Allergology International. 2010;59:21-31
DOI: 10.2332/allergolint.09-RAI-0151

REVIEW ARTICLE

Antigen-Specific Immunotherapy against Allergic Rhinitis: The State of the Art

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ABSTRACT

Allergic rhinitis is the most prevalent type I allergy in industrialized countries. Pollen scattering from trees or grasses often induces seasonal allergic rhinitis, which is known as pollinosis or hay fever. The causative pollen differs across different areas and times of the year. Impaired performance due to pollinosis and/or medication used for treating pollinosis is considered to be an important reason for the loss of concentration and productivity in the workplace. Antigen-specific immunotherapy is an only available curative treatment against allergic rhinitis. Subcutaneous injection of allergens with or without adjuvant has been commonly used as an immunotherapy; however, recently, sublingual administration has come to be considered a safer and convenient alternative administration route of allergens. In this review, we focus on the safety and protocol of subcutaneous and sublingual immunotherapy against seasonal allergic rhinitis. We also describe an approach to selecting allergens for the vaccine so as to avoid secondary sensitization and adverse events. The biomarkers and therapeutic mechanisms for immunotherapy are not fully understood. We discuss the therapeutic biomarkers that are correlated with the improvement of clinical symptoms brought about by immunotherapy as well as the involvement of Tr1 and regulatory T cells in the therapeutic mechanisms. Finally, we focus on the current immunotherapeutic approach to treating Japanese cedar pollinosis, the most prevalent pollinosis in Japan, including sublingual immunotherapy with standardized extract, a transgenic rice-based edible vaccine, and an immunoregulatory liposome encapsulating recombinant fusion protein.

KEY WORDS

allergic rhinitis, biomarker, immunotherapy, pollinosis, regulatory T cell

INTRODUCTION

Allergic rhinitis is the most prevalent type I allergy, and pollen grains are one of the most common causes of respiratory allergies. In western Europe, the prevalence of clinically confirmable allergic rhinitis was estimated to be 23%, with more than 50% of the allergic subjects possessing specific IgE against grass pollen.[1] In Japan, the prevalence of allergic rhinitis was estimated to be 39.4% and that of pollinosis was 29.8%.[2]

Pollinosis is induced by the invasion of pollen grains onto the ocular and nasal mucosa. Pollen grains easily access internal binding sites on contact with the aqueous phases of nasal and ocular mucosal membranes. After pollens are hydrated on aqueous membranes, they swell, rupture, and release their cytoplasmic components. It has been reported that grass pollen grains rupture in water and release large amounts of respirable particles, such as starch granules containing allergens.[3] Although pollinosis patients have a low rate of asthma attacks during pollen season, the attacks that do occur may be attributable to these respirable particles bearing allergens from pollen grains.[4] Pollen grains release not only allergen-bearing particles but also immunomodulatory mediators such as pollen-associated lipid mediators (PALMs) and NADPH oxidases. Proinflammatory PALMs such as leukotriene B4-like substances attract and activate human peripheral blood eosino-

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Received 8 October 2009.

Table 1 Comparison between SLIT and SCIT

	SLIT	SCIT
Administration	Sublingual spitting or sublingual swallowing	Subcutaneous injection with or without adjuvant
Pre-treatment	None	Medication or anti-IgE
Build-up phase	A few weeks, one day for rush protocol, or no up-dosing phase	A few weeks or a few days for rush protocol
Vaccination	Once daily or a few times weekly	A few times weekly or monthly
Adverse event	Local mild reaction in most cases, a few reports of fetal adverse reactions	Sometimes induces fetal adverse reactions

gual treatment also attenuated the symptoms and symptom-reducing drugs intake; however, only the nasal symptom score showed a significant reduction compared to the placebo-control group.[35] Thus, holding the vaccine under the tongue may be an important way to achieve better therapeutic effects with SLIT.

Vaccines for SLIT can also be delivered by two methods: sublingual spitting, in which the vaccine is spat out after being held under the tongue, and sublingual swallowing, in which the vaccine is swallowed after being kept under the tongue. In studies using radiolabeled allergens, most of the allergens remained in the mouth after the vaccine was spat out. However, plasma radioactivity began to increase only after swallowing.[36-38] The author concluded that contact between the allergens and the oral mucosa is a crucial step in the mechanisms of SLIT, and suggested that the more appropriate and advantageous way to administer the allergen sublingually is via the sublingual swallowing procedure.[38]

It has been recommended that the administration of SLIT vaccine be started at least 8 weeks before pollen season for better therapeutic effects.[39] However, an ultra-rush scheme of SLIT treatment for children allergic to grass pollen was reported to significantly improve the symptoms and the medication score compared to the placebo group. In this 2-year randomized, double-blind, placebo-control trial, the authors administered standardized extract of five grass pollen (*Dactylis glomerata*, *Anthoxanthum odoratum*, *Lolium perenne*, *Poa prantensis*, and *Phleum pretense*) beginning 2 weeks before the pollen season started with one day for ultra-rush induction, and followed by daily treatment (120 IR, 10 μg major allergen) for 6 months. It has been reported that SLIT significantly improved the asthma symptom score and reduced the nasal symptom score and the use of rescue medication score compared to the placebo group.[40] The starting point and duration of treatment varied among the clinical trials, and the best procedure for administration remains unclear.[41] (Table 1)

As a novel route to enhance the therapeutic efficacy of the vaccine, direct intralymphatic injection was proposed for the administration of peptide vaccine against viral infection and tumor in the mouse. This paper reported that the direct administration of peptide vaccine into a lymph node induced enhanced immunogenicity compared to subcutaneous and intradermal vaccination.[42] This novel technique was recently applied to patients with hay fever in an open-label, randomized control trial.[43] The authors injected 1,000 SQ-U of aluminum hydroxide-adsorbed grass pollen extract into a superficial inguinal lymph node under ultrasonic guidance. Three intralymphatic injections over 2 months resulted in long-lasting tolerance with the amelioration of hay favor symptoms, reduced skin prick test reactivity, and decreased serum allergen-specific IgE comparable with conventional SCIT. Furthermore, the author reported that there were fewer adverse events than in SCIT, even without premedication with antihistamines, and the injection was less painful than venous puncture.[43] Further clinical trials with a larger population are needed to evaluate the safety, therapeutic efficacy, and duration of tolerance of this treatment.

BIOMARKERS FOR SLIT

The therapeutic effects obtained by antigen-specific immunotherapy are commonly judged on the basis of clinical symptoms according to quality-of-life (QOL) score, symptom diary, and symptom-reducing drugs intake. The biomarkers correlated with the therapeutic effects are still controversial, especially for SLIT.

