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T Cell Receptor (TCR) and Transforming Growth Factor β (TGF-β) Signaling Converge on DNA (Cytosine-5)-methyltransferase to Control *forkhead box protein 3* (*foxp3*) Locus Methylation and Inducible Regulatory T Cell Differentiation*[S]

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Background: TCR and TGF-β signaling regulate the differentiation of Foxp3$^+$-inducible regulatory T cells.
Results: Through posttranscriptional regulation of DNMTs, TCR and TGF-β signaling control *foxp3* promoter methylation.
Conclusion: During antigen-induced proliferation, TCR and TGF-β signaling program T cells epigenetically to achieve heritage maintenance.
Significance: Our results illustrate a single mechanism that can comprehensively underpin the interplay between antigen and environment in guiding iTreg differentiation.

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

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[2] The abbreviations used are: pMHC, peptide major histocompatibility complex; TCR, T cell antigen receptor; Th, T helper; DNMT, DNA (cytosine-5)-methyltransferase; *foxp3*, Forkhead box P3; GSK3β, glycogen synthase kinase 3β; CFSE, carboxyfluorescein succinimidyl ester; nTreg, natural Treg; iTreg, inducible Treg; NFAT, nuclear factor of activated T cell; AP1, activator protein 1;CREB, cAMP response element-binding; CNS, non-coding sequence 1; miR, microRNA.

(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 should 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$^{Rag2tm1Fwa\ H2\text{-}T18a}$ Tg (Tcra5CC7, Tcrb5CC7)lwep). 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-E^k antibody (14.4.4), anti-I-E^k-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 #528116), cyclosporin A (catalog #239835), IκB kinase inhibitor III (catalog #401480), MG-132 (catalog #474790), SB-216763 (catalog #361566), ERK inhibitor II (catalog #328007), JNK 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 miRVana 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 Axiovert-100TV station equipped with a Zeiss 40X EC Plan-Neofluar 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 MethylDetector 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-E^k (27). To determine the role of ligand strength in regulating iTreg differentiation, we stimulated sorted $CD4^+CD25^-$ 5C.C7 T cells using syngeneic antigen-presenting cells loaded with either the strong agonist MCC or a weak agonist 102S (28). To interrogate how the duration of stimulation influences iTreg induction, I-E^k-specific antibodies were added at different time points after the onset of stimulation (*e.g.* 6 and 18 h); this treatment blocks TCR engagement with pMHC within minutes (29). Under these two regimes and without the addition of exogenous cytokines, we analyzed the percentages of $Foxp3^+$ CD4 T cells 72 h after initial TCR stimulation (Fig. 1*A*). Consistent 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-

