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Active Demethylation of the Foxp3 Locus Leads to the Generation of Stable Regulatory T Cells within the Thymus

Aras Toker,* Dirk Engelbert,† Garima Garg,* Julia K. Polansky,* Stefan Floess,* Takahisa Miyao,‡ Udo Baron,§ Sandra Düber,¶ Robert Geffers,** Pascal Giehr,†† Sonja Schallenberg,** Karsten Kretschmer,** Sven Olek,‡ Jörn Walter,‡ Siegfried Weiss,¶ Shohei Hori,‡ Alf Hamann,‡ and Jochen Huehn*  

Stable expression of Foxp3 in regulatory T cells (Tregs) depends on DNA demethylation at the Treg-specific demethylated region (TSDR), a conserved, CpG-rich region within the Foxp3 locus. The TSDR is selectively demethylated in ex vivo Tregs purified from secondary lymphoid organs, but it is unclear at which stage of Treg development demethylation takes place. In this study, we show that commitment to a stable lineage occurred during early stages of murine thymic Treg development by engraving of lineage-specific epigenetic marks in parallel with establishment of a Treg-specific gene expression profile. TSDR demethylation was achieved through an active mechanism and involved enzymes of the ten-eleven-translocation family and hydroxylation of methylated cytosines, a modification that is implicated as an initiating step of mitosis-independent DNA demethylation pathways and has not yet been observed at specific loci during immune cell differentiation. Together, our results demonstrate that initiating TSDR demethylation during early stages of thymic Treg development commences stabilization of Foxp3 expression and guarantees full functionality and long-term lineage stability of Tregs.

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CD4+ regulatory T cells (Tregs) play an essential role in maintaining immune homeostasis and preventing autoimmune reactivity of potentially self-reactive lymphocytes that have escaped central tolerance mechanisms (1). For proper development and function, Tregs crucially depend on the forkhead box transcription factor Foxp3, and loss of Foxp3 function in humans and rodents results in devastating autoimmunity (2–7).

The vast majority of Foxp3+ Tregs is generated during T cell development in the thymus (8). Development of thymic Tregs mainly takes place in medullary regions at the CD4 single-positive (CD4SP; i.e., CD4+CD8−) stage (9, 10). Thymic Treg development requires that CD4SP thymocytes encounter cognate Ag presented by thymic APCs in the context of MHC class II (11–14), and CD4SP thymocytes appear to be predisposed to upregulate Foxp3 expression (15). According to the two-step model of thymic Treg development, this TCR signaling event induces upregulation of IL-2Rα-chain (CD25), rendering these thymocytes receptive to subsequent cytokine signals that foster their development into fully functional Foxp3+ Tregs (16, 17). IL-2 plays a predominant role in this second step of thymic Treg development. However, its loss can be compensated by other cytokines that signal through receptors containing the common γ subunit, such as IL-7 and IL-15 (16, 18, 19).

Stability of Foxp3 expression correlates to DNA demethylation at a conserved intronic CpG-rich region within the Foxp3 gene locus, designated Treg-specific demethylated region (TSDR) (20). Demethylation at the TSDR (also known as CNS2) is not required for initiation of Foxp3 expression, but for its long-term maintenance (21–23). Accordingly, stable Tregs display a fully demethylated TSDR, whereas the TSDR of conventional CD4+ T cells and in vitro–induced Tregs is heavily methylated (22). In addition, forced TSDR demethylation confers stability to in vitro–induced Tregs (21). Hence, understanding the mechanisms that cause TSDR demethylation in developing Tregs could open up ways to manipulate TSDR demethylation and allow safe application of in vitro–generated Tregs for therapeutic approaches (24).

