells in the spleen and a virtual absence of iNKT cells in the liver. In contrast to thymic iNKT cells that are $CD69^{low}$, peripheral iNKT cells of adult mice express a high level of CD69, a feature of activated T cells. But peripheral iNKT cells of young mice are actually $CD69^{low}$. The transition from $CD69^{low}$ to $CD69^{high}$ takes place at two to three weeks after birth. This peripheral maturation step is apparently impaired in the absence of GATA-3 because residual GATA-3-deficient iNKT cells fail to upregulate CD69 once emigrate to peripheral lymphoid organs. More importantly, GATA-3-deficient iNKT cells are impotent in mounting a cytokine storm, produce neither IFN-γ nor type 2 cytokines, and fail to upregulate activation markers, such as CD69 and CD40L, in response to intravenous α-GC. The failure to mount a cytokine storm is mainly due to a defect in TCR signal transduction. This signaling defect probably occurs temporally upstream of protein kinase C and calcium influx because GATA-3-deficient iNKT cells are still capable of producing IFN-γ and upregulating the expression of CD69 normally in response to PMA/ionomycin stimulation. Despite the normal production of IFN-γ, PMA/ionomycin-stimulated GATA-3-deficient iNKT cells still fail to produce IL-4 or IL-13, a result reflecting the cardinal function of GATA-3, i.e., promoting the expression of type 2 cytokines. It is worth pointing out though that GATA-3's role in mediating TCR signal transduction is probably unique to iNKT cells. Deficiency of GATA-3 has little impact on the TCR signaling

of DP thymocytes or peripheral Th cells (46, 80). GATA-3-deficient peripheral Th cells in fact already exhibit features of activated/memory Th cells *ex vivo*. These observations highlight the versatility of GATA-3, the function of which can vary significantly depending on the needs of cells. In agreement with this notion, GATA-3 has been shown to be required for optimal production of both IFN-γ and type 2 cytokines by NK cells (94). Future investigation into how GATA-3 modulates TCR signal transduction and whether GATA-3 is required for cytotoxicity will greatly advance our understanding of the molecular events regulating the function of iNKT cells.

GATA-3 is needed for optimal β-selection

When the GATA-3 gene was deleted at DN2/DN3 stage, but prior to β-selection, by a Cre recombinase driven by the proximal lck promoter, there was a striking reduction in thymic cellularity due to a partial block in the transition from the DN3 to DN4 stage (80). The DN3 to DN4 transition is dependent on successful β-selection, which requires fruitful rearrangement and expression of TCRβ chain to couple with pTCRα. The level of intracellular TCRβ chain was indeed significantly diminished in GATA-3-deficient DN3 cells. Such a result is consistent with the notion that GATA-3 serves as a transactivator of TCR genes by binding to their enhancers. However, the TCRβ loci in GATA-3-deficient DN3 cells, like those in wild type cells, had undergone rearrangement and the level of TCRβ transcripts was comparable between wild type and GATA-3-deficient DN3 cells. These results suggest that GATA-3 actually regulates the expression of TCRβ at a post-transcriptional rather than transcriptional step. Although impaired expression of TCRβ may well explain the block in β-selection, GATA-3 probably has additional roles in regulating the DN3 to DN4 transition. When the DO11.10 TCR transgene was used to substitute for a lack of pTCR, the block in DN3 to DN4 transition was still observed in the absence of GATA-3, despite equivalent transgene expression in wild type and GATA-3-deficient cells. One possible explanation for this finding is that GATA-3 also controls signaling events downstream of pTCR in DN3 cells. This scenario remains to be validated.

