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Initiation and maintenance of Th2 cell identity

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T helper type 2 (Th2) cells produce IL-4, IL-5, and IL-13 and play an important role in humoral immunity and allergic reactions. During Th2 cell differentiation, naïve CD4 T cells acquire 'Th2 cell identity', that is, the capability to produce selectively a large amount of Th2 cytokines. Th2 cell identity is maintained in memory Th2 cells. Significant advances in understanding of the molecular requirement for these processes have been made. The expression of GATA3, a master transcription factor for Th2 cell differentiation, is uniquely regulated by several distinct mechanisms. Molecular analyses of memory Th2 cells revealed that cell survival and the maintenance of Th2 cell function are epigenetically regulated by various nuclear factors, including *Polycomb* and *Trithorax* molecules.

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Introduction

It has been established that effector helper T (Th) cells can be categorized into at least four subsets, Th1, Th2, Th17, and T regulatory (Tr) cells. Th1 cells produce large amounts of IFN γ and direct cell-mediated immunity against intracellular pathogens. Th2 cells produce IL-4, IL-5, and IL-13 and are involved in the humoral immunity and allergic reactions. Recently identified Th17 cells are crucial in certain autoimmune diseases. Tr cells are known to suppress various immune responses including autoimmune responses. Several transcription factors that control the differentiation and the function of these Th cell subsets have been identified. Among them, GATA3 appears to be a master transcription factor for Th2 cell differentiation [1,2], T-bet for Th1 [3], ROR γ t for Th17 [4], and FoxP3 for naturally occurring Tr [5].

Th1/Th2 responses are induced by antigen-specific effector and memory Th1/Th2 cells. Figure 1 illustrates the cellular processes that are required for the generation

of functional memory Th1/Th2 cells from effector Th1/Th2 cells. After antigen recognition by TCR, naïve CD4 T cells undergo clonal expansion and differentiate into effector Th1/Th2 cells within a couple of weeks. After antigen clearance, most of the effector Th1/Th2 cells are thought to undergo apoptotic cell death at the contraction phase [6,7]. Some of the effector cells, however, escape cell death, differentiate into memory type Th1/Th2 cells, and survive for a long time *in vivo*. Several processes, including cell survival/escape from cell death, proliferation/homeostatic proliferation, and the maintenance of Th1/Th2 cell function are all required for the successful generation of functional memory type Th1/Th2 cells. The idea of memory stem cells is also considered, but this has not been experimentally proven at this time.

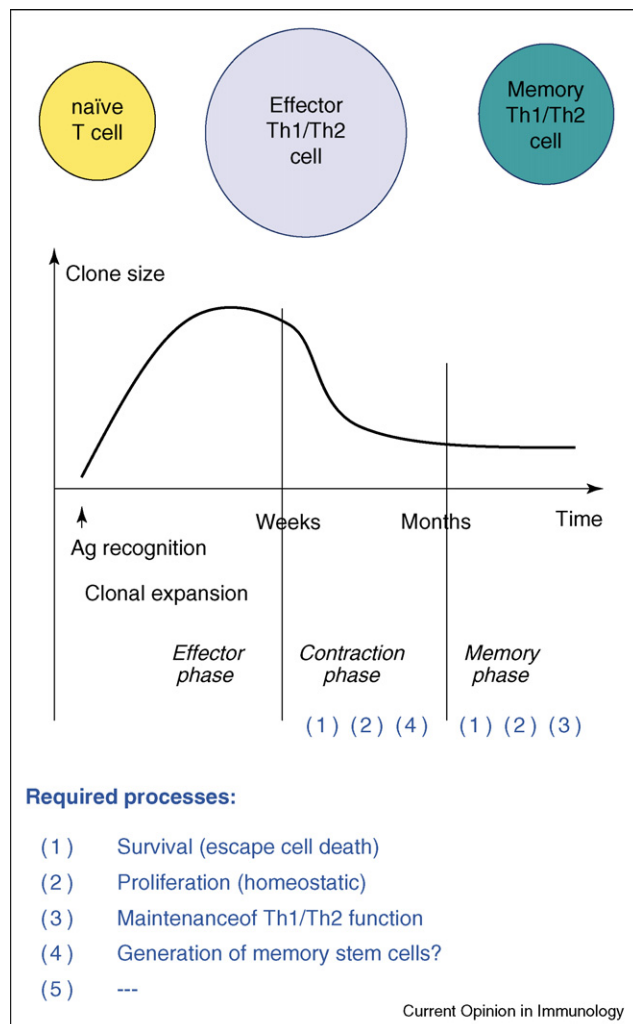
In this review, we present recent findings that provide new insights in our understanding of the molecular mechanisms governing the acquisition and the maintenance of Th2 cell identity.