In this study, we provide direct evidence that commitment to a stable Foxp3-expressing lineage is initiated already during early stages of thymic Treg development and is completed by TSDR demethylation in mature thymic Foxp3+ Tregs, ensuring full functionality and long-term lineage stability. In developing Tregs, CpGs of the TSDR are demethylated through an active mechanism that involves the recently discovered intermediate of active DNA demethylation pathways, 5-hydroxymethylcytosine (5hmC), and enzymes of the ten-eleven-translocation (Tet) family.
Materials and Methods

**Mice**

BALB/c and C57BL/6J mice were purchased from Janvier. BALB/c × Thy.1.1 and Foxp3GFP reporter mice (kindly provided by Alexander Rudensky, Memorial Sloan-Kettering Cancer Center, New York, NY) were bred at the Helmholtz Centre for Infection Research (Braunschweig, Germany). Indu-Rag1fl/fl × Rosa26-CreERT2 mice were obtained by crossing Indu-Rag1fl/fl mice (25) with Rosa26-CreERT2 mice (kindly provided by Prof. Anton Berns, The Netherlands Cancer Institute, Amsterdam, The Netherlands) and bred at the Helmholtz Centre for Infection Research. Lymphocyte development was induced in Indu-Rag1fl/fl mice by oral administration of 400 μl tamoxifen (20 mg/ml; Ratiopharm) in ClinOleic (Baxter). Foxp3GCD2 × Rag1GFP mice were obtained by crossing Foxp3GCD2 (26) with Rag1GFP (27) (kindly provided by Nobuo Sakaguchi, Kumamoto, Japan) and bred at the RIKEN Research Centre for Allergy and Immunology (Yokohama, Japan). RAG2 Gfp mice (28) were bred at the Centre for Regenerative Therapies (Dresden, Germany). Mice were kept under specific pathogen-free conditions in accordance with institutional, state, and federal guidelines.

**Cell sorting**

Peripheral CD4+ T cells were enriched from pooled LN and spleen cells using anti-CD4 microbeads and magnetic separation on an autoMACS separator (Miltenyi Biotec). For thymocyte sorting, total thymocytes were depleted of CD8+ cells using APC-conjugated CD8 Abs and anti-APC microbeads. Depleted cells were stained, and subsets were sorted on a FACS Aria II (BD Biosciences).

**Flow cytometry**

Single-cell suspensions were prepared from lymphoid organs and stained with fluorochrome-conjugated anti-mouse CD4 (clone RM-4), CD8 (53-6.7), CD24 (M1/69), CD25 (PC61), Thy1.1 (HIS11), and anti-human CD2 (RPA-2.10). Intracellular staining was performed with Foxp3 (FJK-16s) and Ki-67 (B56) Abs. Flow cytometric analyses were performed on LSR II (BD Biosciences), and data were analyzed with FlowJo software (Tree Star).

**BrdU incorporation**

Mice received two i.p. injections of 1 mg BrdU in PBS 9 and 3 h before analysis. Thymocytes were stained for cell-surface molecules, followed by fixation, permeabilization, and intracellular staining for Foxp3 and BrdU using the BrdU flow kit (BD Biosciences).

**Adoptive transfers**

Total CD4+ T cells were isolated from pooled LN and spleen cells of BALB/c × Thy.1.1 mice. Isolated cells were i.v. injected into BALB/c recipients in 100 μl sterile PBS. Transferred cells (2 × 10^6 per recipient) were ~95% CD4+.

**Fetal thymic organ cultures**

Thymic lobes from embryonic day 18.5 fetuses of Foxp3GFP mice were placed on a polyethersulfone transwell membrane with a pore size of 0.4 μm (Corning), and the transwell insert was placed in a well containing 400 μl serum-free AIM V medium (Life Technologies) in a 24-well plate. Lobes were incubated for up to 14 d, and medium was replaced every 5 d. Gender of fetuses was determined by PCR as described previously (29).

**Cell culture**

Thymic Treg subsets were stimulated with anti-CD3/anti-CD28–coated microbeads (Life Technologies) and 50 ng/ml recombinant mouse IL-2 (R&D Systems) for 5 d. In some experiments, 125–250 μM t-mimosine (Sigma-Aldrich) was added.