Antigen-specific IgG4 is considered to be a biomarker for antigen-specific immunotherapy; however, the correlation between the induction of IgG4 production and clinical symptoms is controversial.[44] In a report about the use of SLIT against timothy pollinosis, antigen-specific IgG4 was significantly upregulated in the SLIT group compared to the placebo group, and the authors concluded that the upregulation of IgG4 was correlated with the improvement of symptoms compared with the previous year. However, the clinical score and medication score were not significantly different between the SLIT group and the placebo group.[45] A recent study of dairy administration of grass allergen tablets showed dose-dependent efficacy of the SLIT and the induction of blocking IgG. This report showed that the administration of 75,000 SQ-T (15 μg Phl p 5) dose significantly reduced the symptom and medication

scores, and up-regulated specific IgG; however, a 2,500 SQ-T (0.5 μg Phl p 5) dose did not result in amelioration of the symptom and medication scores nor in the induction of IgG.[46] We previously reported that specific IgG4 was significantly increased in pollen season concomitant with improvement of the symptom medication score in the SLIT group compared to the placebo group.[47] The disagreement in results related to the induction of blocking IgG or IgG4 and the improvement of clinical symptoms may depend on the dose and/or the method of administration of the SLIT vaccine.

Other serological parameters have been recently reported to be useful as therapeutic biomarkers for SLIT. A 3-month course of pre-seasonal treatment of patients with grass pollen allergic rhinitis induced a reduction of the serum level of soluble human leukocyte antigen (sHLA)-G. The authors reported a significant relationship among the decrease of the sHLA-G serum level, the increase of interferon (IFN)-γ-producing cells, and the decrease of sHLA-A, -B, and -C after SLIT.[48] Furthermore, the changes of serum sHLA levels were significantly correlated with the clinical symptom score measured using a visual analogue scale (VAS) after SLIT.[49] In this preliminary open-labeled study, the authors suggested that sHLA molecules might be considered as possible biomarkers of the response to SLIT.

Recently, two reports investigated the change of serum reptin levels after SLIT. Leptin is primarily produced by adipocytes and has been reported to protect T lymphocytes from apoptosis, regulate T cell activation, and up-regulate adhesion molecules in endothelial cells.[50] Furthermore, leptin was reported to modulate the hyporesponsiveness and proliferation of human naturally occurring $Foxp3^+CD25^+CD4^+$ regulatory T (nTreg) cells.[51] After a 3-month course of SLIT against pollinosis, serum leptin levels were reported to significantly correlate with symptom severity as assessed by VAS of nasal symptoms in women, the number of peripheral eosinophils in men, the allergen threshold dose for allergen-specific nasal challenge in both men and women, and the medication score in women. This 3-month course of SLIT showed a tendency to increase serum leptin levels compared to the levels before the SLIT, albeit the increase was not significant.[52] After a 2-year course of SLIT, the serum leptin level was significantly increased in men.[53] The relationship between the up-regulation of leptin by SLIT and clinical symptoms remains unclear; however, the difference of the clinical therapeutic efficacy may depend on gender and the presence or absence of obesity.

The reduction of antigen-specific Th2 responses is considered to be an important biomarker for antigen-specific immunotherapy. The increase in the size of the specific Th2 clone, which produces IL4 after being stimulated with Cry j 1 (a major allergen of the Japanese cedar pollen), after pollen season was reported to be significantly reduced in the SLIT group compared with the placebo group in a double-blind, placebo-controlled study of Japanese cedar pollinosis. The increase of specific IL5-producing cells after pollen season was also reduced in the SLIT group, but the reduction was not statistically significant.[47] It has also been reported that after a 2-year course of SCIT against Japanese cedar pollinosis, B and T lymphocyte attenuator (BTLA) expression on $CD4^+$ T cells was down-regulated in untreated patients after Cry j 1 stimulation and up-regulated in SCIT-treated patients. Furthermore, the change of BTLA expression was negatively correlated with IL5 production. The authors concluded that BTLA-mediated coinhibition of IL5 production may contribute to the regulation of allergen-specific T cell responses by antigen-specific immunotherapy.[54]

The therapeutic biomarkers of SLIT in children also remain unclear. In a study of the administration of the SLIT treatment to children with seasonal allergic rhinoconjunctivitis to grass pollen, the authors reported that a 2-year course of SLIT using a standardized 5-grass mixture (1.5 μg/week) did not alter the systemic immunologic reaction of IL4, IL5, and IFN-γ cytokine production, nor the proliferation of PBMC after stimulation with allergens in the SLIT group compared to the placebo group, although a positive effect on rescue medication use was achieved by SLIT treatment.[55] However, another study reported the up-regulation of mRNA expression in PBMC during SLIT in children using SQ-standardized tree pollen extracts. The authors reported that after the stimulation of PBMC with allergen *in vitro*, the mRNA expression of signaling lymphocytic activation molecule (SLAM) was significantly increased from baseline after 1 year in the SLIT group receiving a high-dose (weekly dose of 200,000 SQ-U) treatment. This up-regulation was reported to be correlated with IL10 and transforming growth factor-β (TGF-β) mRNA expression. The IL18 mRNA expression was also increased in the high-dose group over a 1-year treatment compared to the placebo group and was reported to be inversely correlated with the late-phase skin reaction after the second study year. The authors reported that this up-regulation of SLAM and IL18 mRNA expression suggested the down-regulation of Th2-type inflammatory responses by increased Th1-type responses.[56] Another study of SLIT in children using SQ-standardized tree pollen extract (weekly dose of 200,000 SQ-T, 30 μg major allergen containing Bet v 1, Aln g 1, and Cor a 1) reported that specific allergen-induced Foxp3 mRNA expression after a 2-year course of SLIT treatment was significantly increased in PBMCs compared to the placebo group and compared to the level before treatment. Changes in allergen-induced Foxp3 expression that significantly correlated with IL10 mRNA expression

were reported in the whole study group, including the low-dose (weekly dose of 24,000 SQ-T) group and the placebo group, after 1- and 2-year courses of treatment, and correlated with TGF-β1 mRNA after 1 year of treatment. Furthermore, IL17A mRNA expression was significantly correlated with symptom-medication score (SMS) in the whole study group and especially in the high-dose treated group. The authors concluded that IL17 expression may be associated with a poor therapeutic outcome of SLIT.[57]

MECHANISMS OF ANTIGEN-SPECIFIC IMMUNOTHERAPY

Numerous data showing that antigen-specific Th2-type responses are down-regulated and, in contrast, Th1-type and/or regulatory T cell (Treg) responses are up-regulated by immunotherapy have been accumulated. The imbalance of the population among the antigen-specific Th1, dominant Th2, and Treg is considered to induce sensitization and subsequent allergic inflammation in response to invading allergens, and immunotherapy may correct the imbalance of these cells. Actually, the high frequency of IL4-secreting Th2 cells was reported in allergic individuals, as was, in contrast, the dominance of IL10-secreting Tr1 cells in healthy subjects.[58] These authors suggested that the balance between allergen-specific Tr1 cells and Th2 cells causes the development of the allergy.