Expression of GATA3 is required for the initiation and the maintenance of Th2 responses

The initiation of Th2 cell differentiation is dependent on TCR-mediated signaling, and the IL-4 receptor-mediated STAT6 activation and subsequent upregulation of GATA3 transcription. Enforced expression of GATA3 in developing Th1 cells was sufficient to cause the induction of IL-4 producing Th2 type cells in the absence of STAT6, indicating that GATA3 is a downstream target of the IL-4/STAT6 signaling pathway and a master transcription factor for Th2 cell differentiation [1,2]. During Th2 cell differentiation, GATA3 plays a central role in the induction of chromatin remodeling of the Th2 cytokine gene loci (Figure 2a) [8–11]. The differentiated effector Th2 cells now acquire 'Th2 cell identity', that is, the capability to produce selectively a large amount of Th2 cytokines upon TCR re-stimulation. GATA3 is also known to act as a transcription factor for the Th2 cytokine genes, particularly for IL-5 and IL-13. In order to investigate whether GATA3 plays a role in the maintenance of the Th2 cell identity, we established a gene-depletion system in primary *in vitro* differentiated Th2 cells using a site-specific recombination reaction [12]. The depletion of the GATA3 gene resulted in a substantial decrease in the production of Th2 cytokines (IL-4, IL-5, and IL-13) and a significant increase in IFN γ . Two other groups using GATA3 conditional knockout mice demonstrated an important role of GATA3 in Th2 responses *in vivo* [13,14]. *In vivo* deletion of the GATA3

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Figure 1



Cellular processes required for the generation of functional memory Th1/Th2 cells. Details are described in the text.

gene using OX40-Cre eliminated Th2 responses and allowed the development of IFN- γ producing cells in mice infected with *Nippostrongylus brasiliensis*, suggesting that GATA3 serves as a principal switch in determining Th1/Th2 response [13]. The requirement of GATA3 expression in the optimal Th2 cytokine production is also indicated in another line of GATA3 conditional knockout mice [14]. Interestingly, however, the established histone modifications (histone H3-K9/14 hyperacetylation and H3-K4 hypermethylation) at the Th2 cytokine gene loci were not apparently affected by the depletion of the GATA3 gene in the *in vitro* differentiated Th2 cells [12]. Thus, once the Th2 cell identity is established, GATA3 may not be largely involved in the regulation of chromatin status, but still plays an important role as a transcription factor for the Th2 cytokine genes (Figure 2b).