**DNA immunoprecipitation**

Genomic DNA was prepared using the DNA/RNA AllPrep Kit (Qiagen) according to the manufacturer’s recommendations. DNA was sonicated to produce random fragments ranging in size from 200–1000 bp, and 1 μg sheared DNA was used for immunoprecipitation by overnight incubation with Abs against 5-hydroxymethylcytosine (39769; Active Motif) in a final volume of 250 μl IP buffer (10 mM Na-phosphate [pH 7], 140 mM NaCl, and 0.05% Triton X-100) at 4°C. An appropriate aliquot of sheared DNA was kept as input control. Enrichment of Ab-bound DNA was performed using protein A/G-microbeads (Miltenyi Biotec), and magnetic separation was done on corresponding separation columns by several washing steps, the order as follows: low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris [pH 8.1], and 500 mM NaCl), high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris [pH 8.1] and 500 mM NaCl), LiCl buffer (25 mM LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid, 1 mM EDTA, and 10 mM Tris [pH 8]), and TE buffer (10 mM Tris and 1 mM EDTA). Afterwards, Ab-bound fragments were collected with hot elution buffer (1% SDS and 100 mM NaHCO3) and purified using the Nucleospin Extract II kit (Macherey-Nagel) according to the procedure recommended for the purification of sample containing SDS. The amount of immunoprecipitated DNA and input DNA was quantified by real-time PCR using SYBR Green (Invitrogen) with the following primer pair: TSDR forward: 5′-AACC- TTGGGCCCCCTTCGGCA-3′; and reverse: 5′-GGCGGGATGCTTGGGCTTCA-3′. Immunoprecipitated DNA was set in relation to input DNA using the following expression: enrichment = 2^-ΔΔCt = [CtDIP – CtInput]. For normalization, mean enrichment factor for the samples within one experiment was set in relation to the mean enrichment factor for all three experiments.

**Quantitative PCR**

RNA was isolated using the DNA/RNA AllPrep Kit (Qiagen). cDNA was produced from equal amounts of RNA using Superscript II reverse transcriptase (Invitrogen). For quantification, samples were amplified by real-time PCR using SYBR Green (Invitrogen). Relative mRNA expression was calculated by comparative threshold cycle method using hypoxanthine phosphoribosyltransferase for normalization. Primer pairs that were published previously were used for Tet1 and Tet3 (30) or Tet2 (31).

**DNA microarray hybridization and analysis**

RNA was isolated using the DNA/RNA AllPrep Kit (Qiagen), and quality and integrity of total RNA was controlled on Agilent Technologies 2100 Bioanalyzer (Agilent Technologies). A total of 500 ng total RNA was used for biotin labeling according to the 3′ I VT Express Kit (Affymetrix). A total of 7.5 μg biotinylated cRNA was fragmented and placed in hybridization mixture containing four biotinylated hybridization controls (BioB, BioC, BioD, and Cre) as recommended by the manufacturer. Samples were hybridized to an identical lot of Affymetrix GeneChip MOE 430 2.0 (Affymetrix) for 16 h at 45°C. Steps for washing and SA-PE staining were processed on the fluidics station 450 using the recommended FS450 protocol (Affymetrix). Image Analysis was performed on the GCS3000 Scanner and GCOS1.2 Software Suite (Affymetrix). Analysis of microarray data were performed using GeneSpring 11.5.1 (Agilent Technologies). Signal intensities (raw data) were log2 transformed and normalized using RNA. Gene expression values per condition are given as relative expression to mean expression value calculated from intensities of all conditions (mean centralization, normalized data). The entire microarray data set is available under Gene Expression Omnibus accession number GSE42021 (http://www.ncbi.nlm.nih.gov/geo/).

**Methylation analysis**

DNA methylation analysis was performed by bisulphite sequencing as described previously (20). Only cells from male mice were used for methylation analyses.

