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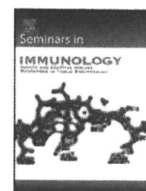
DATABASES
UniProtKB: http://www.uniprot.org
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Seminars in Immunology

Review

The TCR-mediated signaling pathways that control the direction of helper T cell differentiation

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ABSTRACT

In the periphery, upon antigen recognition by $\alpha\beta$TCR, naïve CD4 T cells undergo functional differentiation and acquire the ability to produce a specific set of cytokines. At least four Th cell subsets, i.e., Th1, Th2, Th17 and iTreg cells have so far been identified and the differentiation of each subset is driven by distinct cytokine sets. Antigen recognition by TCR and the activation of the TCR-mediated signaling pathways that follows, however, are most critical for initiating Th cell differentiation. This review focuses on the TCR signal strength and the TCR-mediated signaling pathways that control the differentiation into these four Th cell subsets.

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1. Introduction

After antigen recognition by TCR, naïve CD4 T cells undergo clonal expansion and differentiate into functionally polarized helper T (Th) cell subsets. At least four distinct Th subsets, i.e., Th1, Th2, Th17 and iTreg cells have been identified [1–5]. The direction of functional Th cell differentiation depends on the cytokines present in the priming environment. Naïve CD4 T cells stimulated with antigens differentiate into Th1 cells in the presence of IL-12 and IFNγ [6,7], whereas IL-4 plays an important role for Th2 cell differentiation [8,9]. The activation of downstream signaling molecules of these cytokine receptors, such as STAT4/STAT1 [10–13] or STAT6 [14–16] are required for differentiation into either Th1 or Th2 cells, respectively. Recently, TGFβ was shown to co-operate with IL-6 to induce Th17 cell differentiation [17–19], while TGFβ plus IL-2 induced the generation of iTreg cells [20,21]. Several master transcription factors for differentiation into Th1, Th2, Th17, iTreg cells have been identified; T cell-specific T-box transcription factor (T-bet) [22], GATA3 [23,24], retinoid-related orphan receptor gamma t (RORγt) [25] and Foxp3 [26], respectively.

In addition to appropriate cytokines, antigen recognition by TCR and events triggered by this interaction is most critical for achieving the functional differentiation of all these Th subsets. More specifically, various TCR-mediated signal transduction pathways are activated in naïve CD4 T cells after antigen recognition [27–29]. This review focuses on: (i) the TCR signal strength and the outcome of Th1 and Th2 cell differentiation, and (ii) the role of the TCR-mediated signal transduction pathways or signaling molecules that control the direction of Th cell differentiation.

2. The effect of antigen dosage and signal strength on the differentiation into Th1 and Th2 cell subsets

In addition to cytokines such as IL-12, IFNγ and IL-4, the generation of Th1 and Th2 cells is also controlled by the antigen dosage and overall TCR-mediated signal strength induced in naïve CD4 T cells. More than a decade ago, Bottomly and co-workers [30,31] used moth cytochrome *c* peptide and antigen-specific $\alpha\beta$TCR Tg CD4 T cells, and demonstrated that low antigen doses induced early IL-4 in naïve CD4 T cells and thus preferentially induced Th2 cell generation. Paul and co-workers [32] also reported that the early IL-4 production from naïve CD4 T cells was induced by low antigen doses which appeared to be GATA3-dependent. Independently, O'Garra and co-workers [33] used DO11.10 $\alpha\beta$TCR Tg CD4 T cells and a broad range of antigenic peptide, and demonstrated that Th2 cell development was observed at very high doses and very low doses of antigenic peptides, whereas midrange peptide doses directed the development of Th1 cells. Burstein and Abbas [34] reported that high-dose aqueous protein antigens induced tolerance in Th1 cells, but not in Th2 cells, and thus it appears that high-dose antigens favored enhanced Th2 responses. We used highly purified naïve CD4 T cells and immobilized anti-TCR mAb, and reported a stronger stimulation with higher anti-TCR mAb concentrations in the presence of IL-4 *in vitro* induced an increased Th2 cell differentiation but with a decreased Th1 cell differentiation [35,36]. Similar results were observed even when DO11.10 $\alpha\beta$TCR Tg CD4 T cells and
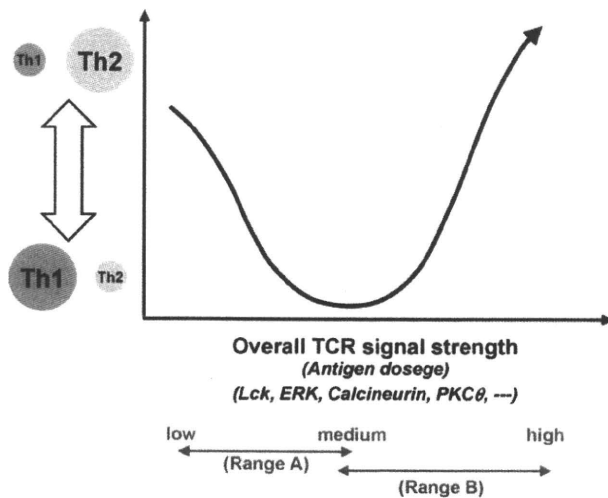
Fig. 1. A bi-phasic Th2 cell differentiation model. After antigen recognition by TCR, naïve CD4 T cells usually undergo differentiation into both Th1 and Th2 cells. The direction of the differentiation into Th1 or Th2 cells is controlled by the overall TCR signal strength. Some reports indicate that low-level antigenic stimulation induced Th2 rather than Th1 cell differentiation (Range A) [30,32,41,42]. However, other experimental results support the notion that low-level TCR stimulation induced Th1 cell differentiation and high-level stimulation induced increased a Th2 cell generation (Range B) [33–35,37,43,44]. These two sets of conclusions seem to be contradictory, but the range of TCR signal strength could be different between these two sets of experiments, although the experimental system used in each study is different, we cannot directly compare them with each other. In this bi-phasic Th2 cell differentiation model, naïve CD4 T cells differentiate into Th2 cells preferentially when naïve CD4 T cells were stimulated with a very low-level TCR signal strength (low dose antigen) and also a very high-level TCR signal strength (high-dose antigen). The levels of activation of major signal transduction pathways, including the ERK/MAPK, calcium/calcineurin, and PKC/NF-κB pathways, may reflect the TCR strength. The ranges of the experiments addressing each signal transduction pathway may be classified as belonging to the Range B, because the decreased signaling activity of these pathways resulted in impaired Th2 cell differentiation.

the specific antigenic peptide were used [35–37]. Several reviews on co-receptors have suggested that stronger activation signals favor Th2 cell differentiation [38–40]. Based on these experimental results, we would like to propose a bi-phasic Th2 cell differentiation model (Fig. 1). A weak stimulation of TCR, such as stimulation with a low dose antigenic peptide, may induce early IL-4 in naïve CD4 T cells, and thereby initiates Th2 cell differentiation in some experimental systems [30,32,41,42]. Under these conditions, although a preferential Th2 cell generation could be observed, the absolute number of effector Th2 cells generated may not be so large, because the extent of T cell expansion is considered to be limited. Relatively higher doses of antigens induced Th1 cells in these experimental settings, where the range of stimulation corresponds to Range A (low-medium) in Fig. 1 [30,32,41,42]. Regarding the signal strength reported in the other studies, they generally correspond to Range B (medium to high) [33–35,37,43,44], where mild infection, low antigen concentration or low dose anti-TCR mAb would favor the induction of Th1 cell differentiation, whereas high-dose stimulations preferentially induced Th2 cells. One characteristic example was observed in experiments using parasites, in which a low dose challenge induced Th1 responses [45,46].

A likely explanation as to why stimulation with high-dose antigen favors Th2 cell differentiation, particularly in the *in vivo* experimental systems is that high-dose antigen may be efficiently presented by APCs, resulting in repeated stimulation of the antigen-specific naïve CD4 T cells and also early developing Th2 cells. Therefore, in addition to early IL-4 production from naïve CD4 T cells, a relatively high amount of IL-4 produced by early developing Th2 cells after stimulation with the same antigen may thus

induce the developing Th2 cells themselves to accelerate both polarization and proliferation. This IL-4 may also induce other surrounding naïve CD4 T cells to undergo Th2 cell differentiation. These autocrine and paracrine IL-4 circuits may function efficiently if high-dose antigen is provided. In contrast, although IFNγ influences the magnitude of Th1 cell differentiation, IL-12 plays an important role in the initiation of Th1 cells [47]. Since IL-12 is not produced by either naïve CD4 or developing Th1 cells, no such enhanced circuit would occur during Th1 cell differentiation. Therefore, Th2 cells would dominate over Th1 cells at the high-doses of antigen. Interestingly, if naïve CD4 T cells are cultured in the presence of both IL-4 and IL-12, they differentiate preferentially into Th2 cells (T.N. and M.Y., unpublished observation) [48].

A high concentration of antigenic peptides is required for the production of Th2 cytokines in effector Th2 cells. The TCR and CD4 are efficiently recruited to lipid rafts in Th1 cells, but not in Th2 cells offering an explanation for the decreased ability of Th2 cells to respond to low-affinity peptide stimulation [43]. The functional significance of the expression levels of CD4 was demonstrated by the experiment with the restoration of high-level expression of CD4 in Th2 cells using a retrovirus gene transfer system [44]. Therefore, particularly in the case of *in vivo* experimental systems, Th2 responses appear to be induced more efficiently if a large amount of antigens is available.

3. Effect of the affinity of antigens and signal strength on the generation of Treg (regulatory T) cells

More recently, low-affinity antigenic peptides have been shown to induce a significant expansion of CD4+Foxp3+ Treg cells, whereas high-affinity antigenic peptides favored the expansion of Foxp3neg Th cells [49]. The inverse correlation of Foxp3 expression and Akt/mammalian target of rapamycin (mTOR) signaling has been reported. Similarly, the premature termination of TCR signaling and the inhibition of the PI3K/Akt/mTOR pathway conferred Foxp3 expression and Treg-like gene expression profiles [50]. Continued TCR signaling and constitutive activation of the PI3K/Akt/mTOR pathway antagonize the expression of Foxp3. In addition, the inhibition of calcineurin by FK506 resulted in the inhibition of the proliferation of conventional T cells, but FK506 less efficiently inhibited the proliferation of Treg cells [51]. Therefore, the activation levels of calcineurin appear to control the proliferation of Treg cells and their numbers.

4. Role of the TCR-mediated signal transduction pathways in Th cell differentiation

After antigen recognition by TCR, the TCR/CD3 complex initiates the activation of various signal transduction pathways. The most proximal biochemical event is the tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) of four components of the TCR/CD3 complex, i.e., CD3γ, CD3δ, CD3ε and TCR-ζ [27–29]. The src family tyrosine kinases including Lck play an important role in the tyrosine phosphorylation of ITAMs. Then, the Zap70 syk family kinase is recruited to the phosphorylated ITAMs through the SH2 domains of ZAP70. The recruited ZAP70 is then phosphorylated and activated by surrounding Lck tyrosine kinase, thus leading to the phosphorylation of LAT molecules. Following these proximal events, the activation of various distinct signaling pathways is initiated, including (i) the Ras/ERK MAPK cascade, (ii) the Ca/calcineurin/NF-AT pathway, and (iii) the PKC/NF-κB pathway (Fig. 2).