IL10-producing regulatory cells are considered to play a crucial role in clinical therapeutic mechanisms in immunotherapy. In a study of SCIT using house dust mite (HDM) extract in patients allergic to HDM, SCIT induced the suppression of PBMC proliferation and the suppression of IFN-γ, IL5, and IL13 production in PBMC stimulated with Der p 1 (a major allergen of HDM) at 70 days after treatment compared to the levels before treatment. In contrast to the suppression of Th1 and Th2 cytokines, the production of both IL10 and TGF-β was significantly increased. The report also showed that the suppression of proliferation was dependent on IL10 and TGF-β and that the source of IL10 is $CD25^+CD4^+$ T cells.[59] It has also been reported that IL10 production was induced by SLIT against HDM. The authors also reported the suppression of the proliferation of PBMC stimulated with extract of mite (*Dermatophagoids farinae*) and the increase of IL10 production compared to non-treated subjects.[60] The IL10 production after 3 years of SLIT treatment was significantly correlated with the improvement of clinical symptoms as assessed by forced expiratory flow between 25% and 75% (FEF_{25-75}).[61]

In a report about the use of SLIT to treat birch pollinosis, the authors investigated the antigen-specific proliferation and mRNA levels of cytokines and Foxp3. They reported that 4 weeks of SLIT induced a reduction in Bet v 1-specific proliferation and induced mRNA expression of IL10 and Foxp3 in $CD3^+$ cells compared to the levels before SLIT. These up-regulations of IL10 and Foxp3 mRNA expression were not seen after 52 weeks after SLIT; however, IFN-γ mRNA expression was significantly induced at 52 weeks after SLIT. The reduced Bet v 1-specific proliferation was significant after both 4 and 52 weeks, and this down-regulation was dependent on IL10 at 4 weeks. It has also been reported that neither TGF-β levels nor cell-cell contact-mediated suppression of $CD25^+CD4^+$ cells were changed during the course of SLIT.[62] Another report shows the significant reduction of IL5 mRNA expression and increased IL10 expression compared to the placebo group after 1 and 2 years of SLIT at a weekly dose of 200,000 SQ-U (30 μg major allergen) in children with tree pollinosis. It has been reported that TGF-β expression remained low after 1 and 2 years of SLIT; however, TGF-β expression was inversely correlated with IL5 and positively correlated with IL10 expression after 1 year of SLIT.[63]

In addition to IL10-secreting Tr1 cells, $Foxp3^+$ Treg cells are also considered to play a crucial role in the therapeutic effects achieved by immunotherapy (Fig. 2). It has been reported that 2 years of SCIT against hay fever significantly induced an increase in the number of $Foxp3^+CD25^+$ and $Foxp3^+CD4^+$ cells in the nasal mucosa compared to the number before SCIT and the number in untreated patients out of season. Twenty per cent of $CD3^+CD25^+$ cells were reported to also be Foxp3-positive, and 18% of $CD3^+$IL10-expressing cells were Foxp3-positive in the nasal mucosa after immunotherapy. This report suggested that the increase of $Foxp3^+CD25^+CD3^+$ cells in the nasal mucosa was associated with the clinical efficacy and suppression of seasonal allergic inflammation. This report also suggested the involvement of different types of regulatory T cells, namely IL10-secreting Tr1 cells and adaptive or induced Foxp3-positive Treg, in the therapeutic mechanisms of immunotherapy.[64] The involvement of Treg cells in immunotherapy was also reported in SCIT against hymenoptera venom allergy. In this report, the authors showed that the numbers of peripheral Treg cells defined as $Foxp3^+CD25^{bright}CD4^+$ T cells were significantly increased by venom immunotherapy, and the increase of circulating Treg cells was significantly correlated with the venom specific IgG4/IgE ratio.[65]

Antigen-specific Tr1 and Treg cells are considered to be involved not only in the suppression of Th2 cells but also, directly or indirectly, in the suppression of peripheral allergic inflammation[24] (Fig. 3). It has been reported that $CD25^+CD4^+$ Treg cells, more than 90% of which are $Foxp3^+$, directly inhibited the FcεR1-dependent mast cell degranulation after crosslinking of IgE, and this inhibition was dependent on cell-cell contact involving OX40-OX40L interactions between Treg and mast cells in the mouse.[66] Furthermore, al-

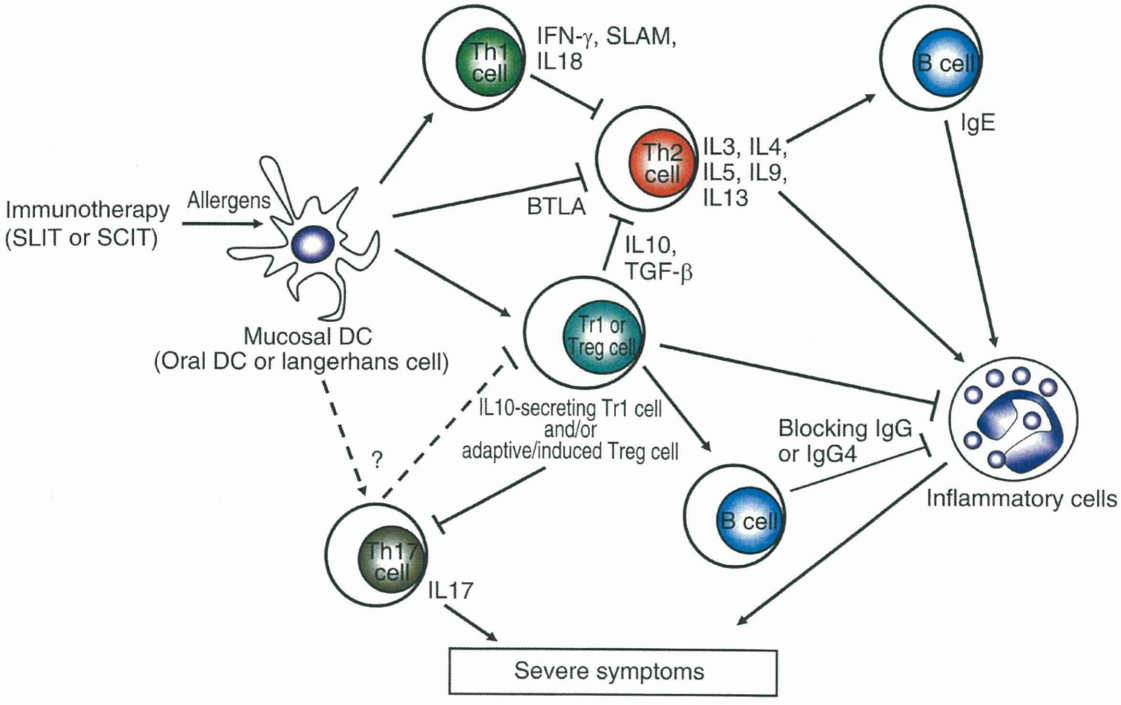

Fig. 2 T cells in antigen-specific immunotherapy. Antigen-specific immunotherapy induces regulatory T cells and Th1 cells via antigen-presentation by mucosal dendritic cells (DC). Th17 cells may be induced in a non-responder population by immunotherapy. The induced Th1 cells and/or regulatory T cells down-regulate the activation of Th2 cells and subsequently the activation of inflammatory cells such as eosinophils and mast cells. The regulatory T cells also activate B cells to produce blocking IgG or IgG4, and the blocking antibody inhibits binding between allergen and surface IgE on inflammatory cells to prevent the secretion of inflammatory chemical mediators.

lergic human eosinophils in peripheral blood and chronically inflamed nasal tissues were reported to express CD40, and the cross-linking of CD40 and CD40L enhanced the survival of eosinophils and induced the release of granulocyte/macrophage colony-stimulating factor (GM-CSF). In this report, IL10 down-regulated the constitutive expression of CD40 mRNA expression in eosinophils.[67] The induction of IL10-producing Tr1 or Treg cells in the nasal mucosa may play an important role in the reduction of nasal symptoms via cross-talk down-regulation of mast cells and eosinophils.