**Results**

**Partial TSDR demethylation in thymic Tregs**

The TSDR contains 14 CpG motifs (Fig. 1A), which are fully demethylated in Tregs isolated from secondary lymphoid organs (20), whereas CD4SP Foxp3+ thymocytes show only partial demethylation at the TSDR (32). We first confirmed this phenotype by genomic and quantitative PCR analysis of samples containing SDS. The amount of immunoprecipitated DNA and input DNA was quantified by real-time PCR using SYBR Green (Invitrogen) with the following primer pair: TSDR forward: 5′-AACC-TTGGGCCCCTTCGGCA-3′; and reverse: 5′-GGCGGGATGCTTGGGCTTCA-3′. Immunoprecipitated DNA was set in relation to input DNA using the following expression: enrichment = 2^-ΔΔCt = [CtDIP – CtInput]. For normalization, mean enrichment factor for the samples within one experiment was set in relation to the mean enrichment factor for all three experiments.

### Partial TSDR demethylation in thymic Tregs

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TSDR demethylation is initiated during thymic Treg development

Activated peripheral T cells are to a low extent capable of recirculating to the thymus (34, 35); hence the possibility was given that TSDR methylation analysis in thymic Foxp3+ Treg subsets was blurred by recirculating peripheral Foxp3+ Tregs. In adoptive transfer studies, we identified essentially no donor-derived Foxp3+ Tregs within thymi of lymphocompetent recipients (data not shown), indicating that recirculation of peripheral Foxp3+ Tregs is very limited. Nevertheless, to formally exclude that recirculating Tregs of peripheral origin were responsible for the partial TSDR demethylation in thymi of untreated mice, we investigated the TSDR methylation status of developing Tregs in fetal thymic organ cultures (FTOCs), where first Foxp3+ cells were observed on day 4 of culture (Supplemental Fig. 1A), corresponding to postnatal days 1 to 2 (36). We used FTOCs from Foxp3GFP fetuses to purify Tregs on day 10 of culture and found that these FTOC-derived Foxp3GFP+ Tregs had a partially demethylated TSDR, akin to their in vivo counterparts, whereas Foxp3GFP− cells isolated from FTOCs displayed a fully methylated TSDR (Fig. 2A). These results showed that the thymic microenvironment is sufficient to trigger TSDR demethylation in developing Tregs.

In a second approach we used an in vivo model of inducible T cell development, Indu-Rag1fl/fl mice, in which inversion of the floxed coding sequence of the Rag1 gene causes lymphopenia (25). In a background that ubiquitously expresses a tamoxifen-inducible Cre recombinase, T cell development can be induced at a given time by tamoxifen administration (Supplemental Fig. 2A). This model allows the interrogation of TSDR demethylation in developing thymic Foxp3+ Tregs without running the risk of including peripheral contaminants to the analysis. Following tamoxifen administration, thymi of Indu-Rag1fl/fl mice were repopulated with T cells (Supplemental Fig. 2B). At day 14, when Foxp3+ Tregs were readily detectable in the thymus but hardly any Foxp3+ Tregs were present in peripheral lymphoid organs (Supplemental Fig. 2B, 2C), we purified CD4SP CD25hi cells from thymi of Indu-Rag1fl/fl mice (Fig. 2B). Foxp3 protein expression in 70–90% of sorted cells was confirmed by intracellular staining (Supplemental Fig. 2D). TSDR demethylation was clearly evident in CD4SP CD25hi cells from both Indu-Rag1fl/fl and wild-type mice, although not as pronounced as in steady-state wild-type thymi (Fig. 2B). In contrast, CD4SP CD25hi cells from both Indu-Rag1fl/fl and wild-type mice displayed a fully methylated TSDR.