We summarize the experimental results that have addressed the role of each signaling pathway or signaling molecules in the Th cell differentiation and Th responses noted below. Interestingly,
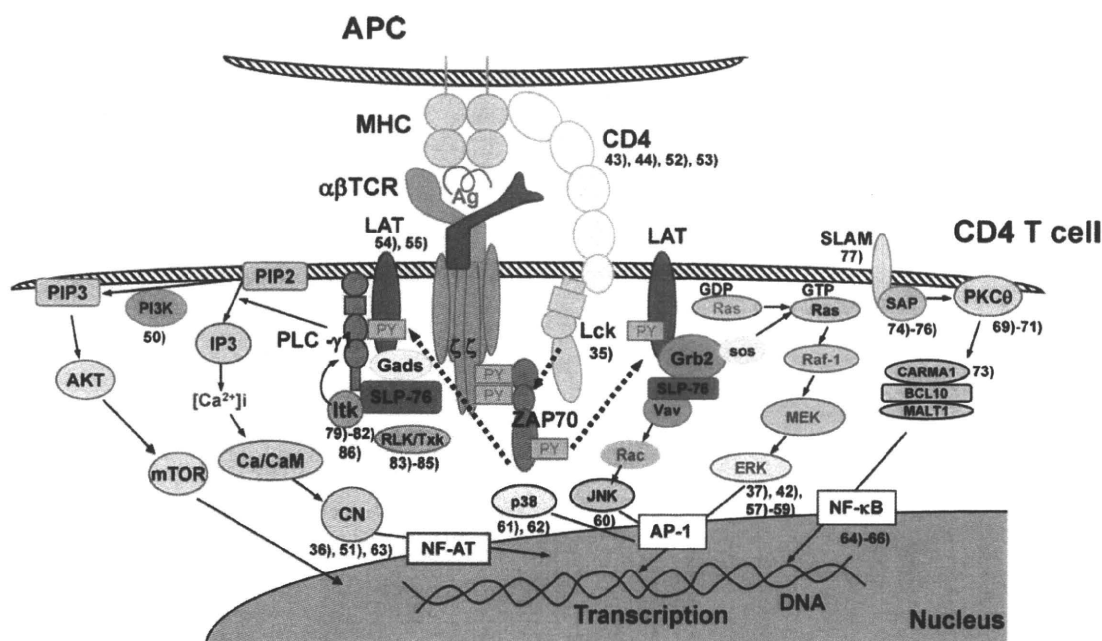
Fig. 2. Major signal transduction pathways downstream of TCR and their roles in Th cell differentiation. The references addressing each molecule in Th cell differentiation are indicated. See text in detail.

the loss-of-function of various signaling molecules tended to lead to the defects in Th2 cell differentiation while leaving Th1 cell differentiation intact although some exceptions have been reported. Therefore, the most of these loss-of-function experiments addressing the role of each signaling pathway may fit within Range B as shown in Fig. 1.

4.1. Lck

We reported that strong Lck kinase activity is required for the efficient generation of Th2 cells [35]. Dominant-negative Lck Tg mice showed impaired Th2 cell differentiation while leaving Th1 cell differentiation intact. It is known that as much as 50% of Lck protein is constitutively associated with the CD4 cytoplasmic domain. Reiner and co-workers [52] demonstrated a critical role of the cytoplasmic domain of CD4 for Th2 cell differentiation. The blockade of CD4/MHC class II interaction by peptides resulted in the inhibition of Th2 responses more efficiently than of Th1 responses [53]. In polarized Th2 cells, the expression levels of CD4 are lower in comparison to Th1 cells, and a decreased CD4 expression resulted in suboptimal TCR-induced phosphorylation and reduced Ca^{2+} signaling [44]. These results are also consistent with the notion that weak TCR stimulation may not efficiently activate Lck and thus favor Th1 cell differentiation, whereas strong stimulation may sufficiently activate Lck to induce Th2 cell differentiation.

4.2. LAT

Gene manipulated mice with mutations of critical tyrosine residues (Y136 and Y132) of LAT showed a severe defect in the development of T cells, and the few remaining T cells caused a spontaneous Th2-like allergic inflammation [54,55]. In MHC class II-deficient mice, a similar spontaneous allergic phenotype accompanied with a severe defect in the CD4 T cell development was observed. The remaining T cells in these animals did not show normal antigen-specific Th2 cell responses [56]. Therefore, the Th2 phenotype observed in these CD4 T cell deficient mice appears

to be unrelated to the strength of the activation of the specific signaling pathways, but more likely due to the secondary effects of the lymphopenic environment, such as abnormal homeostatic proliferation.

4.3. The MAPK cascade

In mammals, there are three distinct MAPK pathways, ERK, JNK and p38MAPK. We previously reported that the activation of the ERK/MAPK cascade was required for Th2 cell differentiation [37]. The inhibition of this cascade by an inhibitor or dominant-negative Ras resulted in impaired Th2 cell differentiation with an increased Th1 cell generation even in the presence of a sufficient amount of exogenous IL-4 (Fig. 3). These results indicate that the inhibition of the activation of the ERK/MAPK cascade induced a shift from Th2 to Th1 fate. This shift has been confirmed in vivo using a Th2-driven murine model of asthma [57]. In addition, we more recently found that ERK controls the level of GATA3 protein post-transcriptionally in Th2 cells through the inhibition of the ubiquitin–proteasome degradation pathway [58]. Interestingly, the activation of the ERK induced increased ribosomal components in Th2 cells to accommodate the production of Th2 cytokines [59]. In contrast, Jorritsma et al. [42] reported that the sustained activation of ERK induced by a high-affinity peptide in naïve CD4 T cells led to Th1 cell differentiation, which can be switched to Th2 differentiation when the activation of ERK was reduced. They suggest that the strong activation of ERK resulted in the down-regulation of IL-4 expression by altering the composition of AP1 complex in naïve CD4 T cells. Thus, the activation of ERK appears to be an important regulator of Th2 cell differentiation and functions at multiple levels. Th1 cell differentiation and Th1 cytokine production are dependent on other MAPK pathways, including JNK and p38, respectively [60,61]. p38 MAPK has also been shown to promote IL-5 expression through the phosphorylation of GATA3 protein [62]. Thus, p38 MAPK appears to be involved in both the Th1 and Th2 responses.
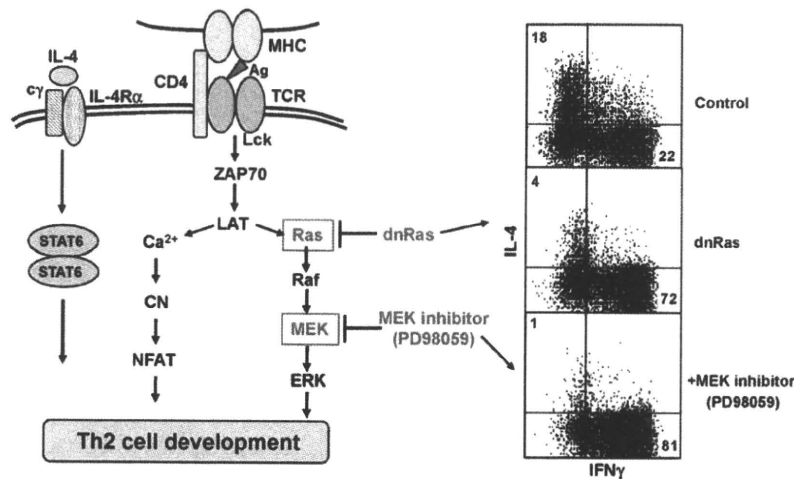
Fig. 3. Decreased activation of Ras and MEK in the ERK/MAKP cascade resulted in decreased Th2 cell differentiation and increased Th1 cell differentiation. Naïve CD4 T cells from normal C57BL/6 mice (control) and dominant-negative Ras Tg mice under the control of Lck distal promoter (dnRas) were stimulated with immobilized anti-TCR mAb in the presence of IL-4, and 5 days later, the cultured cells were harvested and subjected to the cytoplasmic staining with anti-IL-4 and anti-IFNγ. A specific inhibitor for MEK (PD98059) was added to the control C57BL/6 CD4 T cell culture (+MEK inhibitor). The percentages of the cells present in each quadrant are indicated [37].

4.4. The Ca/calcineurin/NF-AT pathway

Ca^{2+}/calcineurin signaling has been reported to play a crucial role in Th cell differentiation. We reported that the inhibition of the calcineurin activity by a specific inhibitor FK506 or by the over-expression of dominant-negative calcineurin Aα chains resulted in the impaired proliferative responses of both Th1 and Th2 cells, although, the inhibition of Th2 cell differentiation was more prominent than Th1 cells [36]. Therefore, when the activity of calcineurin was inhibited, the generation of Th2 cells was affected more profoundly in comparison to Th1 cells (Fig. 4). In this analysis, we identified that the TCR-induced activation of calcineurin modified the IL-4R signaling complex. Efficient calcineurin activation upregulated Jak3 expression and induced both the physical and functional associations of STAT5 with IL-4 receptors. The inhibition of IL-4-induced STAT5 activation resulted in a diminished IL-4-induced proliferation of Th2 cells. This study highlights the crosstalk that exists between the TCR-mediated signaling pathway and the cytokine mediated signaling pathway during Th cell differentiation. In addition, Scheinman and Avni [63] reported that the TCR-mediated activation of NF-AT1 is involved in the transcriptional regulation of the GATA3 expression in both naïve and differentiated Th2 cells. Thus, GATA3 is a common target of the IL-4-and TCR-mediated signaling pathways.

4.5. The PKC/NF-κB pathway

NF-κB appears to be a critical transcription factor that regulates Th2 cell differentiation. NF-κB p50 subunit-deficient mice were unable to induce OVA-induced airway inflammation [64]. The lack of inflammation was not due to defects in T cell priming, T cell proliferation, or the overexpression of important cell adhesion molecules, but rather it was due to the impaired induction of GATA3. We reported that the CD28-mediated activation of NF-κB was required for efficient Th2 cell differentiation [65] and the chromatin remodeling of the IL-5 gene locus [66].

PKCθ is a key component of the T cell immunological synapse whose activity is required for the activation of AP-1, NF-AT and NF-κB [67,68]. Th2 cell-mediated immune responses were markedly impaired in PKCθ-deficient mice, whereas the Th1 immune responses developed normally [69,70]. More recently, the role of PKCθ in Th17 cell-dependent immune responses was

reported [71]. CARMA1 (caspase recruitment domain-containing MAGUK protein 1) is a scaffolding protein, which is thought to link PKCθ to NF-κB [72]. This molecule is also required for the induction of the Th2 responses *in vivo*. CARMA1-dificient mice exhibit impaired Th2 cell-mediated allergic airway inflammation [73]. Signaling lymphocyte activation molecules (SLAM) associated protein (SAP), was shown to activate PKCθ and promote Th2 cell differentiation. SAP-deficient T cells showed impaired TCR-induced GATA3 activation and subsequent Th2 cell differentiation, and increased Th1 cell generation [74–76]. SLAM-deficient CD4 T cells exhibited a similar defect in Th2 cell differentiation [77]. SAP promotes the recruitment of PKCθ to the contact site, which is enhanced by the engagement of SLAM. The TCR-induced phosphorylation of Bcl10 and the NF-κB1/p50 nuclear translocation were both decreased in SAP-deficient CD4 T cells. Thus, SLAM, SAP, PKCθ, Bcl10 and CARMA1 are all required for the induction of Th2 cell differentiation through the activation NF-κB and resulting positive regulation of GATA3 expression.