In a reports on the rush protocol of SCIT against Japanese cedar pollinosis using standardized pollen extract, the percentage of $CD203c^{high}$ cells in $CD3^{-}CRTH2^{+}$ basophils after allergen stimulation was reported to be down-regulated after rush immunotherapy without a decrease of the serum specific IgE titer. Furthermore, the percentage of $CD203c^{high}$ on basophils after *in vitro* stimulation was reported to be significantly correlated with symptom score.[68] The mechanisms which attenuate the sensitivity of peripheral basophils without a change in serum specific IgE remain unclear; however, this attenuation may be partially due to the up-regulation of inhibitory blocking antibody on the surface of basophils.

ANTIGEN-SPECIFIC IMMUNOTHERAPY AGAINST JAPANESE CEDAR POLLINOSIS

In Japan, Japanese cedar pollinosis is one of the most prevalent types of seasonal allergic rhinitis, with a prevalence estimated to be 26.5%.[2] Two clinical trials described the therapeutic effects of SLIT against Japanese cedar pollinosis.[47,69] In both trials, standardized Japanese cedar pollen extract was used at a monthly cumulative dose of 8,000 JAU, which contains approximately 10 μg of Cry j 1. This dosage is less than that reported in Europe, where a dose of 75,000 SQ-T (15 μg of a major grass allergen Phl p 5) was administered once daily for 18 weeks.[46] Unless the monthly cumulative dose is approximately $1/40^{th}$ of the amount required to be considered a major allergen (10/450 μg as a major allergen) in Japan, SLIT with an active treatment group against Japanese cedar pollinosis is still effective for improving quality of life and significantly ameliorates patients' SMS and symptom score during the pollen season. The up-regulation of the IL4-producing clone size specific to epitopes from Cry j 1 and Cry j 2[70] was reported to be significantly attenuated, and Cry j 1-specific IgG4 production was also significantly induced by active SLIT.[47] Furthermore, IL10-producing Tr1 cells were

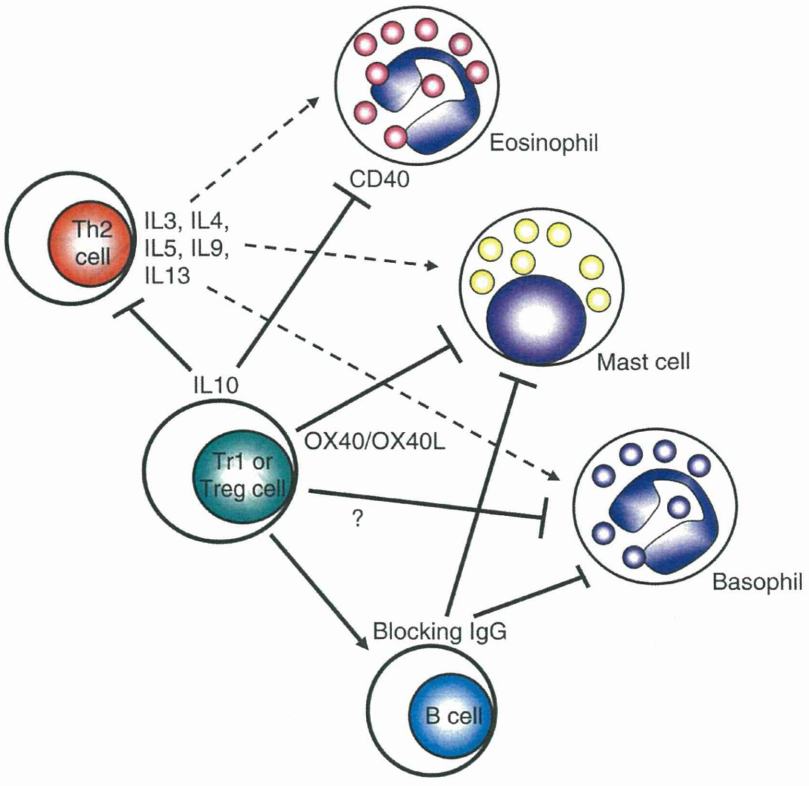

Fig. 3 Proposed roles of regulatory T cells on inflammatory cells in allergen-specific immunotherapy. Regulatory T cells, namely IL10-secreting Tr1 cells or adaptive/induced Treg cells, down-regulate inflammatory cells, directly or indirectly. Regulatory T cells down-regulate the activation of Th2 cells and subsequently Th2-type cytokine secretion. Regulatory T cells suppress the activation of inflammatory cells directly via their surface molecules and by secreting cytokines, and indirectly via the down-regulation of cytokine production in Th2 cells and by the activation of B cells to produce blocking IgG.

reported to be significantly increased in patients treated with SLIT compared with the levels in untreated patients and healthy subjects, and the proliferation of $CD4^+$ leukocytes stimulated with Cry j 1 and Cry j 2 was significantly suppressed by SLIT treatment in an IL10-dependent manner.[71] Supplementation with recombinant or native Cry j-allergens and/or updosing of the extract by bio-engineering may lead to more effective SLIT for treating pollinosis.

Another approach to safer immunotherapy is the use of oral immunotherapy using transgenic rice seed accumulating Cry j 1.[72] The generated transgenic rice plants expressed recombinant, structurally disrupted Cry j 1 peptides but spanned the entire Cry j 1 region as fusion proteins with the major rice storage protein glutenin. These fusion proteins aggregated with cysteine-rich prolamin and were deposited in endoplasmic reticulum-derived protein body I in rice seed. Transgenic rice expressing T cell epitopes from Cry j 1 and Cry j 2 successfully suppressed antigen-specific Th2-mediated IgE responses in a mouse model of allergic rhinitis.[73] Further clinical trials are needed to develop a rice-based edible vaccine as a tool for oral immunotherapy to control allergies.