We next analyzed the TSDR methylation status in recent thymic emigrants (RTEs) by using RAG1GFP mice (27), in which RTEs can be identified in peripheral lymphoid organs as GFPlo cells, due to delayed degradation of GFP protein after cessation of Rag1 gene expression. RAG1GFP mice were crossed to Foxp3hCD2 reporter mice (26). TSDR methylation analysis of peripheral CD4+hCD2+CD25hiGFPlo RTEs revealed complete demethylation (Fig. 2C), similar to CD4+hCD2+CD25GFP+ mature peripheral Tregs. In the RAG2GFP mouse line that also allows tracking of RTEs (28), we observed only slight (<25%) methylation of the TSDR in CD4+hCD2+CD25GFP+ RTEs (data not shown), further supporting the notion that Tregs bear epigenetic marks that confer lineage identity at the time of thymic emigration. In summary, these results demonstrated that TSDR demethylation in Foxp3+ Tregs is initiated during their development in the thymus, and progressive demethylation occurs during maturation of these thymocytes.

Commitment to a stable Treg lineage during early stages of Treg development

We next investigated at which developmental stage commitment to a stable Foxp3-expressing phenotype takes place by assessing
the stability of Foxp3 expression in thymic Foxp3+ Treg subsets of different maturity. Interestingly, even the most immature CD24hi subset among thymic Tregs fully maintained Foxp3 expression upon in vitro stimulation, despite a substantially methylated TSDR (Fig. 3A), and stability of Foxp3 protein expression was accompanied by complete TSDR demethylation during the 5-d culture. In fact, all thymic Treg subsets maintained Foxp3 expression (Fig. 3B), and near-to-complete demethylation was observed in all subsets after in vitro stimulation (Fig. 3C). These findings suggest that commitment to a stable Treg lineage is established during an early stage of thymic Treg development and consists of differentiation programs that initiate Foxp3 expression and subsequently induce TSDR demethylation. We compared gene expression profiles of Foxp3+ Treg subsets of different maturity and could identify a set of genes that were specifically up- or downregulated in Foxp3+ Tregs, but not in Foxp3- conventional T cells, in a maturation-dependent manner (Fig. 3D). Genes that showed the most pronounced differential regulation during maturation were Axl, Bcl2l15, Cxcr5, Il1r2, and Prr5l. Well-known Treg-associated genes such as Socs2, Ebi3, and Ccr6 were also upregulated by thymic Tregs during maturation (Fig. 3D, Supplemental Table I). This finding indicates that Tregs consolidate their unique phenotype in the thymus after Foxp3 expression is initiated by establishment of their specific gene expression profile and functional maturation. Our results suggested that TSDR demethylation is part of this overall consolidation program that is initiated during early stages of Treg development.

We next asked whether thymic Foxp3+CD25+ Treg precursors (16, 17) already have the potential to become stable Foxp3+ Tregs. Thymic CD4SP Foxp3GFP+CD25+ cells displayed a fully methylated TSDR (Fig. 4A). However, when stimulated in vitro via the TCR in presence of IL-2, part of these cells became Foxp3GFP+, and partial TSDR demethylation was observed at the end of the 5-d-culture (Fig. 4B). The two-step model of thymic Treg development suggests that Foxp3+CD25+ Treg precursors only need cytokine signaling by IL-2 to mature into Foxp3+ Tregs (16, 17). We therefore tested whether IL-2 signaling alone would also suffice to induce TSDR demethylation in Treg precursors. When cultured with IL-2 only, 30–40% of Treg precursors became Foxp3GFP+, and mild TSDR demethylation was observed (Fig. 4C). Concomitantly with Foxp3 upregulation, Treg precursors responded to IL-2 by substantial proliferation. However, the percentage of Foxp3GFP+ cells and the extent of TSDR demethylation were similar when Treg precursors were cultured with IL-15, despite a drastically reduced cell-division rate (Fig. 4C). Foxp3GFP+CD25+ Treg precursors also displayed a set of differentially expressed genes compared with conventional Foxp3GFP+CD4SP thymocytes (Fig. 4D, Supplemental Table II), and this differential expression pattern persisted in later Treg differentiation stages (Fig. 4E), indicating that Foxp3-independent components of the Treg differentiation program are already triggered in precursors. Collectively, our results suggest that thymic Treg precursors are already primed by the thymic microenvironment to differentiate into stable Foxp3+ Tregs with a demethylated TSDR along with acquisition of Treg-specific expression programs; and demethylation can be accomplished, albeit less efficiently, in the absence of additional TCR signals.