4.6. TEC family tyrosine kinases

ITK is a member of the TEC family of non-receptor tyrosine kinase and plays a key role in the activation of PLCγ1 (Fig. 2). Therefore ITK-deficient T cells show a defect in the activation of the MAPK signaling pathway, the calcium influx and NF-AT [78]. ITK-deficient mice exhibited defects in the Th2 responses [79–81]. Using IL-4 reporter mice, ITK was found to be dispensable for early IL-4 expression during Th2 cell differentiation, however, it was critical for the IL-4 production in Th2 effector cells [82]. Another TEC family kinase, RLK/Txk, is preferentially expressed in Th1 cells in comparison to Th2 cells [83]. Although RLK mRNA is rapidly down-regulated after TCR stimulation, it is re-expressed in Th1 cells but not in Th2 cells [81]. In human T cells, the overexpression of RLK resulted in the increased production of IFNγ without affecting either the IL-2 or IL-4 production [84]. RLK has been reported to localize in the nucleus after receiving TCR-mediated stimulation, and it may control the transcription of the IFNγ gene [85]. It, therefore, remains to be elucidated whether the activation of RLK promotes Th1 cell differentiation, in addition to enhancing the production of IFNγ in Th1 cells. More recently, ITK was shown to be required for IL-17A production but not for IL-17F production [86]. ITK-dependent activation of NF-AT regulates IL-17A transcription in Th17 cells. The
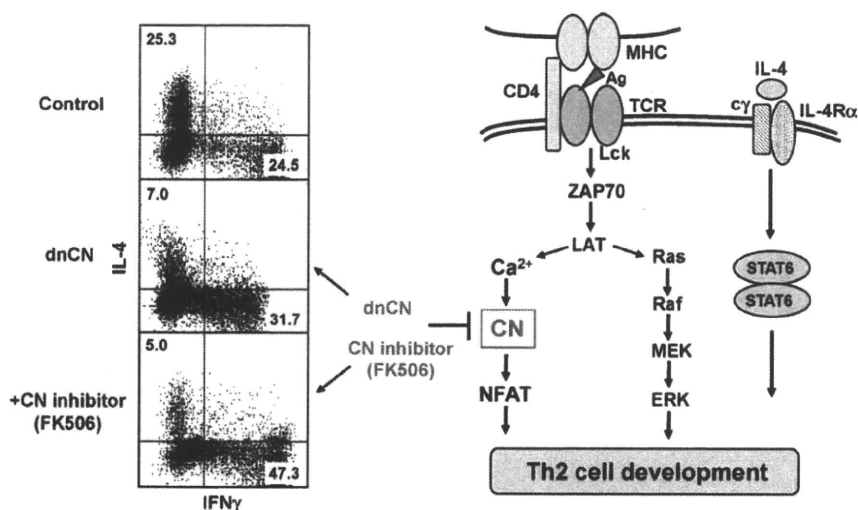
Fig. 4. Decreased activation of calcineurin resulted in decreased Th2 cell differentiation. Naïve CD4 T cells from normal C57BL/6 mice (control) and dominant-negative calcineurin Tg mice (dnCN) were stimulated with immobilized anti-TCR mAb in the presence of IL-4, and 5 days later, the cultured cells were harvested and then were subjected to the cytoplasmic staining with anti-IL-4 and anti-IFNγ. A specific inhibitor for calcineurin (FK506) was added to the control C57BL/6 CD4 T cell culture (+CN inhibitor). The percentages of the cells present in each quadrant are indicated [36].

expression of ROR-γt, which is the master regulator of Th17 cells, did not decrease in ITK-deficient Th17 cells. Taken together, the TEC family tyrosine kinases play important roles in various processes including the induction of the Th1, Th2 and Th17 responses (Fig. 4).

5. Concluding remarks

At least two distinct processes are required for the induction of each Th response, namely the TCR-mediated Th differentiation process and the TCR-induced Th cytokine expression process. The former represents the chromatin remodeling of the Th cytokine gene loci, while the latter involves the activation of specific transcription factors to induce the transcription of appropriate cytokines. Both of them are consequences of the TCR-mediated signaling events. Therefore, some signaling molecule downstream of TCR may only play a role in the former process, while others may be involved in only the latter process, but collectively they are required for the Th responses. In evaluating the experimental results, particularly those observed in the *in vivo* analysis, it will be necessary to clearly elucidate whether a given interesting molecule is involved in either process or both.

Essential for understanding CD4 T cell-mediated immunity will be a complete definition of the role of each signaling molecule in Th cell differentiation. This is becoming quite important from a clinical point of view because various inhibitors have been used clinically, and many other inhibitors can be expected to be developed in the near future. Since the direction of the generation of Th cell subsets is dependent on the overall signal strength, critical awareness is necessary for any inhibitors that modify the CD4 T cell-dependent immune responses in human patients. The effects of the inhibitors may depend on the dose of inhibitors, the duration of the intake, and also on the individual patients themselves.

Acknowledgements

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STAT6-mediated displacement of polycomb by trithorax complex establishes long-term maintenance of *GATA3* expression in T helper type 2 cells

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Polycomb group (PcG) and trithorax group (TrxG) complexes exert opposing effects on the maintenance of the transcriptional status of the developmentally regulated Hox genes. In this study, we show that activation of STAT6 induces displacement of the PcG complex by the TrxG complex at the upstream region of the gene encoding *GATA3*, a transcription factor essential for T helper type 2 (Th2) cell differentiation. Once Th2 cells differentiate, TrxG complex associated with the TrxG component Menin binds to the whole *GATA3* gene locus, and this binding is required for the long-term maintenance of expression of *GATA3* and Th2 cytokine. Thus, STAT6-mediated displacement of PcG by the TrxG complex establishes subsequent STAT6-independent maintenance of *GATA3* expression in Th2 cells via the recruitment of the Menin–TrxG complex.

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Abbreviations used: CBP, CREB-binding protein; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; LCR, locus control region; MLL, mixed-lineage leukemia; mRNA, messenger RNA; PcG, polycomb group; PRE, polycomb response element; RNAPII, RNA polymerase II; TRE, trithorax response element; TrxG, trithorax group; TSA, trichostatin A.

Immunity against different classes of microorganisms is directed by specialized effector CD4 Th subsets, of which the best characterized are Th1, Th2, and Th17 cells (Mosmann and Coffman, 1989; Reiner and Locksley, 1995; Korn et al., 2009). IL-12–induced activation of STAT4 is required for Th1 cell differentiation, whereas IL-4–induced STAT6 activation is crucial for Th2 cell differentiation. Master transcription factors that regulate Th1/Th2/Th17 cell differentiation have been identified. T cell–specific T-box transcription factor (T-bet) appears to be a key factor for Th1 cell differentiation (Szabo et al., 2002), *GATA3* for Th2 (Zheng and Flavell, 1997; Lee et al., 2000; Zhu et al., 2010), and ROR-γt (retinoid–related orphan receptor γt) and ROR-α for Th17 (Ivanov et al., 2006; Yang et al., 2008).

GATA3 is predominantly expressed in T lymphocytes and the embryonic brain (Yamamoto et al., 1990). In peripheral CD4 T cells, the activation of STAT6 induces high-level expression of *GATA3* messenger RNA (mRNA), although the precise mechanisms

underlying the STAT6-induced *GATA3* transcription remain unclear. Changes in histone modification such as H3-K9/14 acetylation and the H3-K4 methylation at the Th2 cytokine gene loci occur during Th2 cell differentiation (Löhning et al., 2002; Ansel et al., 2006; Nakayama and Yamashita, 2008), and this is mediated primarily by *GATA3* in peripheral CD4 and CD8 T cells. High-level expression of *GATA3* is required for producing large amounts of Th2 cytokines in established Th2 cells (Pai et al., 2004; Yamashita et al., 2004; Zhu et al., 2004).

The polycomb group (PcG) complex antagonizes the effect of the trithorax group (TrxG) complex (Ringrose and Paro, 2004). The TrxG complex establishes a chromatin structure permissive for transcription, in part, through the induction of methylation at histone

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H3-K4 (Milne et al., 2002; Nakamura et al., 2002), whereas the PcG complex maintains a repressive chromatin structure via the methylation of histone H3-K27 (Cao et al., 2002). The mammalian TrxG complexes contain RbBP5, Ash2L, and WDR5, which are also related to the components in the yeast Set1 complex, and a catalytic subunit that harbors the SET domain (Yokoyama et al., 2004). In contrast, PcG molecules form multimeric and heterogeneous complexes and maintain the early-determined gene expression patterns of key developmental regulators such as homeobox genes (Satijn and Otte, 1999; van Lohuizen, 1999). There are at least two types of PcG complexes, PRC1 (polycomb repressive complex 1) and PRC2 (Ringrose and Paro, 2004). Ring1B, Ring1A, Bmi1, Mel-18, M33, Pc2, Rae-28/Mph1, and Mph2 are members of a multimeric protein complex that show similarity to the PRC1 identified in *Drosophila melanogaster*. PRC2 is another PcG complex that contains Eed, Suz12, Ezh1, and Ezh2, and it possesses intrinsic methyltransferase activity for histone H3-K27 (Cao et al., 2002). In mature lymphocytes, PcG gene products appear to play several roles in the differentiation process and cell fate. Bmi1 controls the stability of GATA3 protein in developing Th2 cells (Hosokawa et al., 2006) and also memory CD4 T cell survival through the direct repression of the *Noxa* gene (Yamashita et al., 2008). Mixed-lineage leukemia (MLL) is a member of TrxG molecules and controls the maintenance of Th2 cytokine gene expression in memory Th2 cells (Yamashita et al., 2006). Menin was initially identified as a product of the MEN1 tumor suppressor gene and is known to be an essential component for DNA binding of the TrxG–MLL complex (Guru et al., 1998).

This study investigates the molecular mechanisms underlying the PcG complex– and TrxG complex–mediated regulation of *GATA3* transcription. In naive CD4 T cells, the PcG complex bound to the upstream region of the *GATA3* proximal promoter, whereas the accumulation of the Menin–TrxG complex was restricted to a part of the coding region. IL-4–mediated STAT6 activation induced the displacement of the PcG complex by the TrxG complex at the upstream region of the *GATA3* gene locus. After Th2 cell differentiation, the binding of Menin–TrxG complex was required for the maintenance of *GATA3* expression and Th2 cytokine production. This study revealed two distinct molecular processes that are critical in the regulation of *GATA3* transcription in Th2 cells: (1) IL-4/STAT6-mediated displacement of the PcG complex by the TrxG complex leading to the induction of *GATA3* transcription during Th2 cell differentiation and (2) STAT6-independent maintenance of *GATA3* expression and Th2 function via recruitment of the Menin–TrxG complex.

RESULTS

Dissociation of PcG complex and recruitment of TrxG complex to the *GATA3* gene locus during Th2 cell differentiation

The expression of *GATA3* mRNA is regulated in a tissue-specific manner. Naive CD4 T cells express a moderate level of *GATA3*, and in vitro differentiated Th2 cells and fully developed Th2 cells express higher levels of *GATA3*, whereas splenic B cells express little *GATA3* mRNA (Fig. 1 A, left). A similar tissue-specific profile in the protein expression of *GATA3* mRNA was observed (Fig. 1 A, right). Fully developed Th2 cells were established as described in Materials and methods. A schematic representation of the *GATA3* gene locus, with the location of specific primer pairs and probes for quantitative PCR used in this study, is shown in Fig. 1 B. First, the histone modification and the binding of the PcG and TrxG complexes at the *GATA3* gene locus were determined by chromatin immunoprecipitation (ChIP) assays. In B cells, Bmi1 bound to the upstream region of the proximal promoter and the region around exon 1 and exon 2 (primers #1 to #11; Fig. 1 C, dark blue line). The histone H3-K27 trimethylation (H3-K27Me3) signals were enriched at a broader but similar region (primers #1 to #15). Only weak signals for Menin and MLL binding and H3-K4Me3 were detected in B cells. However, in naive CD4 T cells, the Bmi1 signal was enriched only in the upstream region of the proximal promoter (primers #1 to #7; Fig. 1 C, turquoise line) but was low at the proximal promoter and the coding regions (primers #7 to #20). The signal pattern for H3-K27Me3 was almost exactly the same as that of Bmi1. In sharp contrast, Menin and MLL binding and H3-K4Me3 signals were enriched from the downstream region of the proximal promoter to exon 2. Fully developed Th2 cells showed strong enrichment of Menin and MLL signals from the beginning of the distal promoter to exon 5 and H3-K4Me3 signals to exon 3 (Fig. 1 C, red line). No significant Bmi1 accumulation and H3-K27Me3 were detected in fully developed Th2 cells. The real PCR product bands are shown in Fig. S1 (A and B). We detected comparable levels of histone H3 at all regions in these three types of cells (Fig. S1, C and D). The signals for H3-K4Me3 showed a similar pattern to that of Menin and MLL in all cells, whereas the H3-K27Me3 profile was similar to that of Bmi1. These results indicate that the increase in the expression of *GATA3* mRNA correlates with the dissociation of Bmi1 and the association of Menin and MLL to the *GATA3* gene locus, particularly in the upstream region of the proximal promoter (primers #3 to #6).