An immunoregulatory liposome encapsulating the recombinant fusion protein of Cry j 1-Cry j 2 was manufactured as a novel vaccine for Japanese cedar pollinosis without risk of anaphylaxis.[74] The hybrid fusion allergen is expected to provide safer and more effective vaccines for immunotherapy. Vaccines using only T cell epitopes are also safer than native allergens, but there is wide variation among individual T cell epitopes. The fusion protein of major allergens covers all sequential T cell epitopes but is expected to have less IgE-binding capacity because its three-dimensional structure is disrupted in some B cell epitopes. Recombinant hybrid molecules using major allergens of timothy grass pollen induced stronger proliferation of PBMC in timothy-allergic patients than did mixtures of corresponding allergens, but still possess IgE-binding capacity and induce IgG production in sensitized mice.[75] In a mouse model sensitized with native Cry j 1 and Cry j 2, the vaccine that con-

tained Cry j 1-Cry j 2 fusion protein in the immunoregulatory liposome showed suppression of IgE and IgG antibody responses after being challenged with the allergens. Furthermore, oral administration of the vaccine showed efficient suppression of IgE antibody production.[74]

CONCLUSIONS

The standardization of a vaccine enables us to compare the results from varied clinical trials with respect to dose, clinical effects, and changes in biological parameters. Many reports have shown positive clinical therapeutic effects and suppressed effector/inflammatory responses. It is considered that IL10-producing Tr1 and/or adaptive or induced Treg cells may be involved in the suppression of the antigen-specific Th2-responses and local inflammation. However, how immunotherapy induces suppressor cells like Tr1 and Treg cells remains unclear, although the involvement of mucosal dendritic cells has been proposed. High-quality clinical studies are indispensable to clarify the therapeutic biomarkers and the mechanisms of induction of suppressor cells, and the resultant data from the studies may enable us to develop safer and more effective immunotherapy through the modification of the allergens, optimum dose, or administration regimen of a vaccine.

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Published October 18, 2010

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Article

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

The Rockefeller University Press $30.00
J. Exp. Med. Vol. 207 No. 11 2493-2506
www.jem.org/cgi/doi/10.1084/jem.20100760

Supplemental Material can be found at:
http://jem.rupress.org/content/suppl/2010/10/15/jem.20100760.DC1.html

The Journal of Experimental Medicine

Published October 18, 2010

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

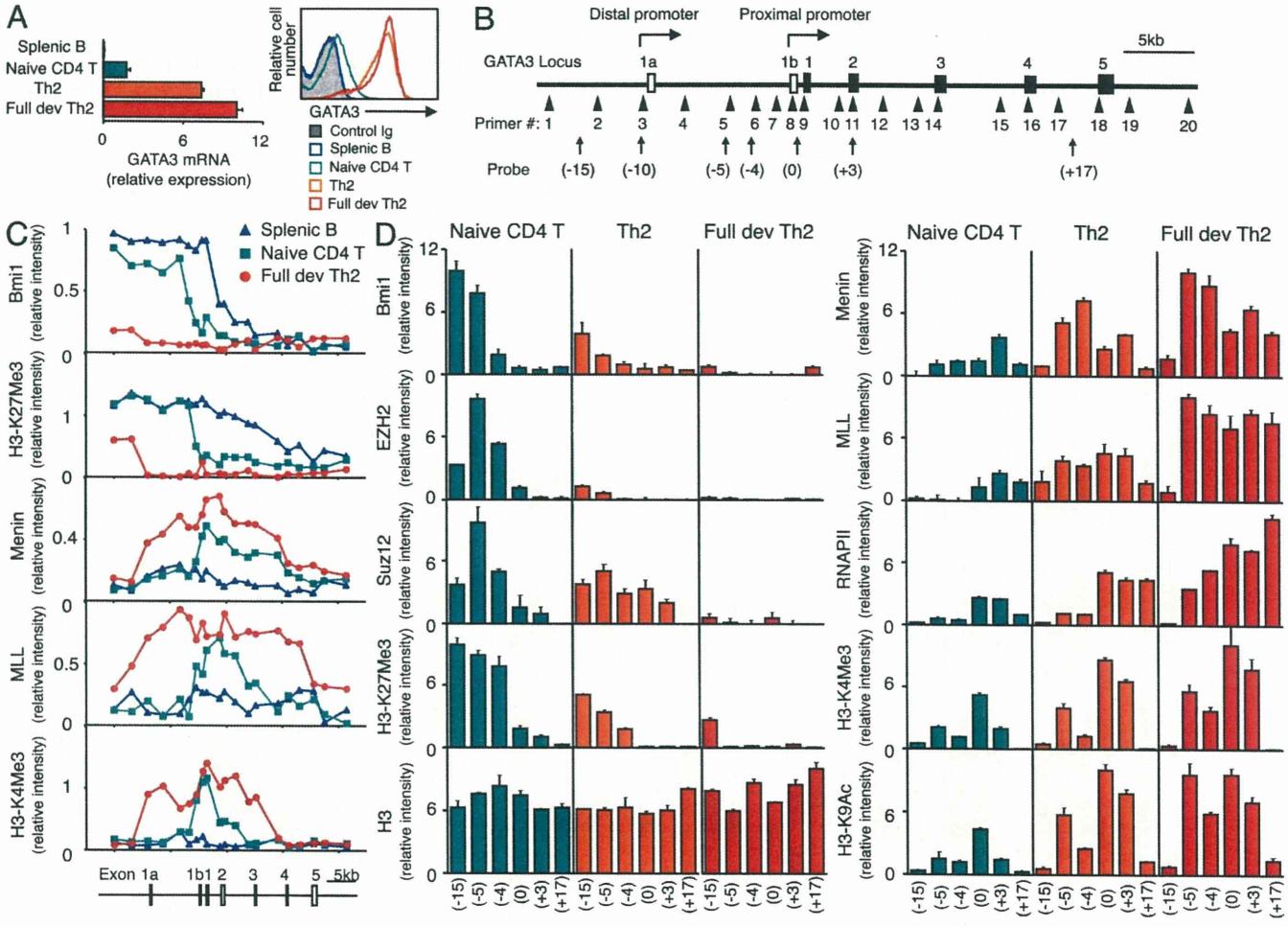

Figure 1. Changes in the histone modifications and PcG and TrxG binding to the *GATA3* locus during Th2 cell differentiation. (A) *GATA3* mRNA was measured by quantitative RT-PCR (left panel shows the relative intensity, compared with HPRT; the mean ± SD of three samples). GATA3 protein expression was 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

