Naïve T cells can be induced to differentiate into Foxp3+ regulatory T cells (iTregs) upon suboptimal T cell receptor (TCR) stimulus or TCR stimulus in conjunction with TGF-β signaling; however, we do not fully understand how these signals coordinately control foxp3 expression. Here, we show that strong TCR activation, in terms of both duration and ligand affinity, causes the accumulation of DNA (cytosine-5)-methyltransferase 1 (DNMT1) and DNMT3b and their specific enrichment at the foxp3 locus, which leads to increased CpG methylation and inhibits foxp3 transcription. During this process the augmentation of DNMT1 is regulated through at least two post-transcriptional mechanisms; that is, strong TCR signal inactivates GSK3β to rescue DNMT1 protein from proteasomal degradation, and strong TCR signal suppresses miR-148a to derepress DNMT1 mRNA translation. Meanwhile, TGF-β signaling antagonizes DNMT1 accumulation via activation of p38 MAP kinase. Thus, independent of transcription factor activation, TCR and TGF-β signals converge on DNMT1 to modulate the expression of foxp3 epigenetically, which marks mother cell iTreg lineage choice within the genome of differentiating daughter cells.

Recognition of a peptide major histocompatibility complex (pMHC)2 displayed on the surface of antigen-presenting cells by a specific T cell receptor (TCR) initiates the T cell response. Upon pMHC-TCR engagement, coordinated downstream signaling cascades promote naïve CD4 T cells to undergo massive expansion and differentiation into distinct T helper (Th) subsets, such as Th1, Th2, Th17, and inducible regulatory T cells (iTreg) (1). Although the requirement for TCR signals in lineage commitment is universal, accumulating evidence indicates that, besides varying cytokine environments, differences in the strength of TCR signaling can also have a tremendous impact on CD4 T cell fate determination. This was initially discovered by Bottomly and co-workers (3, 4) and further confirmed by others (2); in general, weak TCR signals are thought to bias T cells toward the Th2 lineage, whereas strong TCR signals facilitate the formation of the Th1 subset. Recently, it was shown that the differentiation of Th17 cells could also be promoted by weak TCR activation (5). However, the molecular mechanism governing this fate determination remains largely unknown.

In addition to effector Th cells, TCR signal strength influences the differentiation of CD4+ Foxp3+ regulatory T cells (Tregs). Tregs are suppressor T cells that play a dominant role in the maintenance of peripheral tolerance and immune homeostasis (6). These cells express the master transcription factor Foxp3, which is essential for their differentiation, maintenance, and suppressive function (7–10). Mutation of the foxp3 gene in humans and mice results in lymphoproliferative disease that leads to severe inflammation in multiple organs and tissues (11, 12). Based on their origin of development, Tregs have been categorized into two types: thymic natural Tregs (nTregs) generated after thymocyte selection and peripheral inducible Tregs (iTregs) derived from CD4+ CD25+ conventional naïve T cells.
TCR Signal Regulates DNMT to Control foxp3 Methylation

(13). nTregs and iTregs share several common mechanisms in terms of their development and differentiation, such as their reliance on TCR, IL-2, and TGF-β signaling. TCR stimulation leads to the activation of various transcription factors including nuclear factor of activated T cells (NFAT) (14), activator protein 1 (AP1) (14), cAMP response element-binding (CREB) (15), and nuclear factor (NF)-κB (16), all of which have been shown to bind to the foxp3 locus directly and regulate its transcription. Paradoxically, this NFAT-AP1-NFκB panel is also fully or partially employed for effector T cell proliferation as well as the expression of lineage specific cytokines, cytokine receptors, and master transcription factors that control Th1 differentiation (1). How, then, do T cells determine whether they express Foxp3? In a conventional view, this problem is solved by TGF-β signaling, which provides a unique transcription factor, Smad3, as a crucial addition to the NFAT-AP1-NFκB panel in guiding T cell lineage decision (17). However, in the presence of TGF-β, Smad3-deficient T cells only displayed a 50% reduction in iTreg differentiation (18). Thus the currently known transcriptional machinery is inadequate to explain how naïve T cells commit to the iTreg versus Th lineage.

In addition to the regulation of foxp3 by well documented transcription factors, recent studies showed that foxp3 transcription is also regulated by epigenetic mechanisms (19, 20). It was shown that both the promoter and conserved non-coding sequence 1 (CNS1) of the foxp3 gene are more accessible in Tregs than in conventional effector T cells, as indicated by increased local histone acetylation in Tregs. Besides histone modifications, foxp3 expression is also directly regulated at the DNA level by CpG methylation. The CpG islands within the promoter region of foxp3 were almost completely demethylated in nTregs, whereas those in conventional effector T cells showed partial methylation (15, 21). In the foxp3 CNS2 region, the difference in methylation is even more striking; it was fully demethylated in nTregs but completely methylated in effector T cells (15, 22). Interestingly, and consistent with their transient and unstable Foxp3 expression, iTregs had foxp3 CpG islands that were only partially demethylated in the CNS2 region (15). Experiments using inhibitors to block methylation showed that changes in CpG methylation motifs did affect transcription factor binding and foxp3 expression in antigen-stimulated conventional T cells. However, it was not clear how this methylation is regulated during the iTreg differentiation process.

Here, we show that strong TCR signaling, which is elicited by high affinity ligand or by extended ligand exposure, inhibits foxp3 expression in conventional T cells at the epigenetic level. This is coordinately achieved by (i) PLCγ- and PI3K-dependent signaling downstream of TCR, which blocks the GSK3β-dependent, proteasome-mediated degradation of DNMT1 protein and (ii) by dampening miR-148a, the microRNA (miRNA) that targets DNMT1 mRNA. DNMT1 together with DNMT3b is then able to methylate and suppress the foxp3 locus. Meanwhile, TGF-β directly antagonizes these TCR signals by promoting drastic down-regulation of DNMT1 via activation of p38. Thus, DNMT1 represents a crucial node where TCR and TGFβ signals converge to control iTreg fate.