GATA-3 and T progenitor commitment

GATA-3 expression during lymphoid development is considered a canonical sign of early T commitment and has long been known to be essential for this process. Anti-sense oligonucleotides of GATA-3 strongly inhibited the differentiation of T cells from fetal liver progenitors in a fetal thymic organ culture system (95). When GATA-3-deficient lymphoid cells were reconstituted in a RAG blastocyst complementation system, it was discovered that GATA-3-deficient ES cells failed to contribute to the thymocyte compartment in the chimeric mice. No GATA-3-deficient DN thymocytes, the most immature stage of thymic ontogeny, were detected in the chimeric mice, whereas the capacity to reconstituting B cell compartment was comparable between GATA-3-deficient and wild type ES cells (70, 96). These observations suggest that germline deletion of GATA-3 leads to an early and complete block of T cell development. However, the details of this block remain to be investigated. It is possible that deficiency of GATA-3 either prevents early T cell progenitors from seeding the thymus or blocks the differentiation of early thymic progenitors from lymphoid precursors. Not only is GATA-3 essential for the development of T cells, the level and timing of expression of GATA-3 are also critical for the fate of T cell lineage. Overexpression of GATA-3 either in T cell progenitors or early DN thymocytes actually hinders, rather than promotes, the development of T cells partly by interfering with the expression of the RAG genes (97-99). These surprising results indicate that the timing and the level of expression of GATA-3 have to be tightly regulated during the development of T cells. Given the limitations of overexpression approaches, answers to the exact function of GATA-3 during early T commitment must await studies using conditionally deficient or other models of GATA-3 deficiency.

Targets of GATA-3 in the thymus

Unlike mature T cells where some transcriptional targets of GATA-3 have been defined, direct transcriptional targets in the thymus have been elusive. Of note, some of the genes previously regarded as target genes of GATA-3, including CD8α (100) and TCR (11, 13), are normally expressed in GATA-3 deficient thymocytes (80). Indeed, while the level of TCR in the remaining CD8 T cells is slightly reduced, the rearrangement and repertoire of the TCR are intact. With regards to the TCRα locus, a conserved sequence block in the Jα locus and the TCRα enhancer have been demonstrated to bind GATA-3 in chromatin immunoprecipitation (101), though the functional significance of this binding is not known. Direct targets of GATA-3 in mature thymocytes, DN3 cells or in T progenitors are thus poorly characterized and are the subject of ongoing investigation.

Regulating GATA-3

As GATA-3 participates in nearly every stage of T cell development, its activity has to be tightly regulated. Recent studies have indicated that this can be achieved by controlling the transcription of the GATA-3 gene, degradation and post-translational modification of the GATA-3 protein, and interactions with other nuclear proteins. Numerous soluble factors, surface molecules, intracellular signaling pathways, and transcription factors have been shown to modulate the expression of GATA-3 in Th cells. Most of the studies, however, fail to demonstrate a direct effect on the transcription of the GATA-3 gene partly because that the transcriptional regulation of the GATA-3 gene in T cells is poorly understood. Below, we will discuss the recent progress in the post-translation modifications of GATA-3 and its interacting partners in the context of T cell biology.

GATA-3 interacting proteins

GATA-3 has been shown to interact with several nuclear

proteins, including ROG, FOG, Smad3, PU.1, and T-bet, that are expressed in Th cells.

ROG

ROG is a zinc finger protein containing three C2H2-type fingers in its C-terminus. The expression of ROG is rapidly induced in response to TCR stimulation (102). Over-expression of ROG suppresses the function of GATA-3 in several *in vitro* and *in vivo* settings, probably by sequestering GATA-3 from DNA (62, 71, 103). The level of ROG is significantly higher in $CD8^+$ T cells than in Th cells and ROG, along with histone deacetylases, can bind to a ROG response element located in the exon 4 of the IL-13 gene in a type 2 cytotoxic T cell-specific manner (104). It was therefore proposed that the differential expression of ROG might partly explain why $CD8^+$ T cells are poor IL-4 producers. However, ROG-deficient Th cells, although hyper-responsive to anti-CD3 stimulation, can differentiate normally into Th1 and Th2 cells and deficiency of ROG has no impact on the susceptibility to EAE, a Th1 cell-mediated demyelinating disease (105). Moreover, the production of IL-4 by ROG-deficient $CD8^+$ T cells is very comparable to that of wild type $CD8^+$ T cells. These observations suggest possible functional redundancy between ROG and ROG-like proteins.