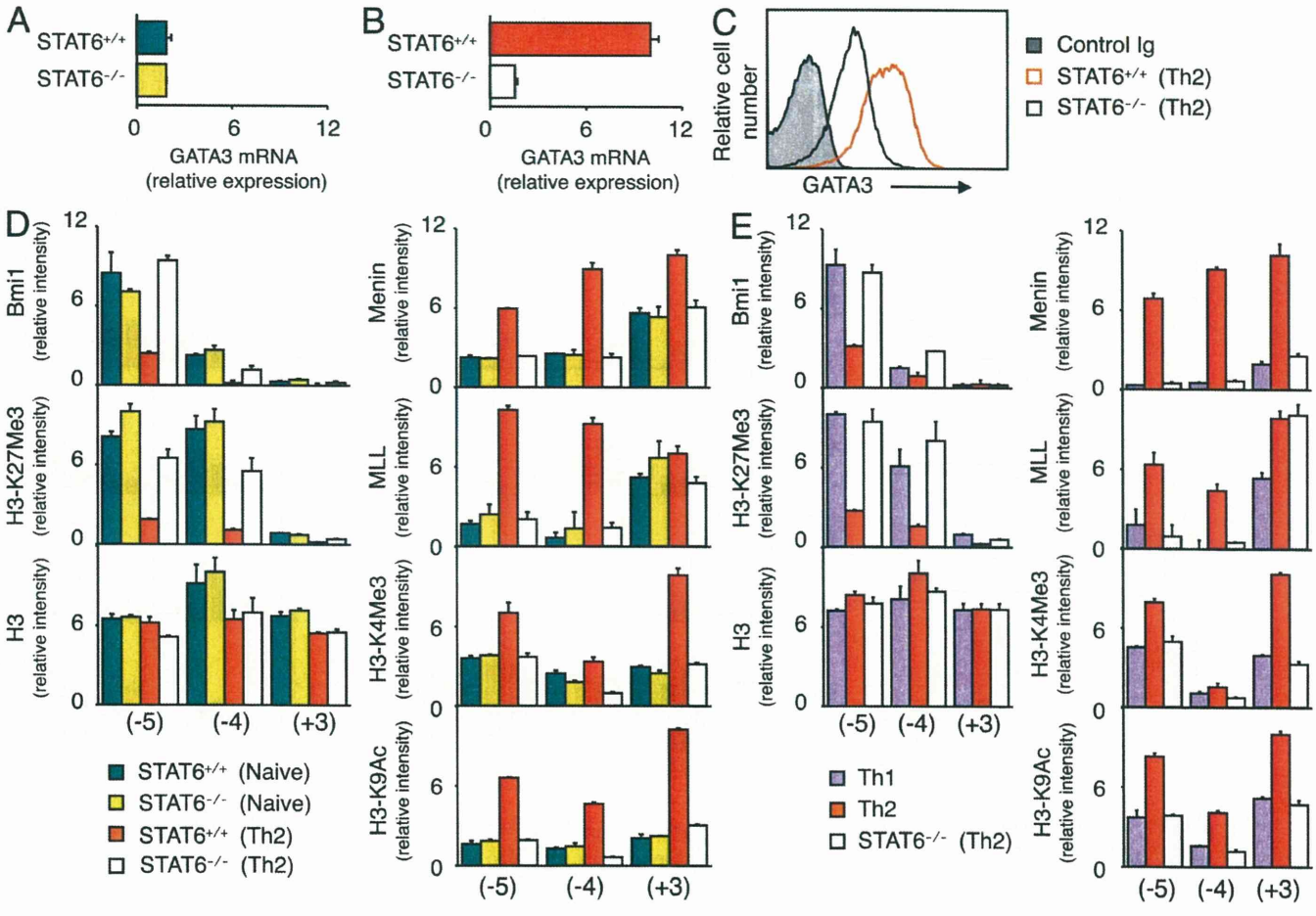

Figure 2. The displacement of the PcG by the TrxG complex at the *GATA3* gene locus was induced in a STAT6-dependent manner. (A and B) *GATA3* expression in STAT6-deficient naive CD4 T cells and Th2 cells was determined by a quantitative PCR. The relative intensity compared with HPRT (mean of three samples 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.

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

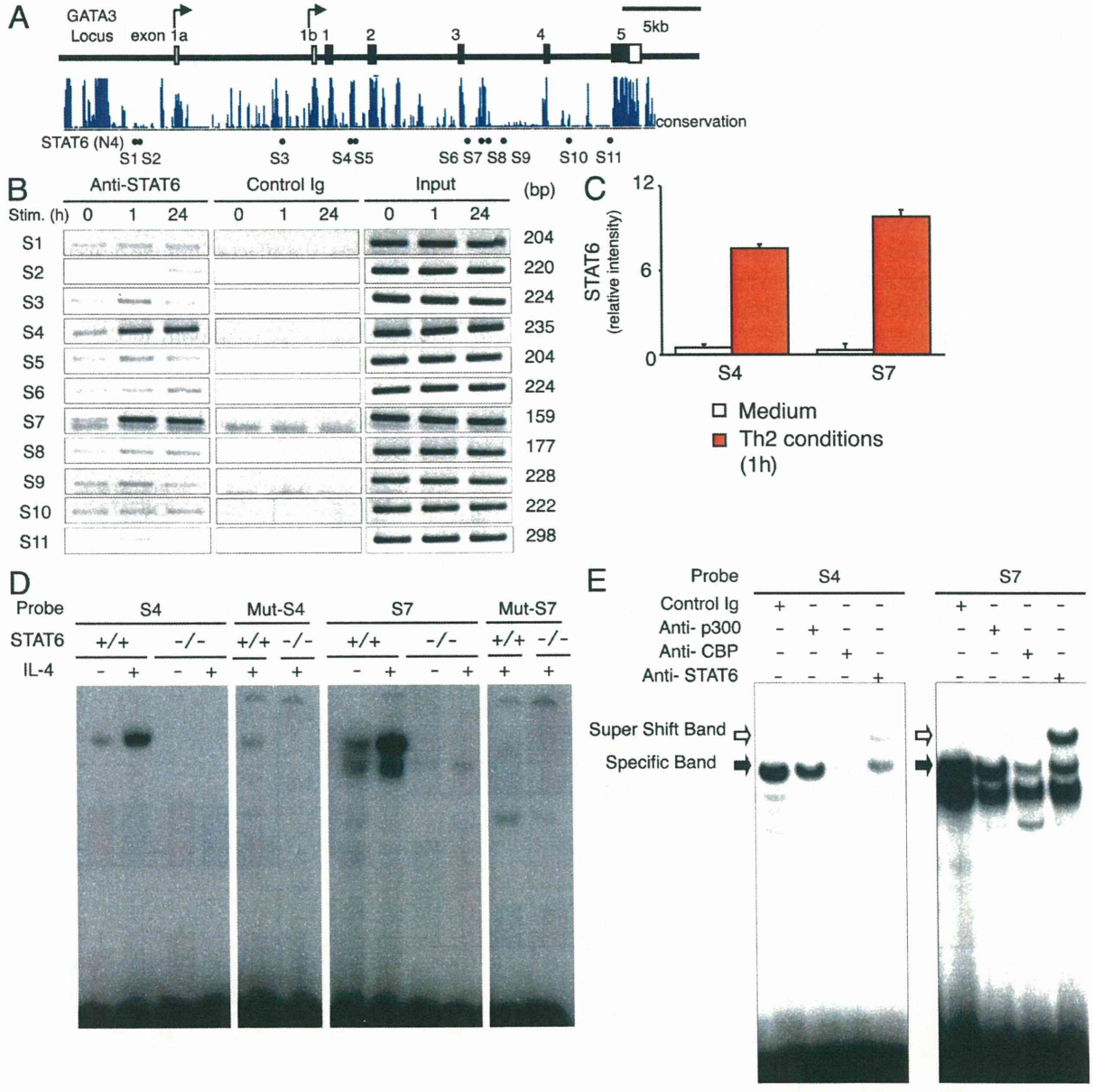

Figure 3. Identification of the binding site of STAT6 at the *GATA3* gene locus. (A) Schematic illustration of the GATA3 gene locus. 11 putative STAT-binding sites designated as S1–S11 (black dots) are indicated. The blue panel shows the conservation track obtained from the University of California, 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.