EXPERIMENTAL PROCEDURES

Mice—All animal work was conducted according to protocols approved by the Institutional Animal Care and Use Committee at Duke University. 5C.C7 TCR transgenic mice were from Taconic (B10.A Rag2tm1Fwa H2-T18a Tg (Tcra5CC7, Tcrlb5CC7)Ijwep). WT B10.A mice were also from Taconic. Wild type C57BL/6 mice were from The Jackson Laboratory. Foxp3-GFP-Cre BAC transgenic mice were kindly provided by Dr. Xiaoping Zhong from the Duke University Medical Center.

T Cell Activation and Differentiation—2 × 10^6 sorted CD4+CD25−LN T cells from 5C.C7 TCR transgenic mice were labeled with 10 μM carboxyfluorescein succinimidyl ester (CFSE) and stimulated by 2 × 10^6 T cell-depleted syngeneic splenocytes loaded with peptides MCC (1 μM) or 102S (1 μM). Anti-I-Ek antibody (14.4.4), anti-I-Ek-MCC (D4), or small molecule inhibitors that block specific pathways were added at different time points. The percentages of CD4+Foxp3+ T cells were analyzed by intracellular staining with eBioscience Foxp3/Transcription Factor Staining Buffer Set (catalog #00-5523-00) and flow cytometry at 72 h. 14.4.4 and D4 antibodies were purified from hybridoma culture supernatant and used at a concentration of 20 μg/ml. LY 294002 (catalog #440202), rapamycin (catalog #553210), PIK-75 (catalog #528110), cyclosporin A (catalog #239835), IκB kinase inhibitor III (catalog #401480), MG-132 (catalog #474790), SB-216763 (catalog #361566), ERK inhibitor II (catalog #328007), INK inhibitor II (catalog #420119), p38 MAP kinase inhibitor III (catalog #506121) were purchased from EMD Biosciences. U-73122 (catalog #U6756) was purchased from Sigma. Recombinant human TGF-β1 and IL-2 were purchased from Peprotech.

Quantitative PCR and Western Blot—Total RNA was isolated with the miRNA extraction kit (Ambion, catalog #AM1561) according to the manufacturer’s instructions. Reverse transcription was performed with qScript™ cDNA SuperMix (Quanta Biosciences, catalog #95048) or qScript™ Flex cDNA kit (Quanta Biosciences, catalog #95049). Gene expression was quantified by SYBR Green-based quantitative PCR analysis. Western blot was performed according to standard protocols with the following primary antibodies: DNMT1 (D63A6) XP® rabbit mAb (Cell signaling, catalog #5032), DNMT3b rabbit polyclonal antibody (Abgent, catalog #Ab1035a), goat anti-β-actin antibody (Sigma). Anti-rabbit-Alexa680 and anti-goat-Alexa680 (Invitrogen) were used as secondary antibodies, and fluorescence intensity was measured on an Odyssey system (Licor).

Intracellular Staining and Fluorescence Microscopy—for flow cytometry analysis, cells were fixed with 2% paraformaldehyde in PBS, permeabilized with 90% methanol in PBS, and stained with anti-DNMT1 mAb, anti-phospho-p44/42 MAPK (Erk1/2) at Thr-202 and Tyr-204, anti-phospho-Akt at Thr-308 (Cell Signaling), or isotype control. A Pacific Blue goat anti-rabbit antibody was used as secondary antibody, and the expression of DNMT1 at the single cell level was measured by flow cytometry. Gating of the DNMT1^+ cells was based on the staining with isotype control antibodies. For imaging of DNMT1 protein in cells, 5C.C7 T cells that were stimulated with different peptides for defined durations were fixed with 4%
paraformaldehyde on coverslips, permeabilized with 0.5% Triton X-100 in PBS, and stained with anti-DNMT1 mAb (Cell Signaling). A Cy3 donkey anti-rabbit antibody was used as secondary antibody for fluorescence microscopy. Imaging was performed on a Zeiss Axiomvert-100TV station equipped with a Zeiss 40x EC Plan-Neofluor objective lens (NA = 1.30), a CoolSNAPHQ CCD camera (Roper Scientific), and a high speed piezzo Z-motor for Z stack recording as described previously (23).

DNA Methylation Analysis and Chromatin Immunoprecipitation—Genomic DNA was purified with GenElute™ Mammalian Genomic DNA Miniprep kit (Sigma, catalog G1N79). Methylation analysis was quantified by sequencing of genomic DNA after bisulfite conversion using the MethylDetect kit (Active Motif), PCR amplification, and cloning. Chromatin immunoprecipitation was done based on a standard protocol with rabbit anti-DNMT1 (H300) antibody, mouse anti-DNMT3b mAb (52A1018) (Santa Cruz Biotechnology), or a nonspecific rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories).

Statistical Analysis—Unpaired two-tailed t tests were utilized to determine whether the difference between a given set of means was statistically significant. Differences with p values of less than 0.05 were considered statistically significant.

RESULTS

iTreg Differentiation Is Controlled by Both Strength and Duration of TCR Signaling through the PI3K-Akt-mTOR and PLC Pathways—While dissecting the functions of the microRNAs within the miR-17–92 cluster, we identified miR-19b as an inhibitor of iTreg differentiation. Further mechanistic analysis indicated that this was mediated through inhibition of PTEN expression and the consequent prolonged PI3K activation upon TCR/CD28 signaling (23). Abbreviated anti-CD3/CD28 stimulation facilitates in vitro iTreg differentiation (24), and previous adoptive transfer studies have shown that low doses of antigen and lack of costimulation favor induction of iTregs in vivo (25).