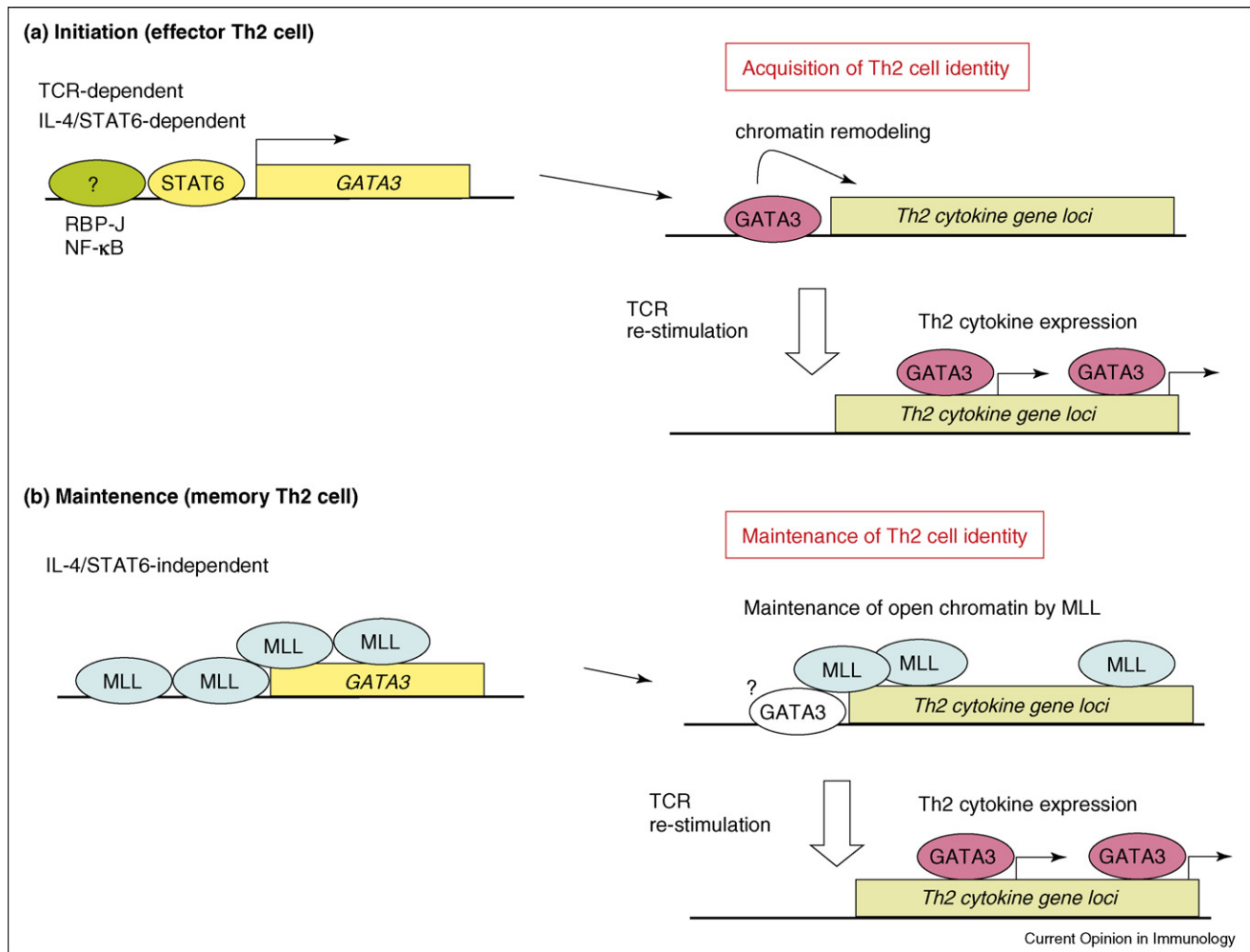
Regulation of GATA3 expression

IL-4/STAT6-mediated signals are required for the upregulation of GATA3 transcription in developing Th2 cells, while the basal expression of GATA3 in naïve CD4 T cells is not dependent on STAT6 activation. In thymocytes, GATA3 expression was shown to be controlled by c-Myb [15], E2A [16], and Notch signaling [17]. Notch signaling was reported to regulate GATA3 expression also in peripheral CD4 T cells [18^{••},19^{••}]. Notch1 ICD (intra cellular domain) and its signaling component, RBP-J, bind to the upstream promoter of GATA3 and induce transcription of GATA3 involving exon 1a. IL-4/STAT6 signaling also induces the GATA3 transcription from exon 1a. Although the relationship between Notch1-mediated signaling and IL-4/STAT6 signaling remains unclear at this time, the Notch1 ICD/RBP-J complex may substitute for STAT6 during Th2 cell differentiation, or the IL-4/STAT6 activation may control the accessibility of the Notch-signaling complex to the GATA3 gene promoter. In fact, Notch was previously reported to play an important role in Th1 cell differentiation [20,21] and both Th1/Th2 cell differentiation [22]. Ectopic expression of Notch1 ICD in CD4 T cells resulted in Th1 cell differentiation, and the suppression of Notch signaling by a γ -secretase inhibitor blocked Th1 cell differentiation [20]. In this report, the Notch ICD/RBP-J complex was demonstrated to directly regulate the T-bet promoter. Thus, Notch/RBP-J signaling may play various roles in the regulation of Th1 and Th2 cell differentiation, and further investigation will be required for understanding the precise role of the Notch signaling pathway.