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)

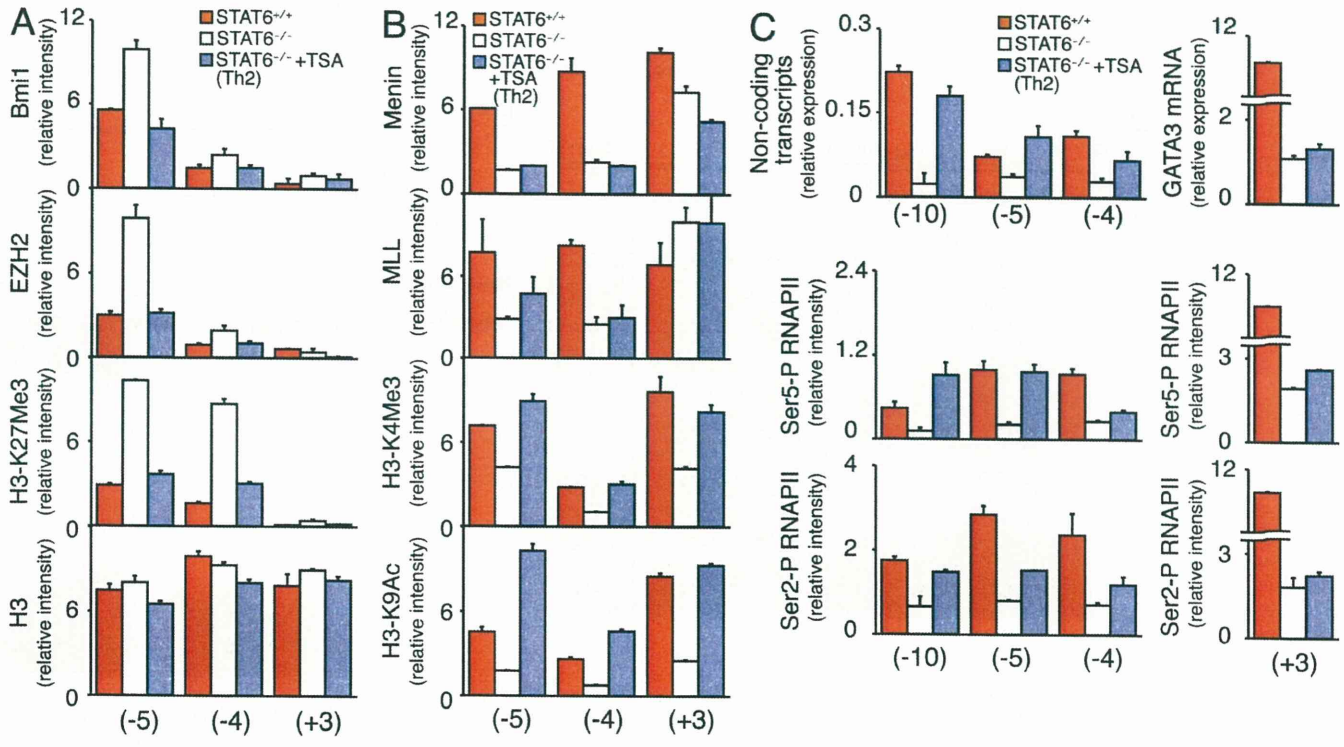

Figure 4. TSA treatment induced dissociation of PcG complex at the upstream region of the *GATA3* gene locus. Freshly prepared WT or STAT6-deficient CD4 T cells were stimulated under Th2 culture conditions, and where indicated, 10 ng/ml TSA was added on day 2. 3 d later, the cells were subjected to ChIP 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.

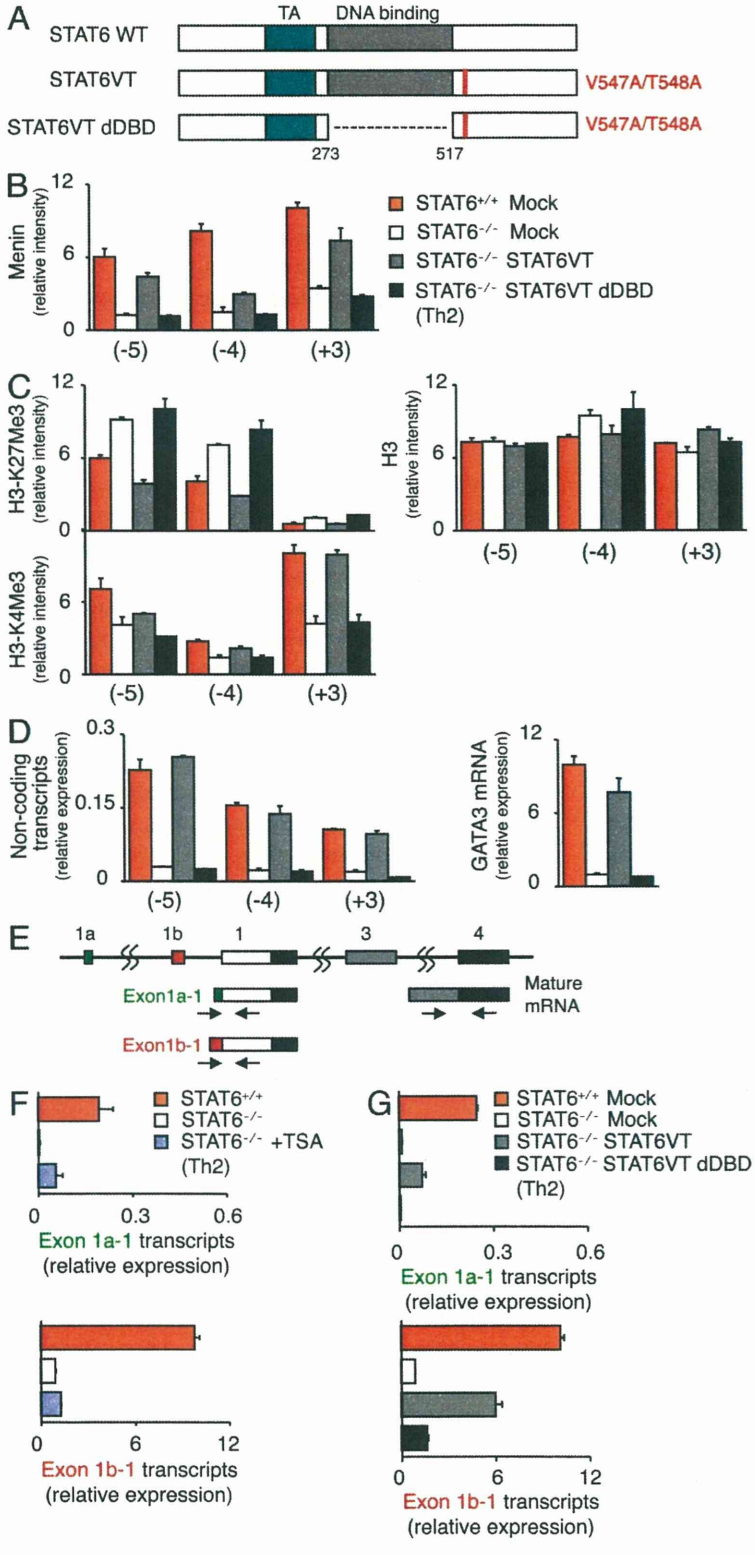

Figure 5. STAT6 activation was necessary and sufficient for recruitment of Menin and up-regulation of mature *GATA3* mRNA during Th2 cell differentiation. (A) Schematic representation of STAT6 WT, full-length STAT6VT, and DNA-binding mutant (dDBD) with location of the transactivation (TA) and DNA-binding 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.