Therefore, we hypothesized that iTreg lineage differentiation would be determined by the integrated strength of TCR signaling based on both pMHC ligand affinity and the duration of ligand availability. To parse out the impacts of ligand affinity and stimulation duration in regulating iTreg differentiation, we utilized CD4 T cells from 5C.C7 TCR transgenic mice, in which every primary T cell carries a unique TCR (26) recognizing a range of biochemically and biophysically well characterized natural and synthetic variants of the moth cytochrome C (88–103) peptide in the context of the MHC II molecule I-Ek (27). To determine the role of ligand strength in regulating iTreg differentiation, we stimulated sorted CD4+CD25−5C.C7 T cells transgenic mice with our hypothesis, a minimal percentage of T cells up-regulated Foxp3 when stimulated for a prolonged period (72 h) regardless of whether a strong or weak antigenic peptide was used. However, when cells were stimulated with the weak ago-
nist 102S for a shorter period of time (6 or 18 h), we observed a substantial frequency of iTreg conversion (Fig. 1, B and D). With both 6 and 18 h stimulation, this elevation of Foxp3 expression occurred at the transcript level (Fig. 1C). In contrast, even with the shortest tested duration of stimulation, MCC was unable to induce Foxp3 expression (Fig. 1, B and D). These data indicate that although brief exposure to weak TCR signaling is required for Foxp3 induction, extensive signaling generated from high affinity ligands and/or a longer duration of antigen exposure actually inhibits foxp3 expression and iTreg differentiation.

We further evaluated the impact of TCR signaling strength and duration in regulating iTreg differentiation with CD4 T cells from wild type C57BL/6 mice upon anti-CD3 and anti-CD28 antibody stimulation. As expected, when sorted CD4+CD25− T cells were stimulated for 72 h, very few Foxp3+ cells were generated. In contrast, when cells were activated for 18 h and then maintained without TCR stimulation for additional 54 h, a substantial fraction of cells differentiated into Tregs (Fig. 1E). Furthermore, reducing the concentration of anti-CD3 antibody further enhanced Foxp3 induction and iTreg differentiation (Fig. 1E). These data confirmed that both TCR signaling strength and duration contribute to the negative regulation of iTreg differentiation by extensive TCR stimulation.

Previous studies have suggested an inverse correlation of cell division and iTreg differentiation after adoptive transfer of CD4+CD25− cells (25). Because T cells stimulated with 102S for 18 h proliferated significantly less than those activated either for a longer period of time (72 h) or with a stronger agonist (MCC for 18 h) (supplemental Fig. S1A), we explored whether the weaker proliferative response could account for improved iTreg conversion in the briefly 102S-stimulated cells. Using a CFSE label to distinguish cell generations, we observed that T cells that had undergone fewer divisions did in fact convert into Foxp3+ cells more efficiently (supplemental Fig. S1B). However, our results also showed that even when considering T cells that had undergone the same number of divisions, brief 102S stimulation conditions produced a higher frequency of Foxp3+ cells; that is, 18 h of 102S stimulation induced 15-fold more Foxp3+ cells than 18 h of MCC stimulation even when considering only singly divided cells. Most importantly, 4-fold more Foxp3+ cells were generated among undivided cells (Fig. 1B and supplemental Fig. S1B). Thus, we reasoned that there are cell cycle-independent mechanisms that inhibit Foxp3 expression and iTreg differentiation under the circumstance of extensive TCR signaling.

Although we do not know precisely how TCR signal strength is translated into cell fate decisions, several previous studies indicate that the PI3K-Akt-mTOR axis downstream of TCR activation might be critical. Blocking of this pathway with LY294002 or rapamycin after 18 h of TCR stimulation resulted in robust Foxp3 induction in vitro (24). In addition, expression of a constitutively active form of Akt in T cells diminished Foxp3 expression in peripheral T cells both in vitro and in vivo, suggesting that the activation of the PI3K-Akt-mTOR axis could contribute to the negative regulation of iTreg differentiation by strong TCR signaling (30). To determine key signaling events preventing foxp3 expression upon extensive antigen stimulation, we repeated our iTreg induction experiments in the presence of a series of small pharmaceutical inhibitors to block specific pathways downstream of TCR activation. We first chose the regime of extended weak TCR stimulus (72 h 102S), which normally results in very little iTreg induction. In this setting, consistent with previous findings that prolonged PI3K-Akt-mTOR activation inhibits iTreg differentiation, we detected a substantial increase in iTreg conversion when cells were treated with LY294002, a small inhibitor that blocks both PI3 kinase and mTOR activity (Fig. 2A). We further dissected these two pathways by treating cells with PIK-75, which specifically inhibits the PI10α and PI110γ subunits of PI3K at the dose used, and with rapamycin, which inhibits mTOR specifically (Fig. 2A). We noted that both inhibitors could significantly potentiate iTreg differentiation. Meanwhile, despite having a dramatic impact on T cell proliferation (31), inhibition of calciunin-NFAT signaling, NFκB function, or ERK activation had a minimal effect on Foxp3 induction (Fig. 2A). Interestingly, we found that U-73122, a specific inhibitor of the PLCγ-dependent hydrolysis of phosphatidylinositol diphosphate to phosphatidylinositol trisphosphate, could also enhance iTreg conversion to a similar extent as PI3K-mTOR inhibition (Fig. 2A).

We next investigated whether inhibition of the PI3K-Akt-mTOR or ZAP70-PLCγ pathway can induce iTregs when TCRs are engaged with strong agonist. As expected, in comparison to 102S peptide, MCC induced stronger Akt activation and calcium signaling as well as ERK1/2 phosphorylation (supplemental Fig. S2). When we inhibited the PI3K and PLC pathway with specific inhibitors at 18 h after TCR engagement, cells stimulated with MCC expressed significantly less Foxp3 than those stimulated with 102S (Fig. 2, B and C). On the other hand, blocking ERK activation did not have any effects on iTreg conversion (Fig. 2, B and C). These data indicated that a short period (18 h) of strong PI3K and PLC activation with MCC peptide is sufficient to inhibit iTreg induction. Furthermore, it strongly suggested that both the PI3K-Akt-mTOR axis and the ZAP70-PLCγ pathway are specifically involved in the negative regulation of iTreg differentiation in response to extensive TCR signaling mediated by higher ligand affinity or prolonged duration.