Next, the change of chromatin status and the PcG/TrxG binding at the *GATA3* gene locus during Th2 cell differentiation were assessed using a quantitative PCR assay system (Fig. 1 D). PcG proteins, Bmi1, EZH2, and Suz12 bound to the upstream region of *GATA3* proximal promoter in naive CD4 T cells (Fig. 1 D, left, probes (−15), (−5), and (−4)). The signal for H3-K27Me3 was also enriched in the upstream region of the proximal promoter. A progressive dissociation of Bmi1, EZH2, and Suz12 and decreased H3-K27Me3 were observed during Th2 cell differentiation. Essentially no PcG association was observed in fully developed Th2 cells (Fig. 1 D, left, probes (−15), (−5), and (−4)). In contrast, the binding of Menin, MLL, and RNA polymerase II (RNAPII) was restricted to the coding region in naive CD4 T cells (Fig. 1 D, right). H3-K4Me3 and H3-K9Ac signals were enriched around the proximal promoter (probe (0)). This accumulation of Menin,

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MLL, and RNAPII was observed in developing Th2 cells accompanied by increased H3-K4Me3 and H3-K9Ac signals across the *GATA3* locus (Fig. 1 D, right, probes (−5) to (+3)). The accumulation was more prominent in fully developed Th2 cells. The total H3 levels were similar in these cells (Fig. 1 D, bottom left). Thus, the displacement of the PcG complex by the TrxG complex occurred during Th2 cell differentiation. Interestingly, the displacement was most characteristic in the region between the *GATA3* proximal and distal promoter (Fig. 1 D, probes (−5) and (−4)). A previous study showed that the reduced expression of the PcG gene caused the dissociation of PcG molecules from the target genes in the epidermal

progenitors (Ezhkova et al., 2009). However, in this study, substantial levels of PcG and TrxG mRNA and protein were expressed in naive CD4 T cells, Th2 cells, and fully developed Th2 cells (Fig. S2), suggesting that the displacement is unlikely to be explained by the expression levels of these molecules.

The displacement of the PcG complex by the TrxG complex was dependent on STAT6

We assessed the role of the IL-4–STAT6 signaling pathway to help elucidate the molecular mechanisms underlying the displacement of the PcG complex by the TrxG complex at the *GATA3* gene locus. Although naive CD4 T cells from
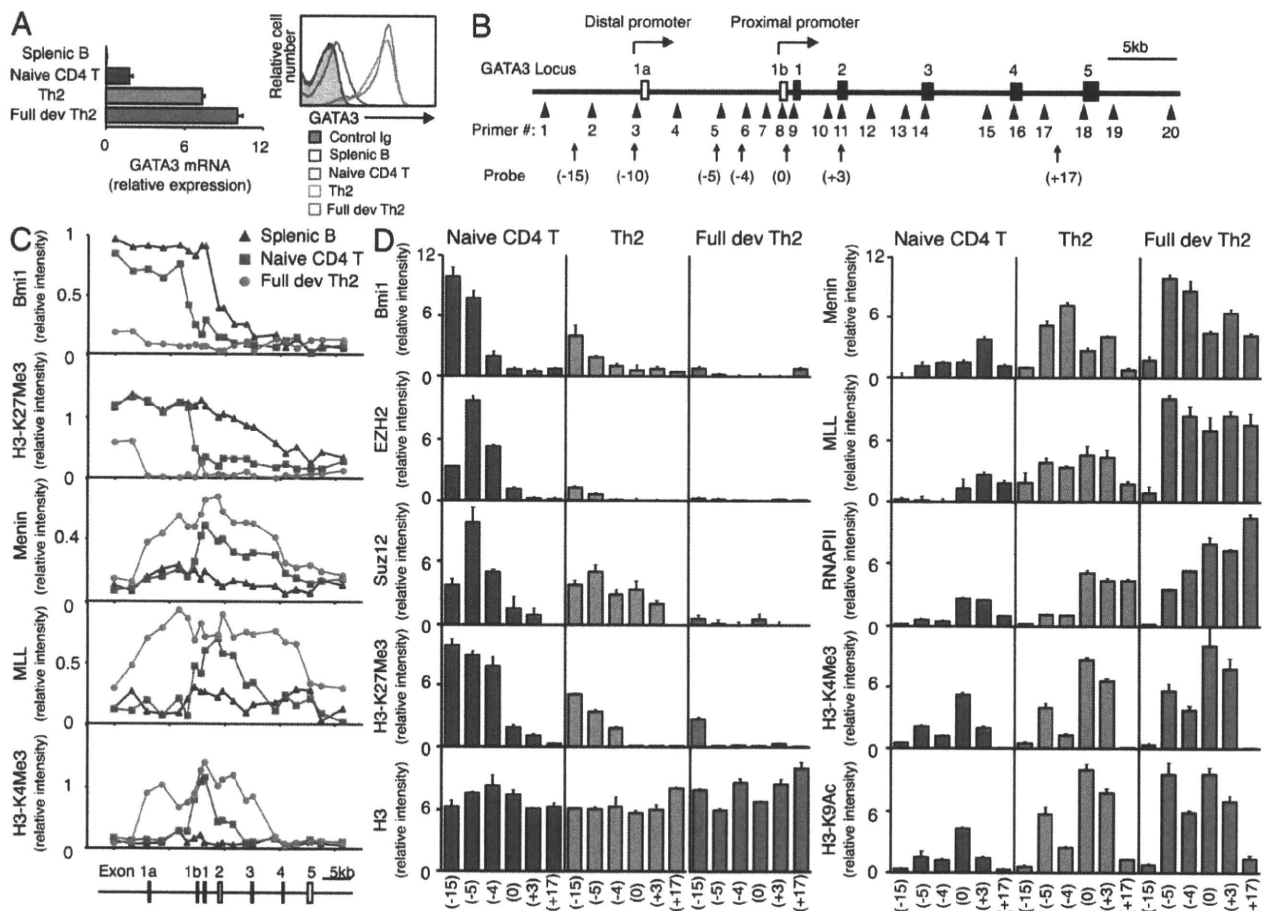

Figure 1. Changes in the histone modifications and PcG and TrxG binding to the *GATA3* locus during Th2 cell differentiation. (A) *GATA3* mRNA was measured by quantitative RT-PCR (left panel shows the relative intensity, compared with HPRT; the mean ± SD of three samples). GATA3 protein expression was determined by intracellular staining (right). Splenic B cells, naive CD4 T cells, effector Th2 cells (Th2), and fully developed Th2 cells (Full dev Th2) were used. Two independent experiments were performed with similar results. (B) Schematic representation of the mouse *GATA3* gene locus. Open rectangles indicate a noncoding exon, and closed rectangles indicate a coding exon. Closed triangles show the locations of PCR primer pairs used in the ChIP assay. Positions of TaqMan probes used for real-time quantitative PCR ChIP are indicated in relative kilobases to the *GATA3* translational start site. (C) A representative result of a ChIP assay using antibodies specific for the indicated proteins, with a series of primer pairs covering the *GATA3* locus. PCR product band intensities relative to input in each primer pair are shown. PCR product bands are shown in Fig. S1. Five independent experiments were performed with similar results. (D) Binding of the PcG complex molecule, Bmi1, EZH2, and Suz12, modifications of histone H3-K27, and total histone H3 levels at several regions around the *GATA3* gene locus were determined by ChIP assays with quantitative PCR analysis (left). Binding of the Menin–TrxG complex and RNAPII and the status of H3-K4Me3 and H3-K9Ac were also determined by quantitative ChIP assays (right). The relative intensity ([specific antibody ChIP− control Ig ChIP]/input DNA; highest signal intensity = 10; mean of three samples) is shown with SDs. Three independent experiments were performed with similar results.

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STAT6-deficient mice expressed equivalent levels of *GATA3* mRNA, they failed to up-regulate *GATA3* mRNA during in vitro differentiation into Th2 cells (Fig. 2, A and B). The reduced expression of GATA3 protein in STAT6-deficient Th2 cells was confirmed by the intracellular staining of GATA3 (Fig. 2 C). WT and STAT6-deficient CD4 T cells were cultured under Th2 conditions for 5 d, and then the cells were subjected to a ChIP assay with quantitative PCR. In the case of WT and STAT6-deficient naive CD4 T cells, equivalent binding of Bmi1, Menin, and MLL and equivalent histone modifications at the upstream region and coding region of the *GATA3* gene (probes (−5) and (−4)) were observed (Fig. 2 D). In contrast, the binding of Bmi1 and the level of H3-K27Me3 at the upstream region of the proximal promoter (probes (−5) and (−4)) were reduced in WT but not in STAT6–deficient effector Th2 cells (Fig. 2 D, left). In addition, the recruitment of Menin and MLL and the induction of H3-K4Me3 and

H3-K9Ac were significantly impaired in STAT6-deficient Th2 cells (Fig. 2 D, right). Although less characteristic, a similar pattern was observed at the coding region (Fig. 2 D, probe (+3)). The total H3 levels were not affected in STAT6-deficient naive and Th2 cells. The displacement of the PcG complex by the TrxG complex and corresponding histone modifications observed in Th2 cells were not observed in Th1 cells (Fig. 2 E). These results indicate that the activation of the STAT6 is crucial for the displacement of the PcG complex by the TrxG complex and that this is accompanied by the alteration in histone modification of the *GATA3* gene locus during Th2 cell differentiation.