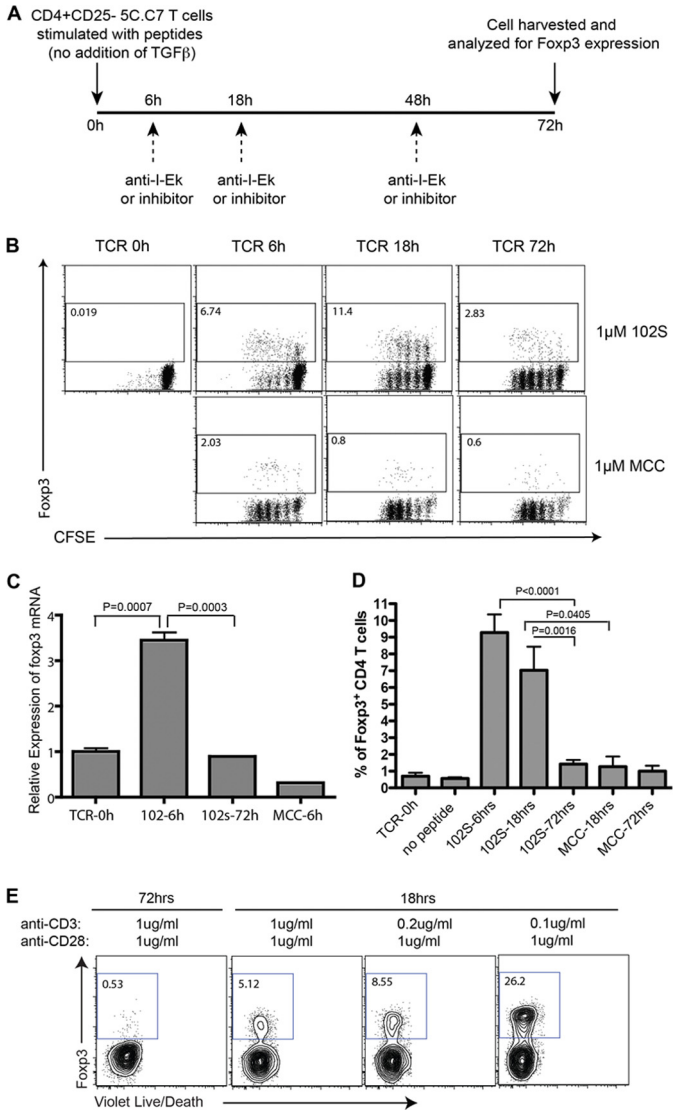

FIGURE 1. **Suboptimal TCR activation in terms of both strength and duration favors iTreg differentiation.** *A*, shown is a schematic view of the workflow for analyzing the role of TCR strength and duration in iTreg differentiation. Briefly, sorted $CD4^+CD25^-$ T cells from the lymph nodes of 5C.C7 TCR transgenic mice were labeled with CFSE, cultured with syngeneic T cell-depleted splenocytes without peptide in the presence of 10 ng/ml recombinant IL-7, or stimulated by syngeneic T cell-depleted splenocytes loaded with a strong agonist peptide MCC (1 μM) or a weak agonist 102S (1 μM). Anti-I-E^k antibody or small molecule inhibitors that block specific pathways were added at the indicated time points. The percentages of $CD4^+Foxp3^+$ T cells were analyzed by intracellular staining and flow cytometry at 72 h. *B* and *D*, percentages of iTregs generated with the indicated TCR stimulatory strength and duration are shown. *B*, a representative FACS plot is shown. *D*, statistical analysis is shown. Data show the means ± S.E. from three independent experiments. *C*, at the end of culture, CD4 T cells were FACS-sorted, and total RNA was extracted for quantitative PCR analysis. Data show the means ± S.E. from three independent experiments. *E*, sorted $CD4^+CD25^-$ T cells from the lymph nodes of C57BL/6 mice were labeled with CFSE and then stimulated with various concentrations of plate-bound anti-CD3 and anti-CD28 antibody for 18 h. After this, the cells were either further stimulated with anti-CD3 and anti-CD28 for 54 h (TCR 72 h total) or maintained without TCR stimulation for 54 h (TCR 18 h). The induction of Foxp3 was then examined by intracellular staining. Data represent three independent experiments.

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. 1*C*). 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. 1*E*). Furthermore, reducing the concentration of anti-CD3 antibody further enhanced Foxp3 induction and iTreg differentiation (Fig. 1*E*). 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. S1*A*), 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. S1*B*). 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. 1*B* and supplemental Fig. S1*B*). 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. 2*A*). We further dissected these two pathways by treating cells with PIK-75, which specifically inhibits the P110α and P110γ subunits of PI3K at the dose used, and with rapamycin, which inhibits mTOR specifically (Fig. 2*A*). We noted that both inhibitors could significantly potentiate iTreg differentiation. Meanwhile, despite having a dramatic impact on T cell proliferation (31), inhibition of calcineurin-NFAT signaling, NFκB function, or ERK activation had a minimal effect on Foxp3 induction (Fig. 2*A*). 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. 2*A*).

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.