FOG

FOG is a multi-zinc finger protein and was originally cloned as a GATA-1 interacting protein (106). It plays a critical but complicated role in the development of erythroid/megakaryocytic lineage by modulating the function of GATA-1 (107). It was subsequently discovered that FOG interacts with the N-terminal finger of GATA proteins (106, 108). Interestingly, FOG is expressed in naïve Th cells but is downregulated in both Th1 and Th2 cells (109, 110). Forced expression of FOG suppressed GATA-3-dependent differentiation of Th2 cells but had a negligible effect on fully differentiated Th2 cells, suggesting that FOG may serve as a repressor of GATA-3 during the differentiation of Th cells. Surprisingly, FOG-deficient Th2 cells, instead of over-producing Th2 cytokines, actually express a somewhat lower level of IL-4 than FOG heterozygous Th2 cells, implying that normally FOG acts as a co-factor for GATA-3. Thus the physiologic role of FOG in Th2 development is unclear.

FOG deficiency affects thymocyte development but does not completely phenocopy GATA-3 deficiency. As deficiency of FOG causes embryonic lethality (111), FOG-deficient T cells were generated through RAG-2 complementation (109). The authors reported a profound block during β-selection, indistinguishable from that of the RAG-2 deficient host, and were not able to determine at which stage of DN development FOG deficient cells were blocked. While a β-selection defect is consistent with what is seen in G3-lckCre mice, the profundity of the block and the fact that CD4 T cells were recovered in the periphery of these mice indicate that FOG at best may act as a cofactor for GATA-3 at the T progenitor or DN thymocyte developmental stages.

Smad3

GATA-3 interacts with Smad3, but not other Smad proteins, and recruits Smad3 to the IL-5 promoter independently of Smad3 binding to DNA (112). Such GATA-3/Smad3 interaction enhances the activity of GATA-3 and provides a molecular explanation for the positive effect of TGF-β on the production type 2 cytokines when administered at miniscule doses. At a higher concentration of TGF-β that is close to the physiological level, TGF-β actually interferes with the differentiation of Th2 cells by inhibiting the expression of GATA-3 (113, 114). The biological significance of the GATA-3/Smad3 interaction therefore remains uncertain.

PU.1

PU.1 is a member of the ETS family of transcription factor and is essential for myeloid and lymphoid development (115). Somewhat unexpectedly, the expression of PU.1, albeit at a low level, was also detected in Th2 cells but not in Th1 cells, and is restricted to low cytokine-secreting subset of Th2 cells that produce a high level of CCL22 (116). Forced expression of PU.1 suppressed cytokine production by Th2 cells, whereas introduction of PU.1 siRNA had an opposite effect. PU.1 physically interacted with GATA-3 and excluded it from DNA. In $IL\text{-}4^{high}$ and $CCL22^{low}$ Th2 cells that express little PU.1, GATA-3 was found to bind to a 3' enhancer of the IL-4 gene. The binding of GATA-3 to this enhancer was prohibited in PU.1-expressing Th2 cells. The expression of PU.1 therefore defines a subset of Th2 cells, but the *in vivo* function of this subset of Th2 cells has yet to be investigated.

T-bet

The latest addition to the list of GATA-3 interactors is T-bet, the master regulator of Th1 cells. It was first reported that GATA-4 or GATA-5 physically interacted with T-box transcription factors, such as Tbx5 and Tbx20, and synergistically transactivated several cardiac specific genes (117, 118). It was subsequently discovered that T-bet could interact with GATA-3 in thymocytes (119). The interaction is dependent on the phosphorylation of T-bet at the tyrosine residue 525 by ITK and inhibits the binding of GATA-3 to its target DNA. Consequently, forced expression of wild type T-bet but not an Y525F mutant inhibited the production of type 2 cytokines in Th2 cells. This observation nevertheless is difficult to reconcile with the finding that ITK-deficient Th cells tend to differentiate into Th1 cells under non-polarizing conditions (120, 121). It was recently reported that GATA-3 could interfere with the transactivation of the promoter of fucosyltransferase VII by T-bet (62). Thus, the functional consequence of T-bet/GATA-3 interactions can be bi-directional, allowing these two opposing transcription factors to counteract each other. Such a counteracting mechanism may be operating at least in cells, such as thymocytes, newly activated but undifferentiated Th cells, or iNK cells, in which both factors co-exist.