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.

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

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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

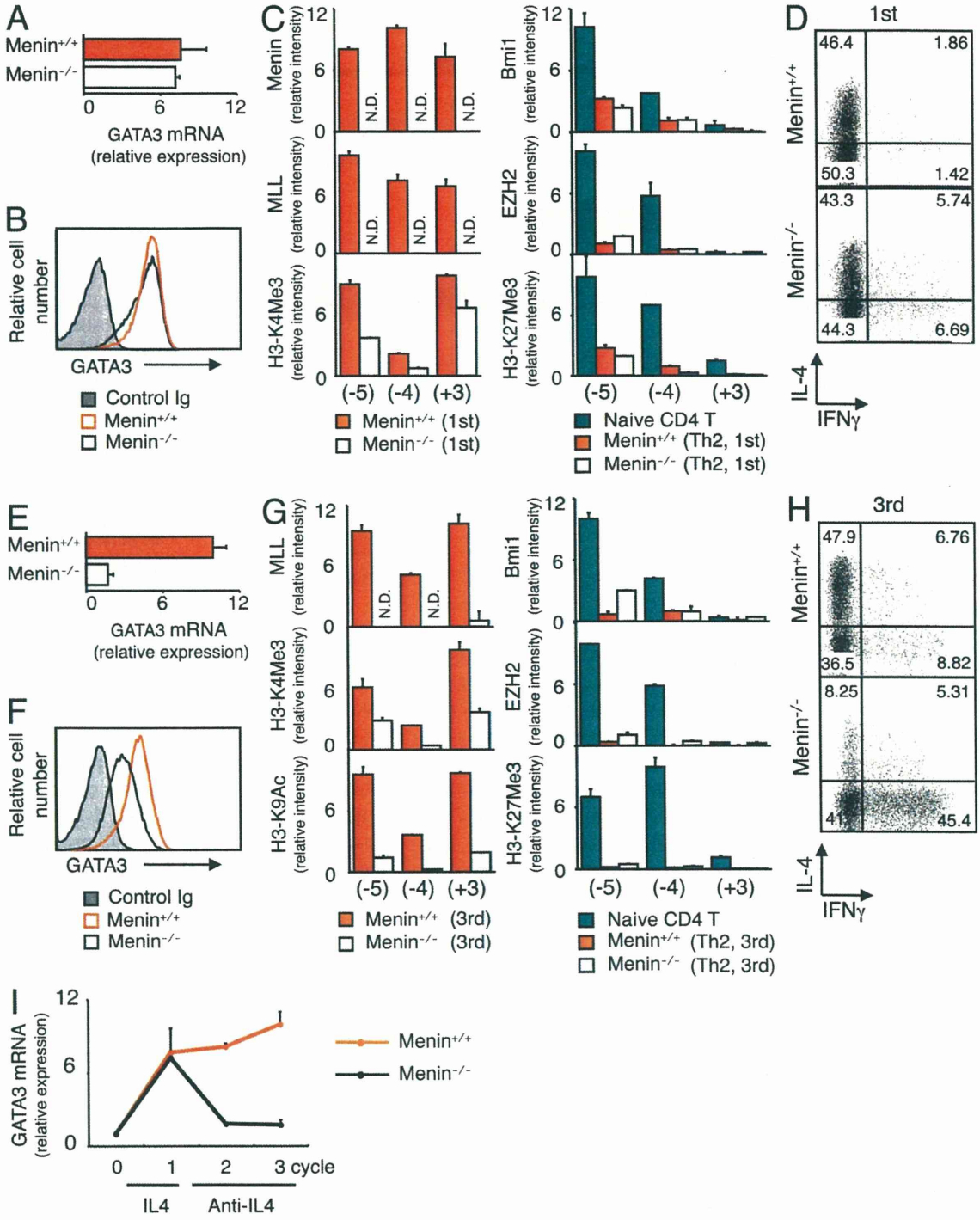

Figure 6. Menin-deficient effector Th2 cells failed to maintain the expression of *GATA3* in the absence of IL-4. (A–D) Naive CD4 T cells from WT or Menin-deficient mice were cultured under Th2 conditions for 5 d (first cycle). These Th2 cells were further cultured for 2 d in the absence of cytokines 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.

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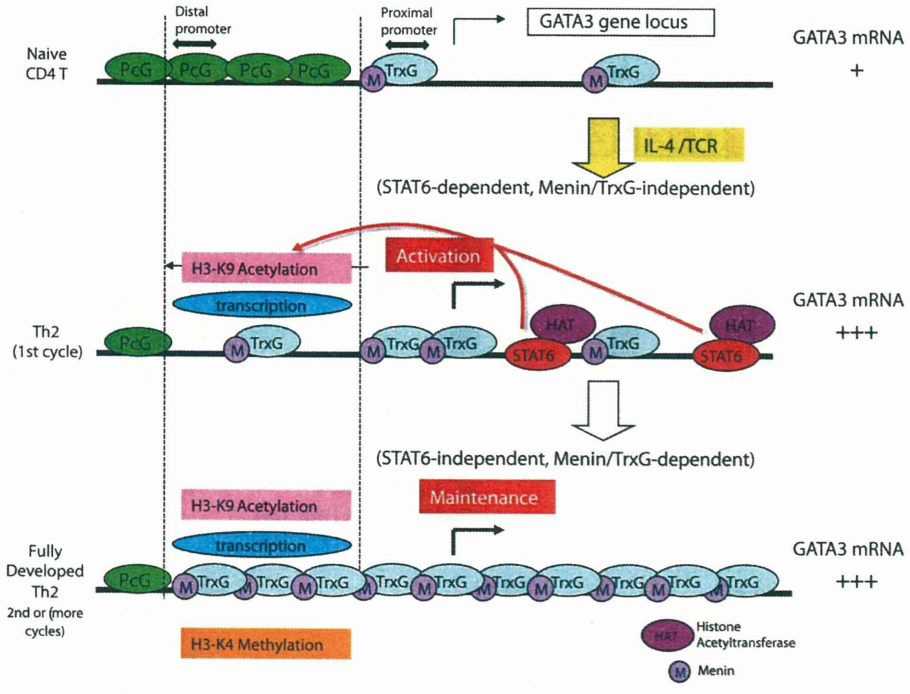

Figure 7. Schematic illustration of the transcriptional regulation of the *GATA3* gene in naive CD4 T cells, developing Th2 cells, and developed Th2 cells. In naive CD4 T cells, the PcG complex binds to the upstream region of the proximal promoter of the *GATA3* gene, and the expression of *GATA3* mRNA 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

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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),