Identification of the STAT6–binding sites within the *GATA3* gene locus

A sequence analysis identified 11 putative STAT6 consensus sites within the *GATA3* locus (from 20 kb upstream to 20 kb
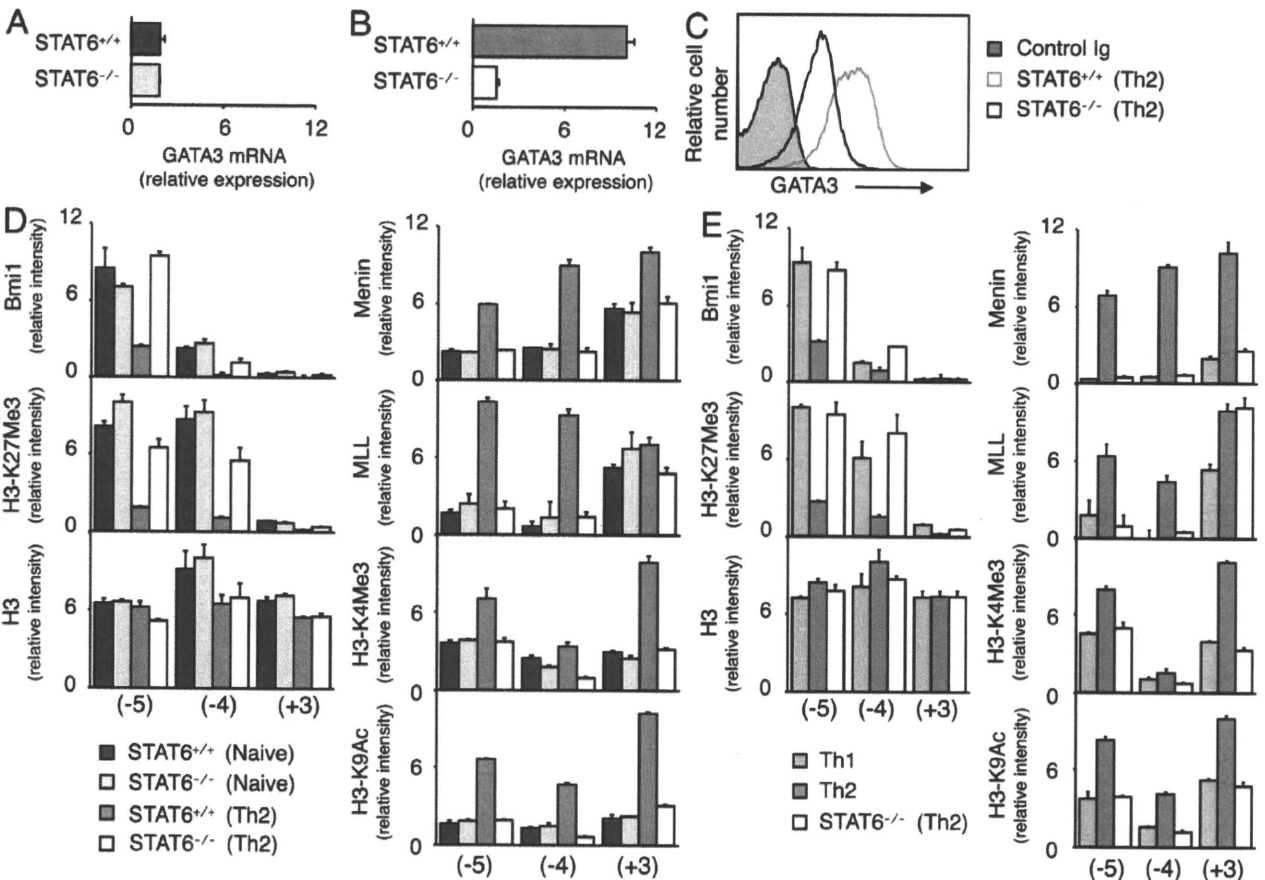

Figure 2. The displacement of the PcG by the TrxG complex at the *GATA3* gene locus was induced in a STAT6–dependent manner. (A and B) *GATA3* expression in STAT6-deficient naive CD4 T cells and Th2 cells was determined by a quantitative PCR. The relative intensity compared with HPRT (mean of three samples with SD) is shown. (C) The levels of GATA3 protein expression were determined by intracellular staining. (D) PcG and TrxG binding to the *GATA3* locus and histone modifications in STAT6-deficient naive CD4 T cells and Th2 cells were determined by a ChIP assay with a quantitative PCR analysis as described in Fig. 1 D. Positions of TaqMan probes described in Fig. 1 B are indicated in the parenthesis. The total H3 levels were included as a control. (E) PcG and TrxG binding to the *GATA3* gene locus and histone modifications in Th1, Th2, and STAT6-deficient Th2 cells were determined by a ChIP assay. Positions of TaqMan probes described in Fig. 1 B are indicated. (D and E) The relative intensity (mean of three samples) is shown with SDs. (A–E) Three independent experiments were performed with similar results.

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downstream relative to the *GATA3* translational start site), and on this basis corresponding primer pairs (S1–S11) were prepared (Fig. 3 A). Splenic CD4 T cells were stimulated with IL-4 for 1 or 24 h, after which these stimulated cells were subjected to a ChIP assay with an anti-STAT6 antibody.

Apparent binding was detected by the S4 and S7 primer pairs (Fig. 3 B). STAT6 binding was observed 1 h after stimulation and maintained for at least 24 h. A ChIP assay and quantitative PCR using CD4 T cells cultured under Th2 conditions for 1 h provided quantitative confirmation of the
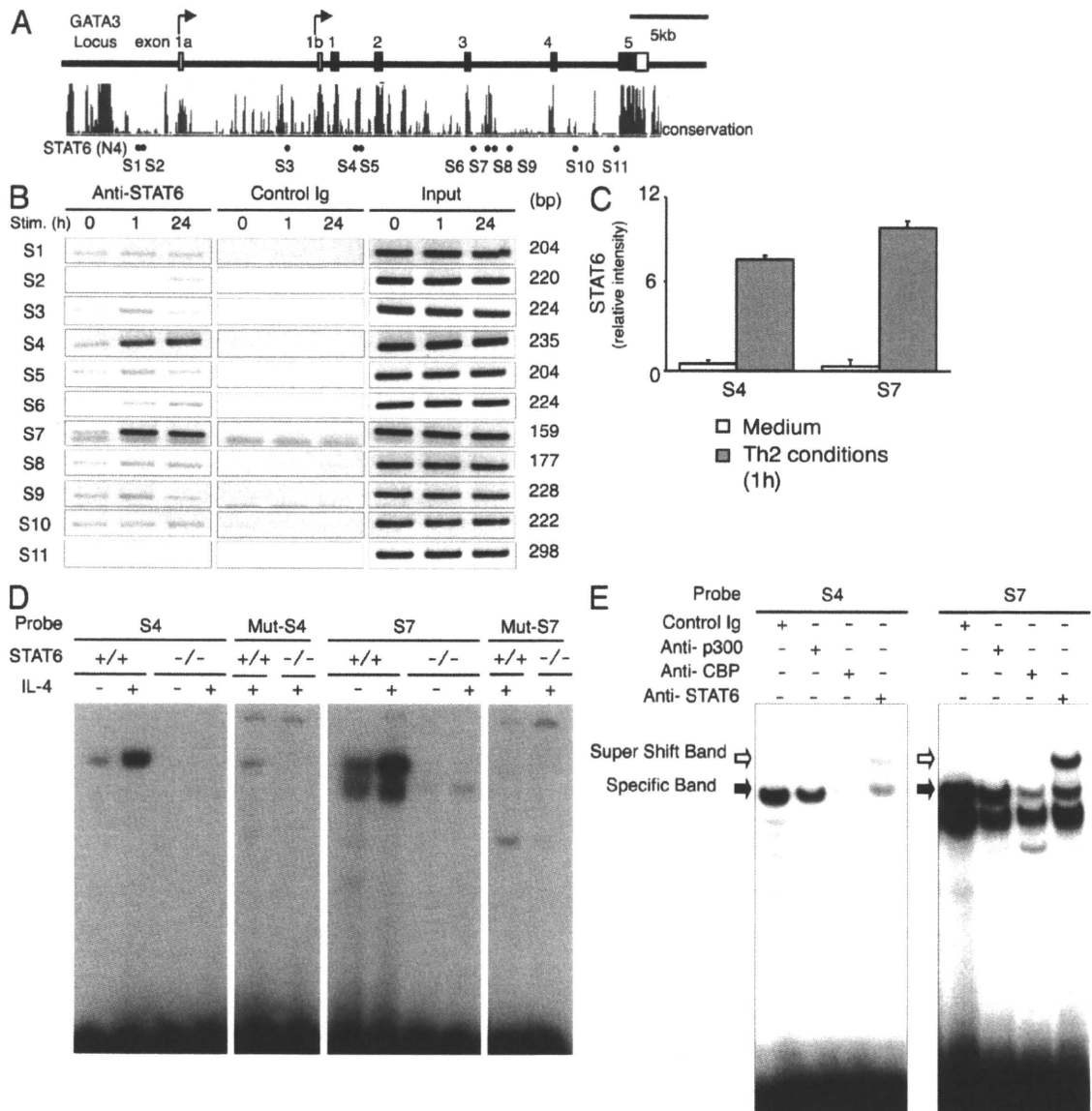

Figure 3. Identification of the binding site of STAT6 at the *GATA3* gene locus. (A) Schematic illustration of the GATA3 gene locus. 11 putative STAT-binding sites designated as S1–S11 (black dots) are indicated. The blue panel shows the conservation track obtained from the University of California, Santa Cruz Genome Browser. (B) CD4 T cells were stimulated with 100 U/ml IL-4 and immobilized anti-TCR mAb for the indicated periods. The stimulated cells were cross-linked with paraformaldehyde and then sonicated. The lysates were subjected to a ChIP assay with anti-STAT6 mAb or control Ig. The results are representative of three independent experiments. (C) CD4 T cells stimulated in medium or under Th2 cell culture conditions for 1 h were subjected to a ChIP assay. The levels of STAT6 binding at the S4 and S7 sites were determined by a quantitative PCR analysis. Three independent experiments were performed with similar results. The relative intensity (mean of three samples) is shown with SDs. (D) EMSA was performed using radiolabeled double-strand probes containing S4, Mut-S4, S7, or Mut-S7 and nuclear extracts from IL-4–stimulated WT or STAT6-deficient Th2 cells. Results are representative of two independent experiments. (E) EMSA with nuclear extracts from WT Th2 cells and radiolabeled S4 or S7 probes. Antibodies against p300, CBP, STAT6, or control mouse Ig were added to the reaction to supershift the STAT6–DNA complex. The results are representative of two independent experiments.

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binding of STAT6 to the *GATA3* S4 and S7 regions (Fig. 3 C). In splenic B cells, phosphorylation and nuclear translocation of STAT6 were induced by IL-4, but STAT6 binding at the *GATA3* gene locus was not detected (unpublished data). The putative STAT6-binding sites are located at positions 1.5 kb (S4) and 10.2 kb (S7) relative to the translational start site (Fig. 3 A). To determine whether these motifs are required for the binding of STAT6, an electrophoretic mobility shift assay (EMSA) was used with nuclear extracts prepared from IL-4–stimulated T cells and incubated with radiolabeled double-stranded DNA oligonucleotide probes containing the putative STAT6-binding sites or mutated STAT6-binding sequence (probes S4, Mut-S4, S7, and Mut-S7). IL-4 treatment resulted in the formation of a nucleoprotein complex at these sites in a STAT6-dependent manner for probes S4 and S7 (Fig. 3 D) but no specific bands for the mutated oligonucleotides (Fig. 3 D, probes Mut-S4 and Mut-S7). Next, a supershift EMSA was performed using WT CD4 T cells stimulated in the presence of IL-4 (Fig. 3 E). Whereas no band shift was observed with control antibodies (Fig. 3, compare D [second through eighth lanes] with E [first through fifth lanes]), bands were supershifted by preincubation with

antibodies against STAT6 (Fig. 3 E, fourth through eighth lanes), indicating that these complexes contain STAT6. Antibodies specific for HAT complex molecules, anti-p300, or anti–CREB-binding protein (CBP) mAb were also included in the supershift analysis. The intensity of the STAT6 complex band was reduced by the addition of anti-p300 mAb (second and sixth lanes) and anti-CBP mAb (Fig. 3 E, third and seventh lanes), indicating that p300 and CBP are components in the STAT6 complex. Thus, STAT6 appears to associate with the p300/CBP-containing HAT complex after IL-4 stimulation.