**The TSDR is demethylated in an active fashion in thymic Tregs**

DNA demethylation mostly occurs in a passive manner by impaired transfer of methyl groups to newly synthesized, unmethylated DNA strands, and thus depends on cell division. Increasing evidence is now available showing that active (i.e., cell-division–independent) DNA demethylation, initially described in plants, also occurs in mammalian cells (37). It was therefore of interest whether cell division was necessary for TSDR demethylation. To this end, we
Stable Foxp3 expression is imprinted during thymic Treg development. (A) Thymic CD4SP Foxp3+CD24hi immature Tregs were purified from Foxp3GFP mice and stimulated with anti-CD3/anti-CD28-coated beads. After 5 d, stability of Foxp3 expression was analyzed by flow cytometry (top panels). TSDR methylation analyses were performed on genomic DNA of ex vivo–isolated as well as stimulated cells (bottom panels). Percentage of methylation is color-coded according to the scale; each horizontal bar represents an individual CpG motif. Percentages indicate average methylation within the TSDR. (B) CD4SP Foxp3GFP+ Tregs were sorted into CD24hi, CD24int, and CD24lo subsets. Sorted cells were stimulated as in (A), and stability of Foxp3 expression was analyzed after 5 d of culture. Pooled data from five independent experiments (n = 5) are shown. (C) TSDR methylation analysis on genomic DNA of ex vivo isolated CD24hi, CD24int, and CD24lo thymic Foxp3GFP+ Treg subsets or after a 5-d in vitro stimulation of the subsets as described in (A). Pooled data from three independent experiments (n = 5) are shown. (D) Gene expression profiling was performed on FACS-purified GFP+ and GFP− CD24hi, CD24int, and CD24lo CD4SP thymocyte subsets from Foxp3GFP mice. Genes that are up- or downregulated in Foxp3GFP+ thymocytes during maturation, but are exempt from such regulation in Foxp3GFP− thymocytes, are displayed in the scatter plot. Only genes showing consistent regulation in three independent RNA microarrays are shown. The outer green lines depict a corridor of 2-fold change. Known Treg signature genes and genes showing highest differential expression are annotated.

FIGURE 3.

Stable Treg induction in the thymus

The family of Tet dioxygenases has recently been shown to catalyze oxidation of 5-methylcytosine (5mC) to 5hmC (30, 39). Enzymes of the base excision repair pathway can then excise 5hmC from the DNA and replace it by an unmethylated cytosine; hence 5hmC acts as an intermediate state during active DNA demethylation. Standard bisulphite sequencing cannot discriminate 5mC from 5hmC (40). Thus, we performed immunoprecipitation of genomic DNA from thymic Treg subsets by an Ab that specifically recognizes 5hmC to examine the relative abundance of 5hmC within the TSDR in developing thymic Tregs. All three thymic Foxp3GFP+ Treg subsets showed substantial enrichment of 5hmC within the TSDR compared with conventional Foxp3GFP−CD25−CD4SP as well as DP thymocytes (Fig. 6A). Notably, thymic Treg precursors (Foxp3GFP−CD25+) and mature Foxp3GFP+CD25hi Tregs from peripheral lymphoid organs did not show enrichment of 5hmC within the TSDR over conventional T cells (Fig. 6A), further supporting the notion that TSDR demethylation is initiated and to a great extent finalized during thymic Treg development.