An important role for NF- κ B activation in the expression of GATA3 was originally reported in the allergic asthma model [23]. Recently, Notch1 and Notch3 were reported to augment the NF- κ B activity in T cells and thymocytes, respectively [24,25]. Notch1 ICD directly interacts with the NF- κ B complex to facilitate its nuclear retention, whereas Notch3 binds IKK α and maintains NF- κ B activation through the p52/RelB pathway. A zinc finger C2H2 type transcriptional repressor, Schnurri-2 (Shn-2) was reported to suppress the expression of GATA3 through the inhibition of NF- κ B activity [26]. In the absence of Shn-2, hyper-activation of NF- κ B is induced in naïve and developing Th2 cells, and the increased expression of GATA3 and augmented Th2 responses were observed [26]. In addition, protein kinase C (PKC) θ appears to regulate NF- κ B activation and GATA3 expression. In PKC θ -deficient CD4 T cells, the expression of GATA3 was severely impaired and Th2 cytokine production decreased [27]. Moreover, PKC θ was reported to be a downstream target of Notch3 [28]. Thus, the expression of GATA3 appears to be controlled by NF- κ B activation in thymocytes and peripheral CD4 T cells.

A high level ubiquitination and efficient proteasome-dependent degradation of GATA3 molecules in primary

Figure 2



Molecular requirement for the initiation and the maintenance of Th2 cell identity. **(a)** The initiation of Th2 cell differentiation is dependent on TCR-mediated signaling and IL-4-receptor-mediated STAT6 activation, and subsequent upregulation of GATA3 transcription. GATA3 plays a central role in the induction of chromatin remodeling of the Th2 cytokine gene loci. GATA3 is also known to act as a transcription factor for the Th2 cytokine genes. **(b)** Once Th2 cell identity is established, GATA3 may not be largely involved in the regulation of chromatin status, but still plays an important role as a transcription factor for the Th2 cytokine genes. In memory Th2 cells, a histone H3-K4 methyltransferase, MLL binds to the GATA3 gene locus and Th2 cytokine gene loci to maintain the hyper-methylation status of H3-K4. MLL histone H3-K4 methyltransferase complex may contribute to IL-4/STAT6-independent maintenance of Th2 cell identity in memory Th2 cells. Once memory Th2 cells are stimulated through TCR, GATA3-dependent Th2 cytokine gene expression is induced.

CD4 T cells have been demonstrated [29]. Mdm2 was found to physically interact with GATA3 and induce multi-ubiquitination and subsequent degradation, indicating that Mdm2 acts as an E3 ligase for GATA3. Interestingly, the ubiquitin/proteasome-dependent degradation of GATA3 protein was counteracted by the TCR-mediated activation of the ERK-MAPK cascade [29]. This may explain the requirement for TCR signals, in addition to the IL-4/STAT6-dependent signal, for the induction of Th2 cell differentiation following GATA3 mRNA expression. Thus, the expression of GATA3 is also controlled at the protein level.

Epigenetic regulation of Th2 cytokine expression

Chromatin structure is an important determinant of gene activation and silencing that is regulated by various epigenetic processes. Changes in chromatin structure at the Th2 cytokine (IL-4/IL-5/IL-13) gene loci occur during Th2 cell differentiation. The chromosome conformation capture (3C) assay is a new technique to analyze the changes of chromatin configuration. Spiliakakis and Flavell applied this technique to the Th2 cytokine gene loci and demonstrated that a recently identified Th2 LCR (locus control region) [30,31] is required for T-cell-specific hub formation [32]. GATA3