Trichostatin A (TSA) treatment was sufficient for the dissociation of the PcG complex from the *GATA3* locus

To further understand the role for histone modification in this process, the effect of histone deacetylase inhibitor TSA in the displacement of PcG complex by TrxG complex was examined. WT and STAT6-deficient CD4 T cells were cultured under Th2 conditions in the absence or presence of TSA for 5 d, and then the cells were subjected to a ChIP assay. The dissociation of the PcG complex (Bmi1 and EZH2) at the upstream region of the proximal promoter (probes (−5)
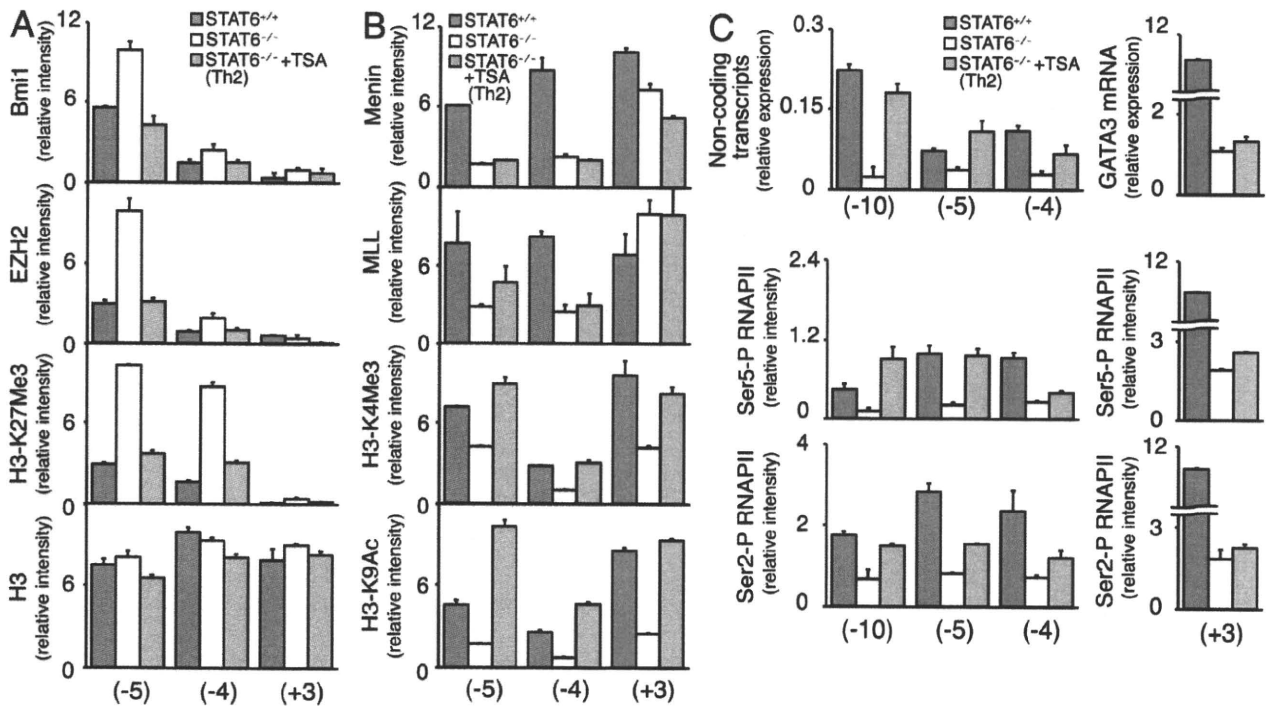

Figure 4. TSA treatment induced dissociation of PcG complex at the upstream region of the *GATA3* gene locus. Freshly prepared WT or STAT6-deficient CD4 T cells were stimulated under Th2 culture conditions, and where indicated, 10 ng/ml TSA was added on day 2. 3 d later, the cells were subjected to ChIP assays using the indicated antibodies. (A) Effects of TSA treatment on the dissociation of Bmi1 and EZH2 and the status of H3-K27Me3. The total H3 levels were included as a control. (B) Effects of TSA treatment on the recruitment of TrxG complexes (MLL and Menin) and the status of H3-K4Me3 and H3-K9Ac. (A and B) The relative intensity (mean of three samples) is shown with SDs. (C) The levels of noncoding transcripts at the *GATA3* gene locus and mature *GATA3* mRNA and HPRT in cells stimulated as in A and B were determined by quantitative RT-PCR. The relative intensity compared with HPRT (mean of three samples with SDs) is shown. Effect of TSA treatment on the recruitment of Ser5-P and Ser2-P RNAPII was determined by a ChIP assay with quantitative PCR (bottom). (A–C) Positions of TaqMan probes described in Fig. 1 B are indicated in the parenthesis. Three independent experiments were performed with similar results.
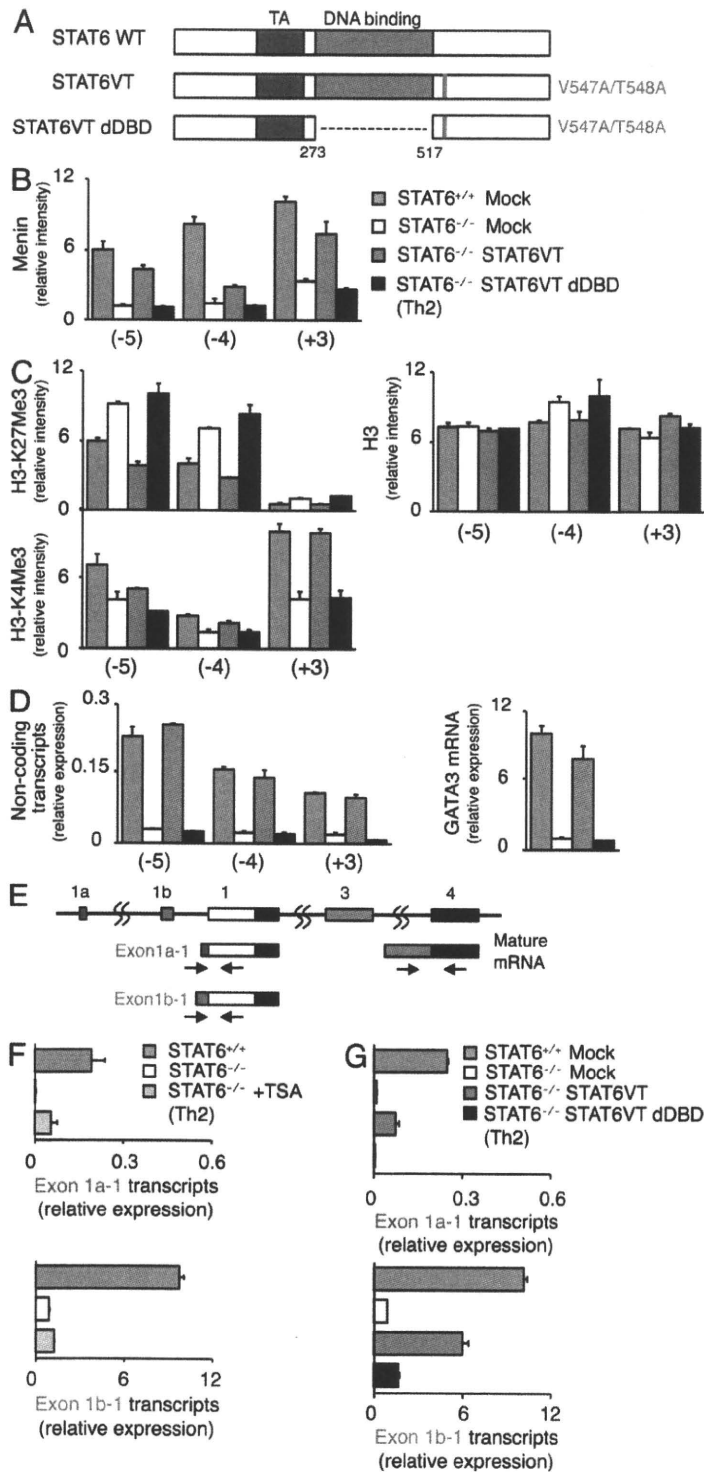
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Figure 5. STAT6 activation was necessary and sufficient for recruitment of Menin and up-regulation of mature *GATA3* mRNA during Th2 cell differentiation. (A) Schematic representation of STAT6 WT, full-length STAT6VT, and DNA-binding mutant (dDBD) with location of the transactivation (TA) and DNA-binding domain indicated. STAT6VT and STAT6VT dDBD contain V547A and T548A mutations. (B–D) Freshly isolated WT or STAT6-deficient CD4 T cells were stimulated under Th2 conditions for 2 d, and then the cells were mock infected or infected with a retrovirus vector containing a STAT6VT cDNA (pMXs-STAT6VT-IRES-hNGFR) or a STAT6VT dDBD cDNA (pMXs-STAT6VT-dDBD-IRES-hNGFR). hNGFR-positive infected cells were enriched by magnetic cell sorting. (B) The levels of Menin binding to the *GATA3* gene locus were assessed by a ChIP assay. (C) Effect of STAT6VT introduction on the histone modifications was determined by a ChIP assay with quantitative PCR. (B and C) The relative intensity (mean of three samples) is shown with SDs. (D) Noncoding transcripts and mature *GATA3* mRNA were measured by quantitative PCR. (E) Schematic illustration of the *GATA3* exon 1a, exon 1b, and exon 1 and the primer sites for the amplification of the 1a (exon 1a-1)- and 1b-containing transcript (exon 1b-1). Arrows represent the position of primer pairs for PCR detection. Squiggly lines represent the break in a long section of the gene. (F and G) The levels of *GATA3* exon 1a–containing transcript and exon 1b–containing transcript were measured by exon-specific quantitative PCR analysis. (F) TSA treatment was performed as described in Fig 4. (D, F, and G) The relative expression (mean of three samples) is shown with SDs. (B–D, F, and G) Two independent experiments were performed with similar results.

In contrast to the dissociation of the PcG complex, no obvious recruitment of Menin or MLL was detected in the TSA-treated STAT6-deficient Th2 cells (Fig. 4 B, top). Interestingly, TSA treatment induced H3-K4Me3 in addition to the H3-K9Ac at the *GATA3* locus (Fig. 4 B, bottom). Next, the effect of TSA on the *GATA3* transcription was examined. The transcripts between the distal and the proximal promoter (probes (−10) to (−4)) were not induced in STAT6-deficient Th2 cells, and these transcripts were induced by the treatment with TSA (Fig. 4 C, top left). However, the TSA treatment did not induce mature *GATA3* transcript (Fig. 4 C, top right).

Recent studies indicate that the phosphorylation status at Ser2 and Ser5 is associated with the transcriptional active and poised status of the RNAPII complex (for review see Brookes and Pombo, 2009). Consequently, we performed ChIP assays to assess the binding of Ser2- and Ser5-phosphorylated RNAPII at the *GATA3* gene locus. No obvious difference in the binding pattern was observed in both WT and STAT6-deficient Th2 cells (Fig. S3). The TSA treatment resulted in the accumulation of RNAPII (both Ser2- and Ser5-phosphorylated RNAPII) between the distal and the proximal promoter (Fig. 4 C, bottom left, probes (−10) to (−4)) but not at the coding region of the proximal promoter (Fig. 4 C, bottom right, probe (+3)). No obvious

and (−4)) occurred normally in STAT6-deficient Th2 cells in the presence of TSA (Fig. 4 A, top). H3-K27Me3 at the *GATA3* locus was also reduced in the presence of TSA. The total H3 levels were not influenced by TSA treatment.

decrease but rather a slightly increased expression of Bmi1, EZH2, Menin, and MLL in the STAT6-deficient Th2 cells was seen with TSA treatment, suggesting that neither PcG complex dissociation nor TrxG complex recruitment in the TSA-treated Th2 cells is controlled by the expression levels of these molecules (Fig. S4). Thus, histone hyperacetylation itself is responsible for the dissociation of the PcG complex accompanied with down-regulation of H3-K27Me3 and noncoding transcripts at the upstream region of the proximal promoter of the GATA3 gene; however, this alone was not sufficient for the recruitment of the TrxG complex.