Expression of all three Tet dioxygenases was detectable in thymic Foxp3+ Treg subsets, and expression of Tet2 and Tet3 was markedly elevated compared with conventional CD4SP thymocytes (Fig. 6B). Tet3 was expressed at much higher levels than Tet1 and Tet2 in all thymic cell populations analyzed, and highest levels of Tet3 expression were detected in CD24hi and CD24int Treg subsets. Hence, 5mC is likely converted to 5hmC by Tet dioxygenases in developing thymic Tregs. Taken together, these results strongly support an active mechanism of TSDR demethylation in developing thymic Tregs, which ensures long-term lineage stability and suppressive potential after thymic egress.

Discussion

Stable Foxp3 expression depends on selective DNA demethylation at the TSDR within the Foxp3 locus. The experiments presented in this study deliver a precise temporal delineation of TSDR demethylation in developing thymic Tregs: the demethylation machinery is triggered in Foxp3+ CD25+ Treg precursors, but demethylation only starts after Foxp3 protein is detectable. It then continues as...
part of the maturation program through which Tregs pass in the thymic medulla and is to a great extent completed at time of egress to the periphery. To draw this conclusion safely, we had to exclude the possibility that recirculating Tregs of peripheral origin contribute to the overall methylation levels we observed in thymocytes. By using several complementary approaches, we could provide clear evidence that recirculating peripheral cells do not significantly contribute to the methylation pattern of thymic Tregs, but rather thymic maturation drives progressive TSDR demethylation.

Somewhat surprisingly, immature CD24^hiFoxp3^+ thymic Tregs, which similar to in vitro TGF-β–induced Foxp3^+ Tregs (21) showed a largely methylated TSDR, almost fully maintained Foxp3 expression when stimulated in vitro, in stark contrast to TGF-β–induced Tregs. Hence, signals that confer stability to Tregs appear to be received at early stages of thymic Treg differentiation, possibly even as early as the Foxp3^+ precursor stage. TSDR demethylation is subsequently accomplished during the consolidation phase in an autopilot state and serves to imprint stable and long-term Foxp3 expression. The early signals are yet to be discovered, though it is tempting to speculate that signals that induce Foxp3 expression at the same time initiate Foxp3-independent pathways of Treg differentiation, including TSDR demethylation (23, 32).

Regulation of DNA demethylation in immune cells mostly has been found to be associated with proliferation [except for a few reports, such as demethylation of the IL-2 gene (41, 42)] and hence has been considered as passive demethylation. Intriguingly, our data show that TSDR demethylation in the thymus occurs independently of proliferation, as CD4SP Foxp3^+ Tregs hardly divide during maturation, and inhibitors of mitosis do not prevent demethylation during in vitro cultures, implying an active mechanism of TSDR demethylation.

It has recently been discovered that 5mC can be modified to 5hmC by Tet family enzymes (30, 39). 5hmC can either iteratively be further oxidized to 5-formylcytosine and 5-carboxylcytosine by Tet enzymes or deaminated to 5-hydroxymethyluracil by AID/APOBEC deaminases (43). 5-carboxylcytosine and 5-hydroxymethyluracil, in turn, are recognized and excised by DNA glycosylases like TDG and SMUG1 (44–47), creating an abasic site that is filled with an unmethylated cytosine by further components of the base excision repair pathway. Alternatively, 5-carboxylcytosine may be excised by a hitherto unknown putative decarboxylase to directly yield unmethylated cytosine (43). Hence, conversion of 5mC to 5hmC by Tet enzymes is a key event that can initiate several mechanisms of DNA demethylation. Recent studies have shown that self-renewal of hematopoietic stem cells as well as their differentiation into myeloid cells is perturbed in mice lacking Tet2, concomitant with altered genomic levels of 5hmC (48, 49). We now demonstrate for the first time, to our knowledge, the presence of 5hmC at a specific gene locus during a defined stage of T cell differentiation. Whether one of the three
Tet enzymes play a predominant role for conversion of 5mC to 5hmC in thymic Foxp3+ Tregs or whether they act in a redundant manner remains to be clarified. At present, very little is known about how expression of Tet enzymes is regulated. It is therefore a highly challenging question, which signals lead to induction of Tet dioxygenases and their specific recruitment to the Foxp3 locus in developing thymic Tregs.