Post-translational modifications of GATA-3

Differential expression of GATA-3 is critical to its function and must be modulated precisely. This is partly achieved by post-translational modification of GATA-3 protein. GATA-3

is a phosphoprotein and can be phosphorylated in Th2 cells by p38 kinase in response to c-AMP (122). Treatment of Th2 cells with a specific inhibitor of p38 attenuated GATA-3-dependent promoter activities and suppressed the c-AMP-induced expression of IL-5 and IL-13. The p38-dependent phosphorylation of GATA-3 may partly explain the synergistic effect between c-AMP and exogenous GATA-3 in converting Th1 cells into type 2 cytokine-producing cells (60). Despite these intriguing observations, the putative c-AMP-induced phosphorylation sites have yet to be identified. GATA-3 has been shown to be a substrate of protein kinase A (PKA), which phosphorylates the serine residue of the KRRLSA sequence in between the two zinc fingers of GATA-3 in breast cancer cells (123). However, such PKA-mediated phosphorylation of GATA-3 has yet to be demonstrated in T cells. GATA-3 is also constitutively acetylated in T cells and the status of acetylation is not altered by stimulation with a mitogen. Coincidentally, the lysine of the KRRLSA sequence is the dominant acetylation site of GATA-3 (45). Conversion of the KRR to AAA, as in the KRR mutant of GATA-3, results in local hypoacetylation and the KRR mutant can function as a dominant negative mutant of GATA-3 in some assays. This observation is consistent with a recent report showing mutation of a key acetylation site of GATA-1 interferes with its *in vivo* chromatin occupancy but not nuclear localization or protein stability (124). Nevertheless, the mechanism of action of the KRR mutant is still unclear. The mutation attenuates the transcriptional activity but does not appear to affect the expression, nuclear localization, capacity of remodeling the Th2 locus, and DNA binding of GATA-3 (43).

More recently, it was shown that GATA-3 was ubiquitinated and degraded by proteasomes. The ubiquitination of GATA-3 is dependent on the polycomb protein Bmi-1 and the E3 ligase Mdm2 (125, 126). Both Bmi-1 and Mdm2 can physically interact with GATA-3, resulting in poly-ubiquitination and degradation of GATA-3. Over-expression of Bmi-1 in Th cells differentiating under non-polarizing conditions inhibited the expression of IL-4 but reciprocally enhanced the production of IFN-γ. In contrast, deficiency of Bmi-1 or suppression of Mdm2 expression has an opposite effect on the differentiation of Th2 cells. This ubiquitination-proteasome degradation pathway of GATA-3 can be counteracted by the TCR-activated Ras-ERK MAPK cascade (126). This observation provides an attractive explanation for the need of TCR signals, in addition to IL-4/Stat6, for the induction of GATA-3 during the differentiation of Th2 cells. Most of the studies of post-translation modification of GATA-3 have been conducted in *in vitro* systems and in the setting of Th cell differentiation. The functional significance of these post-translational modifications of GATA-3 in *in vivo* Th immune response or in the development of thymocytes remains poorly characterized.

Structural and functional analyses of GATA-3

The non-conserved N-terminal half of GATA-3 contains two activation domains and is followed by two zinc fingers that are highly homologous to those of other GATA proteins. Studies of GATA-1 and various mutants of GATA-3 from patients with HDR syndrome have demonstrated that the C-terminal zinc finger is required for DNA binding (127). The N-terminal zinc finger is used for interaction with other nuclear proteins and stabilizing the interactions between DNA and the C-terminal zinc finger. The roles of these functional domains of GATA-3 in the differentiation and function of Th cells have been carefully examined by introducing various mutants of GATA-3 into differentiating Th cells *in vitro*. Deletion of the C-terminal zinc finger or both transactivation domains rendered GATA-3 unable to support the differentiation and function of Th2 cells (128, 129). The inhibitory effect of GATA-3 on the differentiation of Th1 cells was also completely abrogated. Surprisingly, a low level of remodeling of the Th2 locus, particularly at the IL-13 gene, was still induced by these mutants. Most recently, a novel conserved sequence YxKxHxxxRP located immediately downstream of the C-terminal zinc finger was shown to be essential for DNA binding, supporting the differentiation of Th2 cells, inhibiting IFN-γ production, and remodeling of the Th2 locus (130). Computer simulation studies indicate that this novel sequence, particularly the basic residues and the histidine, stabilizes interactions between GATA-3 and DNA. The YxKxHxxxRP sequence is not deleted in the aforementioned GATA-3 mutant that lacks the C-terminal zinc finger, suggesting that both the C-terminal zinc finger and the YxKxHxxxRP sequence are required for stable interaction between GATA-3 and DNA. Deletion of the N-terminal zinc finger also substantially hampered the production Th2 cytokines, particularly IL-5, but had little impact on the GATA-3's ability to induce chromatin remodeling or suppress the expression of IFN-γ (60, 129). Although some of the data are still controversial, these observations indicate that the functional "tasks" of GATA-3 during the differentiation of Th cells are not always inter-dependent and that the mechanism of action of GATA-3 varies depending on the nature of the tasks.