The activation of STAT6 induced TrxG recruitment and up-regulation of mature GATA3 transcript during Th2 cell differentiation

Next, we assessed whether STAT6 activation itself induced the recruitment of Menin and mature transcripts. STAT6VT, an autoactivated STAT6 molecule (Daniel et al., 2000), and a STAT6VT DNA-binding mutant (STAT6VT dDBD) molecule (Fig. 5 A) were introduced into STAT6-deficient CD4 T cells cultured under Th2 conditions. The STAT6VT- and STAT6VT dDBD–introduced cells were harvested 3 d after infection and then were subjected to a ChIP assay. STAT6VT was found to induce the recruitment of Menin between the distal and the proximal promoter and also the coding region (Fig. 5 B, probes (−5), (−4), and (+3)). STAT6VT induced the reduction of H3-K27Me3 and the up-regulation of H3-K4Me3 (Fig. 5 C). However, STAT6VT dDBD did not induce the recruitment of Menin, the reduction of H3-K27Me3, or the up-regulation of H3-K4Me3 (Fig. 5, B and C). The total H3 levels were not influenced by STAT6VT introduction (Fig. 5 C). In addition, the transcripts between the distal and the proximal promoter (probes (−10) to (−4)) were restored by the ectopic expression of STAT6VT in STAT6-deficient developing Th2 cells (Fig. 5 D, left). Furthermore, STAT6VT rescued the mature GATA3 transcript (Fig. 5 D, right), whereas STAT6VT dDBD had no effect on GATA3 transcription (Fig. 5 D). These results indicate that the activation of STAT6 itself induced the recruitment of Menin and the mature GATA3 transcript during the development from naive CD4 T cells to Th2 cells.

The first alternative exons of the GATA3 transcripts (1a and 1b) are spliced to a common exon 1, which contains the translation start site (Fig. 5 E; Asnagli et al., 2002; Scheinman and Avni, 2009). First, the levels of transcripts of both exon 1a-1 and exon 1b-1 were assessed in WT and STAT6-deficient Th2 cells in the presence of TSA (Fig. 5 F). We confirmed that the exon 1b-1 transcript was much more abundant than that of exon 1a-1 (Scheinman and Avni, 2009). The levels of both transcripts were very low in STAT6-deficient Th2 cells. Interestingly, TSA treatment partially rescued the exon 1a-1 transcript but not the exon 1b-1 transcript (Fig. 5 F). The result was similar to that seen with noncoding and mature transcripts, respectively, as shown in Fig. 4 C. Then, we assessed the effect of the ectopic expression of STAT6VT on these two transcripts and observed that rescue was partial for the

exon 1a-1 transcript but was substantial in the case of the exon 1b-1 transcript (Fig. 5 G). Thus, the effect of STAT6VT on exon 1b-1 transcript was similar to that seen on GATA3 mature transcripts, as shown in Fig. 5 D. STAT6VT dDBD showed no obvious effect on exon 1a-1 and exon 1b-1 transcripts (Fig. 5 G). These results indicate that both exon 1a-1 and exon 1b-1 transcripts are dependent on STAT6 activation and that the former is associated with noncoding transcripts of the upstream region of the GATA3 gene and the latter is associated with the mature transcripts.

Menin was required for the maintenance of GATA3 expression in developed Th2 cells

Menin is an essential component for DNA binding of the TrxG–MLL complex (Guru et al., 1998). A CD4-Cre–driven conditional knockout mouse for Menin (Menin-deficient mice) was used to assess the role of Menin in the TrxG recruitment in the expression of the GATA3 gene. In Menin-deficient mice, equivalent numbers of CD4 and CD8 T cells developed in the thymus, but a 50% reduction in the number of splenic CD4 cells was observed in comparison with WT control (Fig. S5 A). The cell surface phenotype of splenic CD4 T cells was found to be within the normal range (Fig. S5 B). Proliferative responses induced with immobilized anti-TCR mAb were not impaired but somewhat slightly enhanced in Menin-deficient CD4 T cells (unpublished data). The lack of Menin mRNA in the conditional Menin-deficient Th1 and Th2 cells was confirmed (Fig. S5 C). Menin-deficient CD4 T cells cultured under Th2 conditions expressed an equivalent amount of GATA3 transcript and protein to WT CD4 T cells (after the first cycle of stimulation; Fig. 6, A and B) and differentiated normally into effector Th2 cells (Fig. 6 D). To further probe the role of Menin, the binding of MLL and the status of H3-K4Me3 at the GATA3 gene locus were assessed in the Menin-deficient Th2 cells. We could not detect the recruitment of MLL at the GATA3 gene locus in Menin-deficient Th2 cells (Fig. 6 C, left). Interestingly, the levels of H3-K4Me3 were decreased at the upstream region of the GATA3 proximal promoter (probes (−5) and (−4)), whereas this was not obvious at the coding region (Fig. 6 C, left, probe (+3)). The binding of the PcG proteins, Bmi1 and EZH2, and those of H3-K27Me3 after the first cycle stimulation were reduced equivalently between the WT and Menin-deficient Th2 cells (Fig. 6 C, right). The total H3 levels were not changed in Menin-deficient Th2 cells (Fig. S6 A). These results indicate that STAT6-dependent but Menin–TrxG-independent GATA3 transcription is induced during the first cycle of Th2 cell differentiation.

Interestingly, although the expression levels of GATA3 transcript in WT Th2 cells was maintained even when the cells were stimulated with anti-TCR mAb in the presence of anti–IL-4 mAb (Fig. S7, second to third cycle), the expression of GATA3 transcript in Menin-deficient Th2 cells was not maintained in the presence of anti–IL-4 mAb (after the third cycle of stimulation; Fig. 6 E). The high expression of GATA3 protein in Menin-deficient Th2 cells was also not
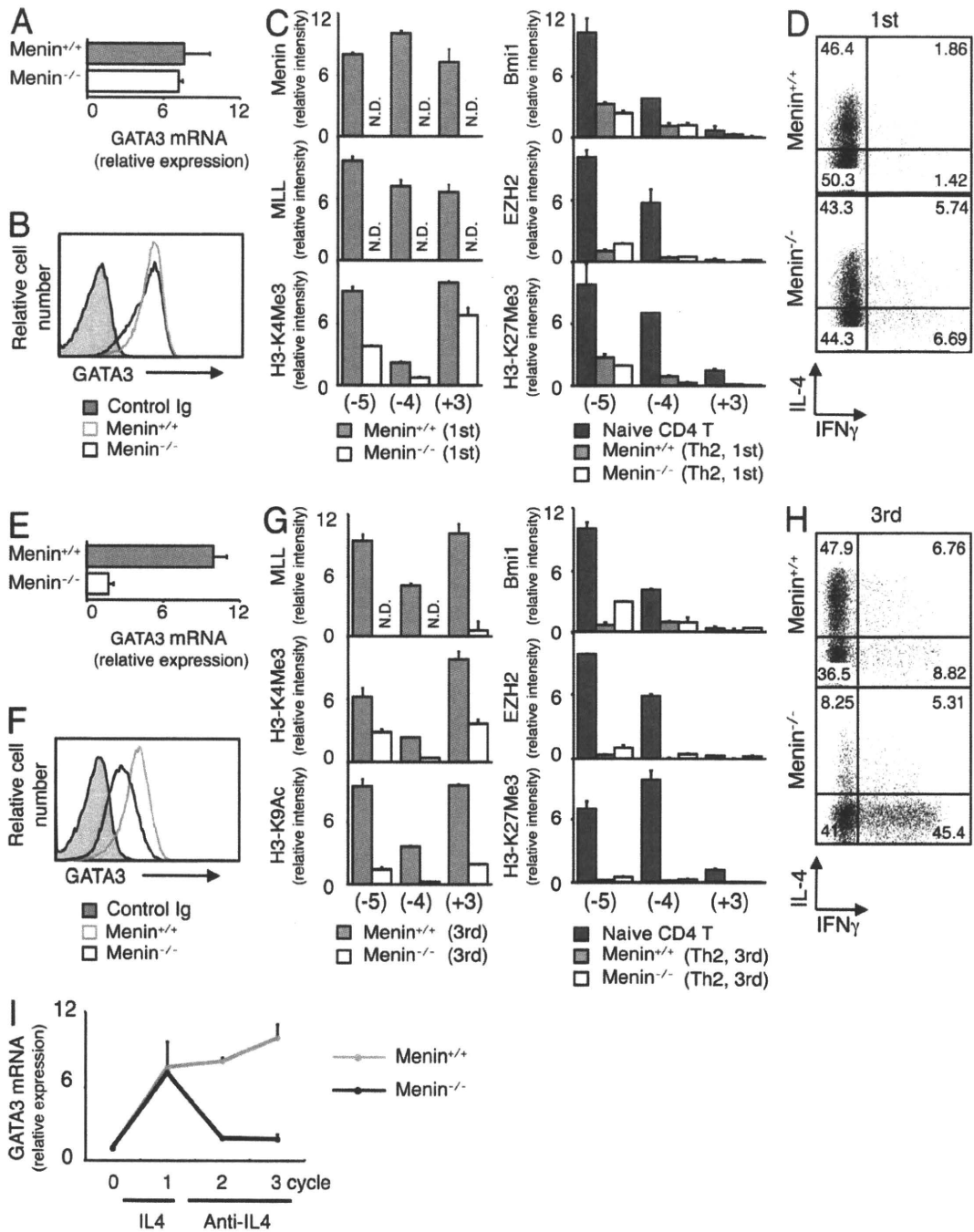
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Figure 6. Menin-deficient effector Th2 cells failed to maintain the expression of *GATA3* in the absence of IL-4. (A–D) Naive CD4 T cells from WT or Menin-deficient mice were cultured under Th2 conditions for 5 d (first cycle). These Th2 cells were further cultured for 2 d in the absence of cytokines and then restimulated with anti-TCR mAb in the presence of IL-2 and anti–IL-4 mAb for an additional 5 d (second cycle). (E–H) This cycle was repeated again (third cycle). (A and E) *GATA3* mRNA was determined by quantitative RT-PCR. The relative intensity compared with HPRT (mean of three samples with the SDs) is shown. (B and F) GATA3 protein expression was determined by intracellular staining. (C and G) Histone modifications and PcG and TrxG binding to the *GATA3* locus were determined by ChIP assays with quantitative PCR analysis as described in Fig. 1 D. The relative intensity (mean of three samples) is shown with SDs. (D and H) The cultured cells were restimulated with immobilized anti-TCR mAb and monensin for 6 h, and intracellular IFN-γ and IL-4 staining profiles were examined. Representative profiles are shown with the percentages of cells in each area. (I) A time course analysis of *GATA3* mRNA expression in WT and Menin-deficient naive CD4 T cells (0 cycle), in vitro differentiated Th2 cells (first cycle), and Th2 cells cultured in the presence of anti–IL-4 (second and third cycles). The relative expression (mean of three samples) is shown with SDs. (A–I) Three independent experiments were performed with similar results.
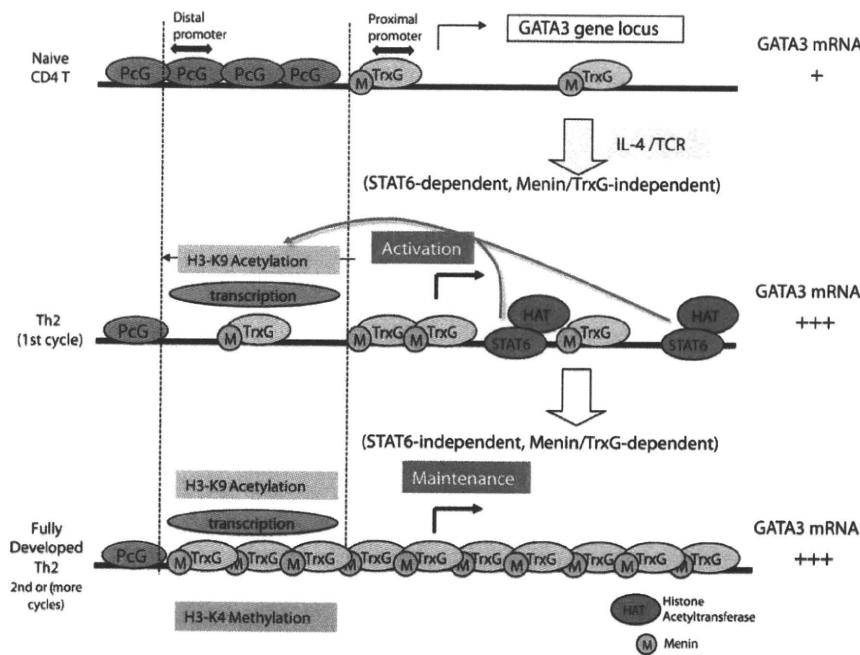
Figure 7. Schematic illustration of the transcriptional regulation of the *GATA3* gene in naive CD4 T cells, developing Th2 cells, and developed Th2 cells. In naive CD4 T cells, the PcG complex binds to the upstream region of the proximal promoter of the *GATA3* gene, and the expression of *GATA3* mRNA is moderate. After stimulation through TCR in the presence of IL-4 (Th2 culture conditions), STAT6 is activated, and STAT6 associated with the HAT complex binds to the *GATA3* gene locus. HAT-dependent histone acetylation spreads to the upstream region, resulting in the dissociation of PcG complex. A high-level *GATA3* mRNA expression is achieved in an IL-4/STAT6-dependent but Menin–TrxG-independent manner. The role of Menin–TrxG recruitment in *GATA3* transcription appears to be minimal at this stage. Once Th2 cells are developed, the Menin–TrxG complex binds to the whole *GATA3* gene region, including the upstream region of the proximal promoter. A broad range of H3-K9Ac and H3-K4Me3 is observed. The Menin–TrxG complex bound to the *GATA3* locus maintains the high expression levels of *GATA3* in the absence of IL-4. IL-4/STA6-independent but Menin–TrxG-dependent regulation of the transcription of *GATA3* is operating.