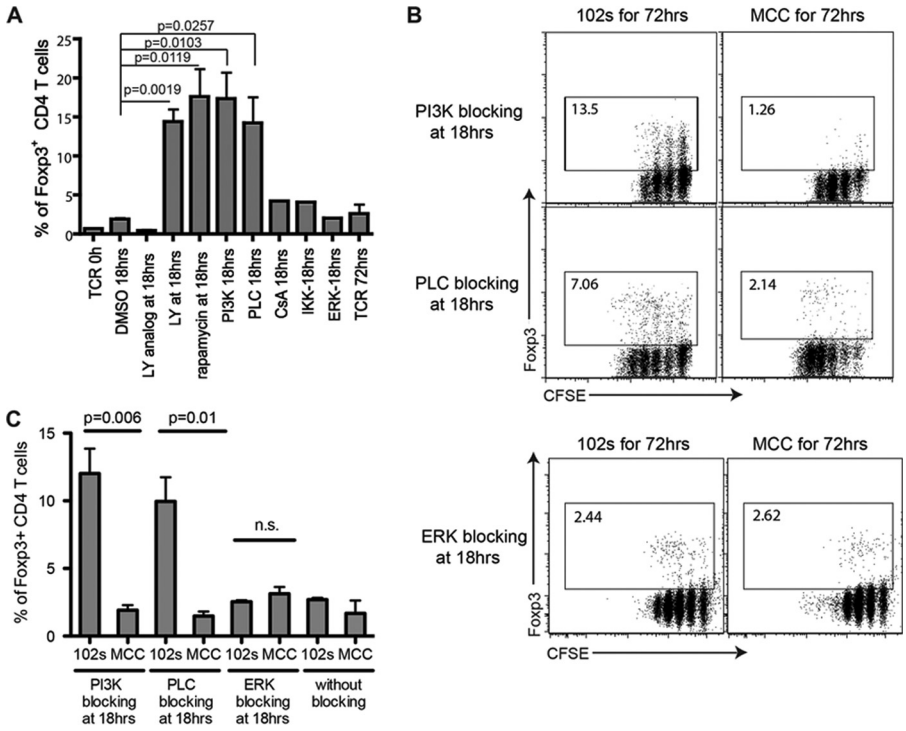

FIGURE 2. **PI3K-Akt-mTOR and PLC pathways downstream of TCR signaling negatively regulate iTreg differentiation.** *A*, sorted $CD4^+CD25^-$ T cells from 5C.C7 transgenic mice were stimulated with 1 μM 102S for 72 h as described in Fig. 1*A*. LY 294002 (10 μM), rapamycin (25 nM), cyclosporin A (*CsA*), 1 μg/ml), IκB kinase inhibitor III (*IKK*, BMS-345541, 1 μM), ERK (ERK inhibitor II, FR180204, 1 μM), PI3K (PIK-75, 100 nM), or PLC (U-73122, 1 μM) were added at the indicated time points to block specific pathways downstream of TCR signaling. The percentages of $CD4^+Foxp3^+$ T cells were analyzed by flow cytometry at 72 h after TCR activation. The *bar graph* shows the means ± S.E. from three independent experiments. *B* and *C*, sorted $CD4^+CD25^-$ T cells from 5C.C7 transgenic mice were stimulated with 1 μM 102S or MCC for 72 h. Inhibitors that specifically block the PI3K, PLC, or ERK pathways were added at 18 h after TCR stimulation. The percentages of $CD4^+Foxp3^+$ T cells were analyzed by flow cytometry at 72 h after TCR activation. *B*, shown are representative FACS plots. *C*, the bar graph shows the means ± S.E. from three independent experiments.