GATA-3 is the only GATA member that is expressed in Th cells. The exclusive expression of GATA-3 in Th cells raises the question whether the critical roles of GATA-3 in T cells can be replaced by other GATA proteins. Mast cells express GATA-1 and GATA-2 but no GATA-3 and are capable of producing a high level of type 2 cytokines upon activation (131, 132). Ectopic expression of GATA-1, GATA-2, GATA-3, or GATA-4 in differentiating Th1 cells all suppressed the expression of IFN-γ and enhanced the production of IL-4 and IL-5 (128). These studies suggest that the role of GATA-3 can be substituted with other GATA proteins. Nevertheless, GATA-4 appears to be less potent than the three hematopoietic GATA proteins in supporting the production of IL-4 and IL-5, a finding in agreement with a few studies showing that not all GATA proteins are functionally interchangeable. Germline deletion of GATA-1 causes a lethal defect in erythropoiesis during embryogenesis. Expression of GATA-1 or GATA-2 under the transcription control of GATA-1 is sufficient to rescue GATA-1-deficient fetuses. Surprisingly, GATA-3 or GATA-4 expressed in the

same or similar manner is unable to rescue GATA-1-deficient fetuses from embryonic lethality (133, 134). Taken together, these observations argue for the presence of intrinsic functional differences at least between GATA-4 and the hematopoietic GATA proteins. The YxKxHxxxRP sequence is conserved in all GATA proteins and cannot explain the differences. Careful structural and functional analyses comparing GATA-3 and the non-hematopoietic GATA proteins, such as GATA-4, in physiological settings should greatly advance our understanding of the mechanism of action of GATA-3.

Epilogue

The functional role of GATA-3 in the biology of T cells has expanded dramatically since the identification and cloning of this versatile transcription factor. A great deal of knowledge regarding the functional consequences of GATA-3 over-expression and deficiency has been accumulated. However, we are still in an early stage of understanding the mechanism of action of GATA-3. Current data clearly indicate that the mechanism of action of GATA-3 goes above and beyond DNA binding and transactivation. Through its interactions with DNA and various nuclear proteins, GATA-3 participates in almost every stage of T cell development. But very little is known as to how these interactions, with either DNA or nuclear proteins, are coordinated and connected with chromatin-remodeling machinery. Detailed structural and functional analyses in combination with crystallography should help address these important questions.

In addition, the genuine target genes of GATA-3, other than the Th2 cytokine genes, still remain elusive. Comparison of target genes in other tissues may prove to be helpful here. GATA-3 not only is required at critical binary lineage decision points such as the T *vs* B, CD4 *vs* CD8, and Th1 *vs* Th2 branchpoints, in the immune system, but also was recently shown to control survival of the mammary luminal epithelial lineage in the breast (135). GATA-3 is also required for normal development of skin, adipocytes, and nervous system in mouse (136-138). It is tempting to speculate that there are common themes of control across tissues, and that GATA-3 acts by orchestrating the carefully timed expression of lineage specific targets as well as proliferative or survival programs. Identifying the GATA-3 target genes that are essential for the generation of CD4SP thymocytes in particular is expected to uncover novel approaches to manipulating the function of the thymus. Such approaches will lead to new treatments to boost the number of $CD4^+$ T cells, a condition that is desirable in numerous clinical situations, such as congenital immunodeficiency, chemo-therapy induced immunosuppression, bone marrow trans-plantation and HIV infection.

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