**TCR Signaling Regulates CpG Methylation at the foxp3 Locus—**

During iTreg differentiation, foxp3 gene expression is driven by the activation of the transcription factors STAT5, Smad3, NFAT, AP1, CREB1, and NFκB (32), the latter four of which are collectively potentiated by strong and sustained TCR signaling. Paradoxically, in the absence of TGF-β, TCR signaling of this magnitude instead suppresses the transcription of foxp3. This apparent conundrum suggested that, in parallel with transcription factor activation, extensive TCR signaling must target a distinct regulatory mechanism. DNA methylation controls the accessibility of general and gene-specific transcription factors toward the regulatory regions of genes, and this has been demonstrated to be one of the central mechanisms controlling foxp3 transcription (19). We hypothesized that, during iTreg differentiation, differences in strength and duration of TCR signaling would result in differential DNA methylation within the foxp3 regulatory regions.
To examine this, we evaluated the methylation status of the foxp3 gene in different T cell populations from male 5C.C7 mice, including naive T cells, T cells activated with 102S or MCC for 6 h, and T cells activated with 102S peptide for 72 h. As we sought modifications that could explain Foxp3 induction before iTreg commitment, we analyzed the whole population (within which the highest ratio of differentiated iTregs is less than 20%) rather than purified iTreg cells under these various conditions. In agreement with previous reports (15, 21), we found that in naive CD4 T cells, CpG islands residing in the foxp3 promoter region were largely unmethylated (Fig. 3A). Although a short and weak stimulation did not alter the overall methylation pattern of the promoter, stronger stimulation in terms of duration and ligand affinity significantly elevated the promoter methylation pattern of the promoter, stronger stimulation in terms of duration and ligand affinity significantly elevated the promoter methylation (Fig. 3B). These data indicate that extensive TCR stimulation suppresses Foxp3 expression by prolonged TCR signaling (Fig. 3C). These data indicate that extensive TCR stimulation suppresses iTreg differentiation by enhancing CpG methylation in the foxp3 gene regulatory regions.

TCR Signaling Augments Levels of DNMT Proteins and Their Bindings to the foxp3 Locus—We next examined how TCR signaling controls DNA methylation within the foxp3 locus. As one of the major DNA methyltransferases in mammalian cells, DNMT1 was recently linked to the regulation of Foxp3 expression in T cells (34). In addition to its well known function in maintaining DNA methylation during cell proliferation, DNMT1 has also been shown to be associated with DNMT3 to induce de novo methylation in CpG islands (35) and silence genes in human cells (36). Because we observed a substantial change in DNA methylation in the foxp3 regulatory regions, we hypothesized that extensive TCR signaling modulates foxp3 gene methylation by controlling the level of DNMTs. Under various stimulatory conditions that we employed for iTreg induction, mRNA levels of DNMT1 (Fig. 4A) and DNMT3b (Fig. 4B) largely remained steady. However, at the protein level,
DNMT1 was slightly elevated with a short duration of 102S stimulation and was dramatically increased when this stimulation was prolonged (Fig. 4C). Similarly, DNMT3b protein was also significantly elevated with as short as 6 h of moderate TCR stimulation (Fig. 4D). Consistent with our hypothesis that DNMT1 mediates iTreg differentiation through interpretation of TCR signaling strength and duration, similar magnitudes of DNMT1 elevation were caused by prolonged stimulus with a weak agonist (102S), as by a shorter stimulation with a strong agonist (102S) and Foxp3 expression are inversely correlated under the permissive condition for iTreg conversion, we directly compared the transcription start site of the foxp3 gene. Open circles, unmethylated CpGs; filled circles, methylated CpGs. Data represent three independent experiments. C, 5C.C7 T cells were activated with 102S for 72 h while also being treated with 5-azacytidine (5-Aza) at 18 h post-stimulation. The percentage of CD4 Foxp3 T cells were analyzed by flow cytometry at 72 h after TCR activation. Data represent three independent experiments.

FIGURE 3. Strong TCR signaling enhances CpG methylation within the foxp3 locus. A and B, CD4 CD25 T cells from male 5C.C7 transgenic mice were stimulated as described in Fig. 1A. The methylation status of CpG islands within the foxp3 promoter (A) or foxp3 CNS2 (B) from these cells was determined by bisulfite sequencing analysis. Each row represents one DNA strand. The number on top indicates the position of CpGs relative to the transcription start site of the foxp3 gene. Open circles, unmethylated CpGs; filled circles, methylated CpGs. Data represent three independent experiments. C, 5C.C7 T cells were activated with 102S for 72 h while also being treated with 5-azacytidine (5-Aza) at 18 h post-stimulation. The percentage of CD4 Foxp3 T cells were analyzed by flow cytometry at 72 h after TCR activation. Data represent three independent experiments.

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protein levels; PI3K prohibits GSK3β-mediated phosphorylation of DNMT1 and thus protects it from ubiquitin-mediated proteasomal degradation (42). We examined whether this DNMT1 stabilization mechanism is exploited by TCR signaling. T cells were stimulated with 102S peptide antigen for 18 h and then treated with a specific proteasome inhibitor, MG-132 or SB-216763, a specific inhibitor of GSK3β activity (42). When these cells were analyzed at the 72-h end point, both inhibitor treatments partially but significantly enhanced the level of DNMT1 protein in response to weak TCR signaling (Fig. 6A).