To examine this, we evaluated the methylation status of the *foxp3* gene in different T cell populations from male 5C.C7 mice, including naïve 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 naïve CD4 T cells, CpG islands residing in the *foxp3* promoter region were largely unmethylated (Fig. 3*A*). 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 *foxp3* promoter methylation (Fig. 3*A*), and this methylation pattern mirrored the final expression level of Foxp3 protein (Fig. 1*B*). In addition, the CpG islands within the *foxp3* CNS2 region were completely methylated in naïve T cells and T cells that had experienced various TCR stimulations (Fig. 3*B*). It was not surprising to observe that the methylation status in CNS2 was largely unchanged in conventional CD4 T cells. It has been shown that even in fully differentiated iTregs induced by TGF-β and IL-2 treatment, the CpG islands within CNS2 still remains largely methylated (15). Furthermore, although the genetic modification demonstrates that CNS2 is rather essential for the maintenance of Foxp3 expression in mature nTregs (33), our data validate that CNS2 is likely dispensable for Foxp3 induction during iTreg induction. To further functionally determine whether extensive TCR stimulation blocks *foxp3* expression through DNA methylation, we stimulated 5C.C7 T cells with 102S for 72 h while also treating samples at 18 h post stimulation with 5-azacytidine, a cytosine nucleoside analog that inhibits DNA methylation. In agreement with our methylation data, 5-azacytidine treatment abrogated the inhibition of Foxp3 expression by prolonged TCR signaling (Fig. 3*C*). 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. 4*A*) and DNMT3b (Fig. 4*B*) largely remained steady. However, at the protein level,

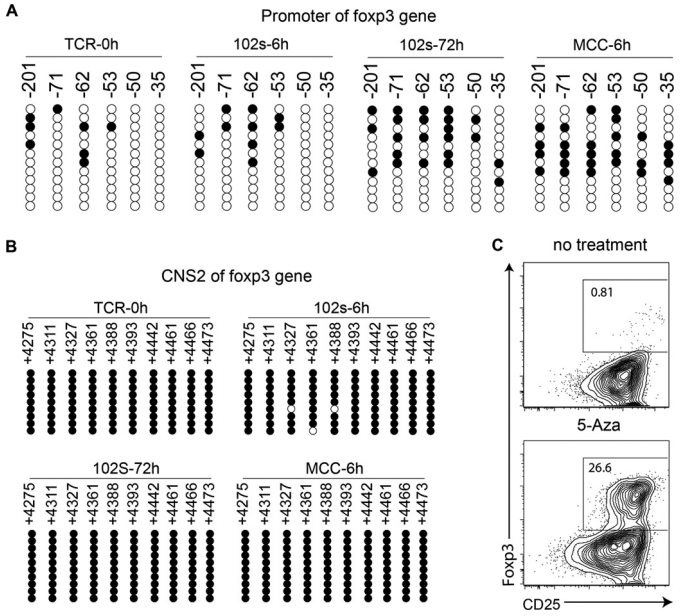

FIGURE 3. **Strong TCR signaling enhances CpG methylation within the *foxp3* locus.** *A* and *B*, $CD4^+CD25^-$ T cells from male 5C.C7 transgenic mice were stimulated as described in Fig. 1*A*. The methylation status of CpG islands within the *foxp3* promoter (*A*) or *foxp3* CNS2 (*B*) from these 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 percentages of $CD4^+Foxp3^+$ T cells were analyzed by flow cytometry at 72 h after TCR activation. Data represent three independent experiments.

DNMT1 was slightly elevated with a short duration of 102S stimulation and was dramatically increased when this stimulation was prolonged (Fig. 4*C*). Similarly, DNMT3b protein was also significantly elevated with as short as 6 h of moderate TCR stimulation (Fig. 4*D*). 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 (MCC) (Fig. 4, *E* and *F*). To examine whether DNMT1 and Foxp3 expression are inversely correlated under the permissive condition for iTreg conversion, we directly compared DNMT1 levels between $Foxp3^+$ and $Foxp3^-$ populations in CD4 T cells with the same TCR priming. We employed BAC transgenic mice expressing the GFP-Cre fusion protein under the control of the foxp3 promoter, in which GFP expression faithfully reflects endogenous Foxp3 expression (37). We then stimulated sorted $CD4^+GFP^-$ conventional T cells from these mice with the permissive condition optimized in Fig. 1*E*. GFP^- ($Foxp3^-$) and converted GFP^+ ($Foxp3^+$) CD4 T cells were then sorted to determine DNMT1 expression (Fig. 4*G*). In agreement with a critical role of DNMT1 in negatively controlling Foxp3 expression, we detected significant lower DNMT1 levels in GFP^+ cells as compared with the GFP^- population (Fig. 4*H*).