maintained (Fig. 6 F). The recruitment of MLL at the *GATA3* gene locus was not detected in Menin-deficient Th2 cells after third cycle cultivation (Fig. 6 G, left). H3-K4Me3 and H3-K9Ac levels were decreased at both the upstream and the coding regions after stimulation in the presence of anti–IL-4 mAb (Fig. 6 G, left). Interestingly, no significant increase in the binding of Bmi1 and EZH2 and in the level of H3-K27Me3 was observed even after stimulation in the presence of anti–IL-4 mAb (Fig. 6 G, right). The total H3 levels were not changed in Menin-deficient Th2 cells even after the third cycle cultivation (Fig. S6 B). These results indicate that the PcG complex did not reassociate with the *GATA3* gene locus in Th2 cells even in the absence of the binding of the Menin–TrxG complex. The ability to produce IL-4 was assessed after restimulation with anti-TCR mAb and was found to be diminished in the Menin-deficient Th2 cells after the two-cycle stimulation in the presence of anti–IL-4 mAb (Fig. 6 H). The antigenic stimulation with IL-4 for 1 wk did not restore the production of IL-4 in the Th2 cells that had received two-cycle stimulation in the presence of anti–IL-4 mAb (unpublished data), indicating that the ability to become IL-4–producing cells was lost in the absence of Menin. Menin-deficient naive CD4 T cells expressed an equivalent amount of *GATA3* transcript to WT CD4 T cells (Fig. 6 I, 0 cycle). The expression of *GATA3* transcript in Menin-deficient Th2 cells was not maintained in the presence of anti–IL-4 mAb (Fig. 6 I, second cycle). Collectively, once Th2 cells have successfully developed, they maintain the *GATA3* expression and Th2 function via the recruitment of the Menin–TrxG complex to the *GATA3* gene locus even in the absence of IL-4–mediated STAT6 activation.

DISCUSSION

This study dissects two distinct processes that are critical in the regulation of transcription of the *GATA3* gene in Th2 cells based on the binding of PcG and TrxG complexes (Fig. 7). In naive CD4 T cells, the PcG complex binds to the upstream region of the *GATA3* proximal promoter, whereas the accumulation of the Menin–TrxG complex is restricted to a part of the coding region. In developing Th2 cells, IL-4–mediated STAT6 activation induces the dissociation of the PcG complex, which is accompanied by the acetylation of H3-K9 and noncoding transcripts at the upstream region of the *GATA3* gene locus. Histone hyperacetylation is sufficient to cause the dissociation of the PcG complex and the induction of noncoding transcripts. High-level transcription of the *GATA3* gene is induced in these cells (Fig. 7, middle). The role of binding of the Menin–TrxG complex in *GATA3* transcription appears to be minimal at this stage. In fully developed Th2 cells, high-level expression of *GATA3* can be maintained in the absence of IL-4 (Fig. 7, bottom). IL-4/STAT6-independent but Menin–TrxG-dependent regulation of *GATA3* transcription is operating. A broad range binding of Menin was confirmed by a ChIP-seq assay (Fig. S8). The binding of Menin–TrxG complex to the *GATA3* gene is required for the long-term maintenance of *GATA3* expression and Th2 cytokine production. Because the supply of IL-4 is limiting in the body after the clearance of infectious organisms, this could be the mechanism by which memory Th2 cells can maintain Th2 function for a long time in vivo.

STAT6 plays several distinct roles in the regulation of *GATA3* transcription. Although STAT6 is well known to be

required for the expression of the *GATA3* gene, STAT6-binding sites that are critical for the induction of *GATA3* transcription have not been identified. We identified two STAT6-binding sites within the intron 1 and intron 3 regions of the *GATA3* gene locus at which STAT6 rapidly accumulated after IL-4 stimulation, resulting in the recruitment of the HAT complex (Fig. 3). Therefore, Th2-specific *GATA3* transcription appears to be mediated by the direct binding of STAT6. These binding sites are located several kilobases downstream from the transcriptional start site. However, this is not unusual because there are several examples in which transcription is induced by the binding of STAT family proteins at the distal locus control region (LCR; Jothi et al., 2008; Liao et al., 2008).

We also demonstrate that STAT6 binding recruited the HAT complex to the *GATA3* gene and induced the spreading of histone hyperacetylation with the resultant dissociation of the PcG complex at the upstream region of the *GATA3* gene. The two STAT6-binding sites are also located several kilobases downstream from the region where the displacement of PcG by TrxG occurs. Two likely mechanisms can be entertained. First, these STAT6-binding sites may act as cis-regulatory elements and exert enhancer activity for *GATA3* mRNA expression. At the β-globin gene locus, communication between the LCR and the active β-globin genes is generally thought to occur via a looping mechanism whereby the LCR and the β-globin genes come into direct physical contact through their interacting proteins and the intervening DNA sequences are looped out (Dean, 2006). The STAT6-binding sites may be close to the proximal or distal promoter through the high-ordered chromatin configuration, and thus, the STAT6 complex may control the dissociation of the PcG complex from the distant region of the STAT6-binding sites. Alternatively, the STAT6–HAT complex may spread from its binding sites toward the upstream region during Th2 cell differentiation and induce the dissociation of the PcG complex. This model is called the tracking or spreading model (Bondarenko et al., 2003; Dean, 2006). For example, erythroid activators NF-E2 and *GATA1* are important for the recruitment of the HAT complex to the LCR and for histone acetylation at the LCR and the distant β-globin gene (Johnson et al., 2001). The accumulation of STAT6 was observed at only two sites at the early phase of Th2 cell differentiation (Fig. 3, B and C), whereas a few days after the initial stimulation, the binding of STAT6 was broadly detected throughout the *GATA3* gene by a ChIP assay (unpublished data). Thus, we would favor the second model.

The induction of Th2-specific up-regulation of *GATA3* transcription has been suggested to result from the activation of the distal promoter of *GATA3* (Amsen et al., 2007; Fang et al., 2007). However, recent studies by other investigators have indicated that the Th2-specific increase of *GATA3* transcripts was dependent on the proximal promoter (Scheinman and Avni, 2009; Yu et al., 2009). As shown in Fig. 5 (F and G), we demonstrated that exon 1b-1 transcripts induced by the proximal promoter were much more abundant

in comparison with exon 1a-1 transcript, and thus, it would appear to us that the majority of the Th2-specific increase of mature *GATA3* transcripts is dependent on the proximal promoter. In contrast, the transcript induced by the distal promoter may reflect the existence of noncoding transcripts, which may play an important role in the dissociation of the PcG complex at the *GATA3* gene (Hekimoglu and Ringrose, 2009). In fact, the induction of noncoding transcripts and that of exon 1a-1 junction-containing transcripts occurs in similar fashion. The induction of noncoding transcripts at the upstream region of the proximal promoter including the region of the distal promoter was STAT6 dependent, and the noncoding transcripts were induced by the treatment with TSA (Fig. 4 C). The transcripts from the distal promoter are spliced and form the exon 1a-1 junction (Fig. 5 E, middle). These exon 1a-1 junction-containing transcripts were also STAT6 dependent and significantly up-regulated by the treatment with TSA (Fig. 5 F). The polycomb response element (PRE) and the trithorax response element (TRE) have been well established in *Drosophila* (Schwartz and Pirrotta, 2008). Noncoding transcripts have been detected around the PRE/TRE, and they play an important role for the binding of the PcG and TrxG complexes (Schmitt et al., 2005; Hekimoglu and Ringrose, 2009). Therefore, the identification of the PRE/TRE in the region between exon 1a and exon 1b of the *GATA3* gene locus would be an interesting issue.

In summary, this study provides the first evidence indicating that the activation of the transcription factor STAT6 induces the displacement of the PcG complex by the TrxG complex, which then leads to control of the expression of the *GATA3* gene in Th2 cells. STAT6-induced histone acetylation appeared to be responsible for the dissociation of the PcG complex. The recruitment of the Menin–TrxG complex was found to be crucial for the maintenance of high *GATA3* expression in developed Th2 cells. Therefore, STAT6-dependent displacement of the PcG by the TrxG complex would allow for a permissive chromatin status of the *GATA3* gene locus in the developed Th2 cells in which a STAT6-independent regulation of transcription operates.

MATERIALS AND METHODS

Mice. C57BL/6 and BALB/c mice were purchased from CLEA. STAT6-deficient mice (Takeda et al., 1996) were provided by S. Akira (Osaka University, Suita, Osaka, Japan). Menin-deficient mice (Crabtree et al., 2003) were purchased from The Jackson Laboratory. CD4-Cre transgenic mice were purchased from Taconic. All mice used in this study were maintained under specific pathogen-free conditions and ranged from 6 to 8 wk of age. All experimental protocols using mice were approved by the Chiba University animal committee. All animal care was performed in accordance with the guidelines of Chiba University.

Reagents. Recombinant mouse IL-12 was purchased from BD, and recombinant mouse IL-4 was purchased from TOYOBO. The OVA peptide (residues 323–339; ISQAVHAAHAEINEAGR) was synthesized by BEX Corporation.

Antibodies. The antibodies used for the ChIP assay were anti–acetyl-histone H3-K9 (Millipore), anti–trimethyl-histone H3-K4 (LP Bio), anti–trimethyl-histone H3-K27 (Millipore), anti–histone H3 (Abcam),