Reciprocally, whereas blocking TCR engagement, PI3K activation, or PLC activity at the 18 h point led to a substantial Foxp3 induction, the addition of GSK3β inhibitor could partially diminish this effect (Fig. 6B). Overall, these data suggest that extensive TCR signaling stabilizes DNMT1 protein by inhibiting GSK3β-mediated phosphorylation and proteasomal degradation of DNMT1.

TCR Signaling Elevates DNMT1 Protein levels by Dampening Its MicroRNA Modulator, miR-148a—The fact that MG-132 and GSK3β inhibitor could only partially rescue the level of DNMT1 protein led us to speculate that there is another layer of control, possibly at the level of DNMT1 translation. miRNAs are small non-coding RNAs that regulate gene expression posttranscriptionally via a combination of mRNA degradation and/or translational repression, depending on the particular microRNA (43). Because DNMT1 transcript levels were not
altered in our T cells, we explored the possibility that TCR signaling could release miRNA-mediated translational inhibition of DNMT1 expression. Two members of the miR-148 family, miR-148a (44) and miR-152 (45), were previously suggested to be direct modulators of DNMT1 expression, and we verified this in our transgenic T cell system (supplemental Fig. S3A). Upon TCR engagement, expression levels of all three miRNAs within this family, miR-148a, miR-148b, and miR-152, were

FIGURE 5. Strong TCR signaling causes enhanced enrichment of DNMT1 and DNMT3b at the foxp3 locus. A, representative images show the nuclear localization of DNMT1. CD4+ CD25+ T cells from 5C.C7 transgenic mice were stimulated as described in Fig. 1A. The cells were then fixed with 4% paraformaldehyde on cover slips and stained for intracellular DNMT1. DAPI was used to label the nucleus. Data represent three independent experiments. B–F, shown is chromatin immunoprecipitation analysis for the enrichment of DNMT1 at foxp3 promoter (B), foxp3 CNS2 (C), and Ifng CNS-6 (D) or DNMT3b at foxp3 promoter (E) and foxp3 CNS2 (F) in 5C.C7 transgenic CD4+ CD25+ T cells that were left unstimulated (TCR-0h) or stimulated as in A. The amount of DNA immunoprecipitated by the DNMT1 or DNMT3b-specific antibody or a nonspecific control IgG antibody was quantified by quantitative PCR using primers specific for the indicated gene-regulatory regions and normalized to the input before immunoprecipitation. Data show the means ± S.E. from three independent experiments. ns, not significant.
suppressed (Fig. 5C). When ectopically expressed using a ret-
roviral tool during T cell activation, all three failed to suppress
DNMT1 mRNA levels (supplemental Fig. S3B). However, one
of the three, miR-148a, significantly suppressed DNMT1
expression at the protein level (Fig. 6D). Furthermore, CD4 T
cells that forcibly overexpressed miR-148a enhanced their
Foxp3 induction significantly (Fig. 6, E and F). This suggested
that dampening of miR-148a expression is a complimentary
pathway that contributes to TCR-mediated epigenetic regulation of the foxp3 gene.

**TGF-β Antagonizes TCR Signaling by Targeting DNMT1 for Degradation via p38 Activation**—In addition to TCR signal strength, TGF-β signaling also strongly modulates iTreg induction. Although 72 h of 102S stimulation normally leads to very few iTregs, TGF-β can exert a dominant effect that increases the proportion of iTregs substantially (Fig. 7A). It is known that TGF-β acts through its receptor complex to trigger the activation of Smad3 protein, which then translocates to the nucleus and promotes foxp3 transcription (17). However, this classical pathway cannot explain how TGF-β overcomes methylation-mediated transcriptional silencing within the foxp3 locus under the circumstance of strong TCR signaling. We thus investigated the direct impact of TGF-β on epigenetic regulation of the foxp3 gene. When strong signals from both the TCR and TGFβ...
receptors were induced simultaneously, the TCR-mediated accumulation of DNMT1 protein was abolished (Fig. 7, A and B). The dampening of DNMT1 protein levels was also not related to the TGF-β inhibitory effects on T cell proliferation; a reduction of DNMT1 was observed in each successive generation when TGF-β was present (Fig. 7B). The regulation of DNMT1 by TGF-β mainly occurred at the protein level, as the DNMT1 mRNA level was not affected by TGF-β treatment (supplemental Fig. S4A). As could be expected from the reduced protein level of DNMT1, TGF-β treatment also resulted in reduced CpG methylation within both the promoter (Fig. 7C) and CNS2 region of foxp3 (supplemental Fig. S4B).

We also examined whether TGF-β signaling can effectively antagonize strong agonist-induced DNMT1 accumulation and iTreg differentiation. CD4 T cells stimulated with MCC alone for 72 h have more DNMT1 protein accumulated than those stimulated with 102S for 72 h (supplemental Fig. S5A). Similarly, in contrast to a relatively strong impact of TGF-β signaling on DNMT1 accumulation in 102S-stimulated cells, TGF-β could only moderately down-regulate the DNMT1 protein in cells stimulated with MCC (supplemental Fig. S5A). In agreement with this, when same concentrations of TGF-β were supplemented, MCC induced significantly less Foxp3+ cells (supplemental Fig. S5B). This suggested that excessive TCR signaling can antagonize TGF-β effects on iTreg differentiation.

In addition to Smad-mediated transcriptional regulation, TGF-β can also initiate alternative signaling via the Ras-ERK, TAK-MKK4-JNK, and TAK-MKK3–p38 pathways (46). During TCR stimulation of naive T cells, ERK activation is inhibited by TGF-β treatment (47), and this curtailed-ERK signaling failed to increase foxp3 expression (Fig. 2A). We thus investigated the potential roles of the other two MAPK pathways in potentially linking TGFβ receptors to DNMT1 using well established specific inhibitors. Whereas treatment with a specific JNK inhibitor had no effect, treatment with a p38 inhibitor completely abolished TGF-β-induced DNMT1 down-regulation, as shown by both the frequency of DNMT1+ cells and the intensity of DNMT1 staining at the single-cell level (Fig. 7D). Consistent with these increased DNMT1 protein levels, blockade of the p38 pathway also resulted in a significant reduction of Foxp3 induction by TGF-β (Fig. 7E). These data suggest that TGF-β signaling antagonizes the effect of TCR signaling on DNMT1 stabilization and foxp3 gene methylation through the activation of p38.