Signals that initiate functional differentiation of thymic Tregs are better known. Upregulation of CD25 upon TCR ligation is the first step of Treg differentiation in CD4SP thymocytes (16). Because first signs of TSDR demethylation are already observed in Foxp3+CD25+ Treg precursors upon exposure to IL-2, one might consider IL-2, or other cytokines that signal through common γ subunit–containing receptors, as being one of the signals involved in

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** TSDR demethylation occurs in absence of cell division. (A) Thymic CD4SP GFP+ Tregs were sorted from Foxp3GFP mice. Left panels show postsort purity and ex vivo TSDR methylation analysis. Sorted cells were stimulated with anti-CD3/anti-CD28–coated microbeads in the presence or absence of the late G1-phase inhibitor L-mimosine. After 5 d, TSDR methylation status was analyzed by bisulphite sequencing. Percentage of methylation is color-coded according to the scale; each horizontal bar represents an individual CpG motif. Numbers indicate average demethylation within the TSDR. (B) Summary TSDR methylation analyses performed as in (A) from three independent experiments (n = 5). (C and E) BALB/c mice received two i.p. injections of 1 mg BrdU. Nine hours after the first and 3 h after the second injection, BrdU incorporation of thymocyte subsets was analyzed by flow cytometry. Histograms are gated on indicated subsets, and numbers indicate the percentage of BrdU+ cells. (E) Summary of three mice. (D and F) Ki-67 staining of thymocyte subsets. Histograms are gated on indicated subsets and numbers indicate percentage of Ki-67+ cells. Shaded histograms represent isotype controls. (F) Summary of five mice. Results are representative for two independent experiments. DP, Double-positive.

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** 5hmC is enriched in thymic Treg subsets. (A) Indicated cell populations were purified from thymi or pooled spleens and lymph nodes (LN) of Foxp3GFP mice. Genomic DNA was extracted from sorted cells and subjected to DNA immunoprecipitation with an anti-5hmC Ab. Quantitative PCR was performed on precipitated DNA with TSDR-specific oligonucleotides. Pooled data from three independent experiments (n = 7) are shown. (B) Total RNA was extracted from indicated cell populations and quantitative RT-PCR was performed to assess the expression levels of Tet1, Tet2, and Tet3. Pooled data from three independent experiments (n = 7) are shown. DP, Double-positive; HPRT, hypoxanthine phosphoribosyltransferase.
driving epigenetic fixation of Foxp3 expression. IL-2 signals have been recently suggested to contribute to TSDR demethylation in vitro–generated Tregs after their adoptive transfer (50). Nevertheless, it appears unlikely that IL-2 alone is sufficient to induce full TSDR demethylation, as only very mild epigenetic changes were observed when Foxp3/CΔ25 Treg precursors were cultured with IL-2 in the absence of TCR signaling.

In this study, we present evidence that commitment to the Treg lineage takes place in the thymus through induction of stable Foxp3 expression that is consolidated through demethylation at the Foxp3 locus to catalyze hydroxylation of 5mC as well as the induction of Tet enzymes and their recruitment to the system or other somatic tissues. The clarification of signals that epigenetic imprinting of lineage identity in cells of the immune system or other somatic tissues. The clarification of signals that induce expression of Tet enzymes and their recruitment to the Foxp3 locus to catalyze hydroxylation of 5mC as well as the factors that further process 5mC to yield demethylated CpG motifs warrant intensified research. It can be expected that identification of these factors will pave the way for improved strategies to generate and expand Tregs for therapeutic applications, for which the lack of stability is still a major concern.

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Disclosures

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References