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and STAT6 are required for the establishment and/or maintenance of the interactions [32]. Targeted deletion of the Th2-specific LCR (LCR-C/RHS7 HS) was associated with decreased long-range interchromosomal interactions between this LCR and the promoters of the Th2 cytokine genes [33]. More recently, Cai *et al.* reported that TCR-mediated induction of SATB1 (special AT-rich sequence binding protein 1) was required for the coordinated expression of Th2 cytokines [34^{••}]. SATB1 organizes cell-type-specific nuclear architecture by anchoring specialized DNA sequences and recruiting chromatin remodeling factors, and controls various gene transcriptions. After TCR stimulation, SATB1 was rapidly induced and bound to several specific regions (CNS1, CNS2, etc.) at the Th2 cytokine gene loci including the RAD50 and KIF3A genes. A 3C assay and ChIP-loop assay [34^{••}] revealed that SATB1 is crucial for the formation of higher order chromatin structure around the Th2 cytokine gene loci and regulates the accessibility of RNA polymerase II, Brg1, and c-Maf.

In Th1 cells, the stable inhibition of the expression of IL-4 has been attributed to epigenetic regulation. DNA hypersensitive (HS) site IV is located at the 3' end of the IL-4 gene locus and plays a crucial role in the Th1-specific silencing of the expression of IL-4 [35]. Runx3 has emerged as a potential regulator of the HS IV-mediated IL-4 gene silencing in Th1 cells [36[•],37[•]]. Runx3 was upregulated during Th1 cell differentiation in a T-bet-dependent manner, and Runx3 and T-bet cooperatively bound to the IFN- γ promoter and the IL-4 silencer. Th1 cells that lack the HS IV region were less sensitive to the repression of IL-4 by T-bet and Runx3 [36[•]]. Interestingly, in naïve CD4 T cells, Runx1 complex binds to the IL-4 silencer instead of Runx3. The overexpression of GATA3 in Th1 cells induced the dissociation of the Runx complex from the IL-4 silencer [37[•]]. An analysis on the chromatin configuration around the Th2 cytokine loci may provide a clearer view of the molecular events underlying the Runx-dependent repression of the IL-4 gene in Th1 cells.

Maintenance of the expression of the GATA3 gene locus and Th2 cytokine gene loci by a *Trithorax* group gene product, MLL

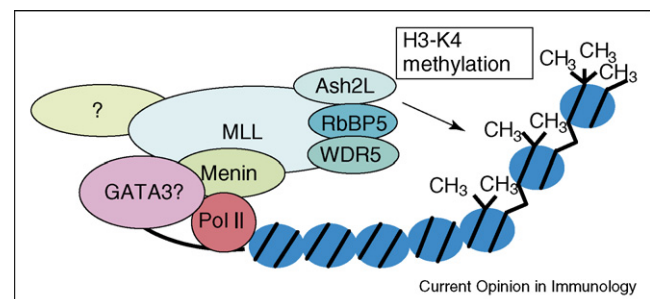
Once a high level expression of GATA3 is induced in *in vitro* differentiated Th2 cells via the activation of IL-4/STAT6-mediated signaling, the high level GATA3 expression is maintained in an IL-4-independent manner (Figure 2b). It is known that the dimethylation and trimethylation of histone H3-K4 are associated with the transcriptionally active status of chromatin [38]. In *in vitro* established Th2 cells, histone H3-K4 methylation and H3-K9/14 acetylation of the upstream region of GATA3 promoter (between exon 1a and exon 1b) are detected [39^{••}]. A genome-wide analysis of the H3-K4

methylation pattern in human and mouse fibroblast and tumor cell lines was previously reported [40]. However, the molecular dynamics governing the changes in H3-K4 methylation during cell differentiation has not been well analyzed. Since the expression of GATA3 is crucial for inducing Th2 responses, it is important to study the mechanisms governing the induction and the maintenance of H3-K4 methylation at the GATA3 gene locus.

MLL, mixed lineage leukemia is a member of the *Trithorax* group gene. MLL possesses a histone methyltransferase activity for H3-K4, and forms a huge protein complex containing RNA polymerase II [41]. Figure 3 illustrates the proposed MLL complex [41] (MY and TN, unpublished observation). Recently, we have reported a crucial role for MLL for the maintenance of the expression of the GATA3 gene [39^{••}]. A substantial decrease in the transcription of GATA3 accompanied by decreased methylation levels of H3-K4 at the GATA3 gene locus was detected in MLL-knockdown Th2 cell lines and in *in vivo* generated memory Th2 cells with an *MLL* heterozygous background. Thus, the maintenance of transcriptional expression of GATA3 in established Th2 cells including memory Th2 cells is epigenetically regulated by MLL (Figure 2b, left).