DISCUSSION

Because it was first appreciated that Tregs could be induced from naive T cell precursors (48), the combinatorial roles of strong TCR signaling and strong TGF-β receptor signaling have been well established as important determinants of foxp3 transcriptional activation (14–17). Here we have identified multiple pathways by which signaling through TCR and TGF-β receptor converge to control the protein level of DNMT1, an epigenetic modifier that we and others (34) have shown to strongly influence foxp3 locus accessibility and iTreg differentiation. Our data suggest that DNMT1-mediated methylation in foxp3 locus is likely aided by DNMT3b. Upon strong TCR stimulation, before strong DNMT1 elevation, a significant accumulation of DNMT3b protein were observed (Fig. 4, C and D). However, this cellular accumulation does not translate into increased DNMT3b occupancy within the foxp3 promoter region, which was only observed in cells receiving a prolonged stimulation (Fig. 5E). This suggests that there are additional factors required for the recruitment of DNMT3b to the foxp3 promoter. Previous studies showed that DNMT1 and DNMT3b co-localize and directly associate with each other through the N-terminal domain (35), which indicates that sufficient accumulation of DNMT1 triggered by a prolonged TCR stimulation may be required to recruit or anchor DNMT3b to the foxp3 locus. Therefore, our model suggests DNMT1 serves as the key modulator controlling transcriptional accessibility to the foxp3 regulatory regions.

One important function of DNMT1 is to maintain CpG methylation during DNA replication in the S-phase (49, 50), and accordingly, the transcription of DNMT1 is regulated in a cell-cycle dependent manner (51). We observed a gradual enrichment of DNMT1 protein with the progression of cell divisions (Figs. 4C and 7B) and a corresponding reduction of foxp3 expression in T cells from late generations (supplemental Fig. S1B). However, in the absence of TGF-β and under different stimulatory conditions, strong TCR signaling suppresses Foxp3 expression to a similar extent in every generation, including undivided cells and the first generation of daughter cells (supplemental Fig. S1B). This indicates that DNMT1 could begin to accumulate in response to TCR signals even before the earliest cell cycle, which is sufficient to eventually suppress foxp3 expression. In addition, under various conditions of antigen stimulation, we did not detect any change in the mRNA level of DNMT1. We cannot exclude the contribution of cell cycle-dependent transcriptional regulation (especially in late T cell generations) (32, 34), and the detailed mapping of GSK3β- and p38-targeted phosphorylation sites on DNMT1 is still ongoing. However, taken together, our results argue for molecular mechanisms that involve direct post-translational modification of DNMT1 downstream of TCR and TGF-β receptor signaling.

In its role as a signal integrator, we believe that DNMT1 represents the node where TCR-based self/non-self discrimination converges with environmentally cued danger signals. TCR signals mitigate miR-148a-mediated DNMT1 translation inhibition and also relieve GSK3β-mediated DNMT1 protein degradation via PI3K and PLC-γ signaling (Fig. 6). Because TCRs with high avidity for self-antigens are preferentially deleted or converted to nTregs in the thymus, stronger TCR signaling can be interpreted via higher DNMT1 levels as an indication of foreignness, which then favors foxp3 methylation. CD28 costimulation can provide an independent indication of foreignness, as its ligands are induced on antigen-presenting cells by signaling in response to microbial and viral products. CD28 signaling can then also feed into regulation of DNMT1 by enhancing TCR-induced PI3K activity, inhibiting GSK3β, stabilizing DNMT1, and further favoring foxp3 methylation. Meanwhile, naïve T cells maintain an only partially methylated foxp3 promoter, presumably because the level of DNMT1 attained by tonic/homeostatic TCR signaling is below the threshold needed for foxp3 methylation. Finally, healthy or
tumor tissues can exert influence on T cell priming by secreting TGF-β, which can act via p38 to antagonize strong TCR signaling by diminishing DNMT1 protein, impeding foxp3 methylation, and pushing the balance of immunity toward iTreg-mediated tolerance.

Whereas transcription factors such as NFAT can be activated within minutes and less than 1 h of TCR signaling is sufficient to drive T cells into the proliferative cycle (29), DNMT1 accumulates and methylates foxp3 over the course of days. This mechanistic and temporal segregation of epigenetic control from transcription factor-mediated control could allow T cells to gauge the duration of TCR signaling over long periods (which likely represent the persistence of antigen). Furthermore, by segregating the commitment to proliferation from the commitment to silence foxp3, T cells can make the decision for clonal expansion shortly upon antigen encounter but can integrate signals over the following days before finalizing their iTreg versus Th fate. This dichotomous commitment process may thus support the adaptive immune response dual requirement for rapidity of response on the one hand and accuracy of pathogen discrimination on the other. In line with this possibility, it is noteworthy that human naïve T cells transiently express Foxp3 during priming, even when their eventual fate is an effector Th lineage (52, 53). In the DNMT1-centric view, this would represent the early activity of NFAT/NFκB/AP1 on a naïve T cell partially methylated foxp3 promoter, which would only be completely repressed by DNMT1-mediated methylation after a much longer course of TCR signaling.

Overall, it is a well appreciated concept that T cells interpret subtle differences between antigens and between antigen contextual cues to enact their fate decision and that it is epigenetic modifications that enforce the heritage of differentiated T cells (54–57). Our data illustrate a mechanism whereby TCR signaling and environmental cues can target the epigenetic machinery directly to instruct differentiating T cells. Unlike most cell types, in which differentiation and proliferation are in general mutually exclusive, T cells acquire their identities in the midst of rapid proliferation. It would be difficult to imagine how T cells could employ transcription factor activation as a heritable mechanism; first, in the absence of ligands, the activation status of TCR (29) or transcription factors (e.g. NFAT (58)) can only be maintained within the range of minutes after ligand withdrawal and so would be unlikely to preserve their activation state between mother and daughter cells; second, the newly divided daughter cell makes brief contact with antigen-presenting cells in vivo (59), which likely gives them different antigen experience than their mother cells. In contrast, direct TCR-driven epigenetic reprogramming can mark mother cell antigen experiences in the genome during the commitment to cell division, which then keeps daughter T cells poised according to their mothers’ lineage choice. These features are also not likely to be exclusive to the iTreg lineage choice. The Th2 and Th17 lineages are also antagonized by strong TCR signal strength, and il-4 and il-17 are both expressed in a methylation-sensitive manner (2–5). Thus, upon TCR activation, it may be possible that DNMT1 or another epigenetic mechanism also controls master transcription factors or signature cytokines of Th2 and Th17 lineages in a manner analogous to foxp3.

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REFERENCES

1. Zhu, J., Yamane, H., and Paul, W. E. (2010) Differentiation of effector CD4+ T cell populations (*) Annu. Rev. Immunol. 28, 445–489
2. Yamane, H., Zhu, J., and Paul, W. E. (2005) Independent roles for IL-2 and GATA-3 in stimulating naïve CD4+ T cells to generate a Th2-inducing cytokine environment. J. Exp. Med. 202, 793–804
3. Tao, X., Grant, C., Constant, S., and Bottomly, K. (1997) Induction of IL-4–producing CD4+ T cells by antigenic peptides altered for TCR binding. J. Immunol. 158, 4237–4244
4. Constant, S., Pfeiffer, C., Woodard, A., Pasqualini, T., and Bottomly, K. (1995) Extent of T cell receptor ligation can determine the functional differentiation of naïve CD4+ T cells. J. Exp. Med. 182, 1591–1596
5. Purvis, H. A., Stoop, J. N., Mann, J., Kozijn, A. E., Hambleton, S., Robinson, J. H., Isaacs, J. D., Anderson, A. E., and Hilkens, C. M. (2010) Low-strength T-cell activation promotes Th17 responses. Blood 116, 4829–4837
6. Sakaguchi, S., Yamaguchi, T., Nomura, T., and Ono, M. (2008) Regulatory T cells and immune tolerance. Cell 133, 775–787
7. Fontenot, J. D., Gavin, M. A., and Rudensky, A. Y. (2003) Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat. Immunol. 4, 330–336
8. Hori, S., Nomura, T., and Sakaguchi, S. (2003) Control of regulatory T cell development by the transcription factor Foxp3. Science 299, 1057–1061
9. Khattri, R., Cox, T., Yasayko, S. A., and Ramsdell, F. (2003) An essential role for Scurfen in CD4+CD25+ regulatory T cells. Nat. Immunol. 4, 337–342
10. Williams, L. M., and Rudensky, A. Y. (2007) Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. Nat. Immunol. 8, 277–284
11. Bennett, C. L., Christie, J., Ramsdell, F., Brunkow, M. E., Ferguson, P. J., Whitewell, L., Kelly, T. E., Saulsbury, F. T., Chance, P. F., and Ochs, H. D. (2001) The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat. Genet. 27, 20–21
12. Brunkow, M. E., Jeffery, E. W., Hjerrild, K. A., Paeper, B., Clark, L. B., Yasayko, S. A., Wilkinson, J. E., Galas, D., Ziegler, S. F., and Ramsdell, F. (2001) Disruption of a new forkhead/winged-helix protein, scurfyn, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nat. Genet. 27, 68–73
13. Curotto de Lafaille, M. A., and Lafaille, J. J. (2009) Natural and adaptive foxp3+ regulatory T cells. More of the same or a division of labor? Immunity 30, 626–635
14. Mantel, P. Y., Ouaked, N., Rüegg, B., Karagiannis, C., Welz, R., Blaser, K., and Schmidt-Weber, C. B. (2006) Molecular mechanisms underlying FOXP3 induction in human T cells. J. Immunol. 176, 3593–3602
15. Kim, H. P., and Leonard, W. J. (2007) CREB/ATF-dependent T cell receptor-induced Foxp3 gene expression. A role for DNA methylation. J. Exp. Med. 204, 1543–1551
16. Long, M., Park, S. G., Strickland, I., Hayden, M. S., and Ghosh, S. (2009) Nuclear factor-kappaB modulates regulatory T cell development by directly regulating expression of Foxp3 transcription factor. Immunity 31, 921–931
17. Tone, Y., Furueuchi, K., Kojima, Y., Tykocinski, M. L., Greene, M. L., and Tone, M. (2008) Sma3 and NFAT cooperate to induce Foxp3 expression through its enhancer. Nat. Immunol. 9, 194–202
18. Martinez, G. I., Zhang, Z., Chung, Y., Reynolds, J. M., Lin, X., Jetten, A. M., Feng, X. H., and Dong, C. (2009) Sma3 differentially regulates the induction of regulatory and inflammatory T cell differentiation. J. Biol. Chem. 284, 35283–35286
19. Huehn, J., Polansky, J. K., and Hamann, A. (2009) Epigenetic control of FOXP3 expression. The key to a stable regulatory T-cell lineage? Nature Rev. Immunol. 9, 83–89
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20. Lal, G., and Bromberg, J. S. (2009) Epigenetic mechanisms of regulation of Foxp3 expression. Blood 114, 3727–3735

21. Janson, P. C., Winerdal, M. E., Marits, P., Thörn, M., Ohlsson, R., and Jomaa, H. (2008) FOXP3 promoter demethylation reveals the committed T cell population by foreign antigen. Nat. Immunol. 9, 523–544