The histone modifications including histone H3-K9 hyper-acetylation at the IL-4 gene locus were also maintained in an IL-4-independent manner in memory Th2 cells. In addition to the GATA3 gene locus, MLL is also found to be involved in the maintenance of activated chromatin status at the Th2 cytokine gene loci (Figure 2b, right) [39^{••}]. The direct binding of MLL at specific regions on the IL-4 and IL-13 gene loci was detected. Th2 cytokine production was severely reduced in MLL-knockdown Th2 cell lines and MLL heterozygous memory Th2 cells accompanied by decreased levels of H3-K9/14 acetylation and H3-K4 methylation.

Figure 3



A proposed MLL complex. MLL is reported to be associated with Ash2L, RbBP5, WDR5, and Menin. Ash2L is a *Trithorax* group molecule. RbBP5 and WDR5 are WD40 repeat-containing protein. Menin is a *MEN1* tumor suppressor gene product. Several other molecules appear to be associated with MLL.

Maintenance of memory Th2 cell numbers

In order to establish sufficient memory Th1/Th2 cell responses, effector Th1/Th2 cells must escape apoptotic cell death and survive at the contraction phase (Figure 1). In a series of studies on the role of Shn-2 in the CD4 T cell immunity, we have found that Shn-2 is required for the generation of sufficient numbers of both memory Th1 and Th2 cells [42^{*}]. The expression levels of Shn-2 were high in naïve CD4 T cells, but they were decreased after TCR stimulation, and increased again in memory Th1/Th2 cells, particularly in memory Th2 cells. Shn-2-deficient effector Th1 and Th2 cells showed hyper-activation of the NF- κ B signaling pathway and tended to die at the contraction phase, partly owing to the increased susceptibility to apoptosis via the Fas/FasL pathway. Thus, after antigen clearance, the successful generation of memory Th1/Th2 cells appears to require Shn-2-mediated deactivation of the NF- κ B signaling pathway at the contraction phase.

Memory T cells have several features associated with stem cells, and the similarity of the gene expression patterns between memory T/B cells and long-term hematopoietic stem cells was reported [43]. The *Polycomb* group gene *Bmi1* has been implicated in the maintenance of hematopoietic, neural, and cancer stem cells. We recently found that *Bmi1* supports memory Th1/Th2 cell survival via the repression of the expression of the *Noxa* gene [44]. *Noxa* is a member of the proapoptotic BH3 only protein. Although *Bmi1* was reported to promote cell proliferation, cell survival and stem cell self-renewal by the repression of the *Ink4a/Arf* gene locus, *Bmi1*-mediated regulation of the expression of the *Noxa* gene and the survival of memory CD4 T cells were found to be *Ink4a/Arf*-independent. Although less is known about the molecules that control the cell survival of memory CD4 T cells, further analyses on the nuclear factors that are preferentially expressed in memory T cells may provide new insights in our understanding of the generation and the maintenance of memory Th2 responses.

Conclusion

In the past few years, new insights into the molecular requirement for the epigenetic regulation of Th2 cell identity have emerged. A role for GATA3 expression has been more highlighted both in the initiation and the maintenance of Th2 cell identity. GATA3 may control the Th2 cytokine gene expression in a variety of ways through the association with distinct functional nuclear complexes including Polycomb, Trithorax, HAT, HDAC, Pol II, and those containing chromatin modifying factors such as SATB1. The elucidation of the nature of GATA3 complexes, particularly that of cell-type-specific differences of the GATA3 complex may provide a more comprehensive view of the regulation of Th2 cell identity. *Polycomb* and *Trithorax* group gene products counteract

each other in function during the development in *Drosophila* and mammals. The analyses of precise molecular actions of these two interesting molecules in the initiation and the maintenance of Th2 cell identity would also be also intriguing.

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