22. Vignali, D. A., Zinkernagel, R. M., and Uhr, J. (2001) T cell expansion required for synapse maintenance and full effector function. J. Immunol. 166, 16878–16882

23. Ebert, P. J., Ehrlich, L. I., and Davis, M. M. (2008) Low ligand requirement for induction of Foxp3 in T cells mediated through inactivation of ERK. J. Immunol. 180, 2757–2761

24. Chien, Y. (1998) Ligand recognition by T cell receptors. Annu. Rev. Immunol. 16, 523–544

25. Reay, P. A., Kantor, R. M., and Davis, M. M. (1994) Use of global amino acid replacements to define the requirements for MHC binding and T cell recognition of myo cytoplasmic c (93–103). J. Immunol. 152, 3946–3957

26. Reay, P. A., Kantor, R. M., and Davis, M. M. (1994) Differential regulation of glycogen synthase kinase-3 by protein kinase C isotypes. J. Biol. Chem. 267, 16878–16882

27. Sun, L., Zhao, H., Xu, Z., Liu, Q., Liang, Y., Wang, L., Cai, X., Zhang, L., Hu, L., Wang, G., and Zha, X. (2007) Phosphatidylinositol 3-kinase/protein kinase B pathway stabilizes DNA methyltransferase I protein and maintains DNA methylation. Cell. Signal. 19, 2255–2263

28. Goode, N., Hughes, K., Woodgett, J. R., and Parker, P. J. (1992) Differential regulation of glycogen synthase kinase-3 by protein kinase C isotypes. J. Biol. Chem. 267, 16878–16882

29. Wang, J., Ioan-Facsinay, A., van der Voort, E. I., Huizinga, T. W., and Toes, R. E. (2007) Transient expression of FOXP3 in human activated nonregulatory T cells by directly and indirectly targeting DNA methyltransferase I. J. Immunol. 178, 6763–6781

30. Fabian, M. R., Sonenberg, N., and Filippowicz, W. (2010) Regulation of mRNA translation and stability by microRNAs. Annu. Rev. Biochem. 79, 351–379

31. Jiang, S., Li, C., Olive, V., Lykken, E., Feng, F., Sevilla, J., Wan, Y., He, L., and Li, Q. J. (2011) Molecular dissection of the miR-17–92 cluster’s critical dual roles in promoting Th1 responses and preventing inducible Treg differentiation. Blood 118, 5487–5497

32. Luo, X., Zhang, Q., Liu, Y., Xia, Z., Pothoven, K. L., and Lee, C. (2008) Cutting edge. TGF-β-induced expression of Foxp3 in T cells is mediated through inactivation of ERK. J. Immunol. 180, 785–789

33. Sauer, S., Bruno, L., Hertweck, A., Finlay, D., Leleu, M., Spivakov, M., Knight, Z. A., Cobb, B. S., Cantrell, D., O’Connor, E., Shokat, K. M., Fisher, B. A., and Wahl, S. M. (2003) Conversion of peripheral CD4+CD25+ naive T cells to CD4+CD25+ regulatory T cells by TGF-β induction of transcription factor Foxp3. J. Exp. Med. 198, 1875–1886

34. Smith-Garvin, J. E., Koretzky, G. A., and Jordan, M. S. (2009) T cell activation. Annu. Rev. Immunol. 27, 591–619

35. Pillai, V., Ortega, S. B., Wang, C. K., and Flavell, R. A. (2006) Transforming growth factor-β regulation of immune responses. Annu. Rev. Immunol. 24, 99–146

36. Koh, M., Yang, C., and Inoue, K. (2007) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell 69, 915–926

37. Jackson-Grusby, L., Beard, C., Possemato, R., Tudor, M., Fambrough, D., Csankovszki, G., Dausman, J., Lee, P., Wilson, C., Lander, E., and Jaenisch, R. (2001) Loss of genmic methylation causes p53-dependent apoptosis and epigenetic deregulation. Nat. Genet. 27, 31–39

38. Kretschmer, K., Apostolou, I., Haviger, D., Khazai, K., Nussenzweig, M. C., and von Boehmmer, H. (2005) Inducing and expanding regulatory T cell populations by foreign antigen. Nat. Immunol. 6, 1219–1227

39. Huse, S., Sprengler, C., Feil, E., Fries, B., and Wahl, S. M. (2003) Epigenetic control of the foxp3 locus in human T cells. Blood 101, 205, 7797–7802

40. Chen, C., Jiang, S., Li, C., Olive, V., Lykken, E., Feng, F., Sevilla, J., Wan, Y., He, L., and Li, Q. J. (2011) Molecular dissection of the miR-17–92 cluster’s critical dual roles in promoting Th1 responses and preventing inducible Treg differentiation. Blood 118, 5487–5497

41. Cui, H., Feinberg, A. P., Lengauer, C., Kinzler, K. W., Baylin, S. B., and Vogelstein, B. (2002) DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. Nature 416, 552–556

42. Zhou, X., Jeker, L. T., Fife, B. T., Zhu, S., Anderson, M. S., McManus, M. T., and Blustone, J. A. (2008) Selective miRNA disruption in T reg cells leads to uncontrolled autoimmunity. J. Exp. Med. 205, 1983–1991

43. Schoenborn, J. R., Dorschner, M. O., Sekimata, M., Santer, D. M., Shynevra, M., Fitzpatrick, D. R., Stamatoyannopoulos, J. A., Stamatoyannopoulos, J. A., and Wilson, C. B. (2007) Comprehensive epigenetic profiling identifies multiple distal regulatory elements directing transcription of the gene encoding interferon-γ. Nat. Immunol. 8, 732–742

44. Cohen, P., and Frame, S. (2001) The renaissance of GSK3. Nat. Rev. Mol. Cell Biol. 2, 769–776

45. Cross, D. A., Alesi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378, 785–789