We next examined whether the overall accumulation of DNMT1 and DNMT3b protein by strong TCR signaling leads to enhanced enrichment of these two enzymes at the *foxp3* locus, which could account for the increased CpG methylation status in the promoter of the *foxp3* gene. As shown by our immunocytochemistry experiments, DNMT1 protein resides in small punctate structures within naïve CD4 T cell nuclei (Fig. 5*A*). In agreement with our Western blot results, the total signal intensity of DNMT1 staining rose sharply upon stimulation in a TCR signal strength-dependent manner. Moreover, in contrast to a few concentrated DNMT1 punctae observed in naïve cells (*TCR-0h*) or cells given a short and weak priming (*102S-6h*), T cells with strong TCR signaling (*102S-72h*, *MCC-6h*/*72h*) had significantly increased nuclear DNMT1 staining not only with respect to the intensity of each individual puncta but also the number of punctae (Fig. 5*A*). We predicted that this increased-quantity and broadened distribution would impact the occupancy of DNMT1 on the *foxp3* gene regulatory regions. The locus-specific recruitment of DNMT1 and DNMT3b was quantified by chromatin immunoprecipitation. Consistent with the overall demethylated status of promoter CpG islands in unstimulated and weakly stimulated CD4 T cells, we did not detect any specific DNMT1 or DNMT3b binding to the *foxp3* promoter in these two populations (Fig. 5, *B* and *E*). In contrast, cells that were suboptimally stimulated, either with weak agonist for a long duration or with strong agonist for a short duration, showed significantly enhanced binding of DNMT1 and DNMT3b to *foxp3* CpG islands (Fig. 5, *B* and *E*). Interestingly, strong TCR signaling also enhanced DNMT1 occupancy at the CNS2 region but not DNMT3b occupancy (Fig. 5, *C* and *F*). Importantly, as implied by the local accumulation of DNMT1 at punctae within the nucleus, we could demonstrate a degree of specificity in the local recruitment of DNMT1 to the *foxp3* locus. In agreement with the fact that strong stimulation of 5C.C7 T cells favors their Th1 lineage differentiation, no significant DNMT1 binding was detected within the CNS-6 region of the *ifng* gene after MCC stimulation (Fig. 5*D*), which was methylated in naïve cells but completely demethylated and accessible to support IFNγ production in Th1 cells (38). This biochemical evidence, combined with the associated epigenetic and functional outcomes, indicates that strong TCR signaling blocks the accessibility of the *foxp3* locus through the elevation of DNMT1 and DNMT3b protein levels, delivery of these two enzymes to the *foxp3* regulatory region, and the resultant enhanced local methylation.

TCR Signaling Stabilizes DNMT1 Protein through Inhibition of GSK3β Activity and Protection from Proteasome-mediated Degradation—Based on the data presented above, we hypothesized that there must be a signaling node that is capable of receiving signals from both PI3K and PLCγ pathways downstream of TCR and then integrating these signals to post-translationally modify the level of DNMT1 protein. Within the TCR signaling network, a good candidate for such a modulator is glycogen synthase kinase 3β (GSK3β). GSK3β is a constitutively active serine/threonine protein kinase in resting cells (39). Upon receptor signaling, its activity can be silenced by phosphorylation mediated through PI3K-Akt (40) and/or PLCγ-PKCθ signaling (41). Interestingly, in some human tumor cell lines suppression of GSK3β activity upon PI3K activation has been implicated as the cause for stabilized DNMT1

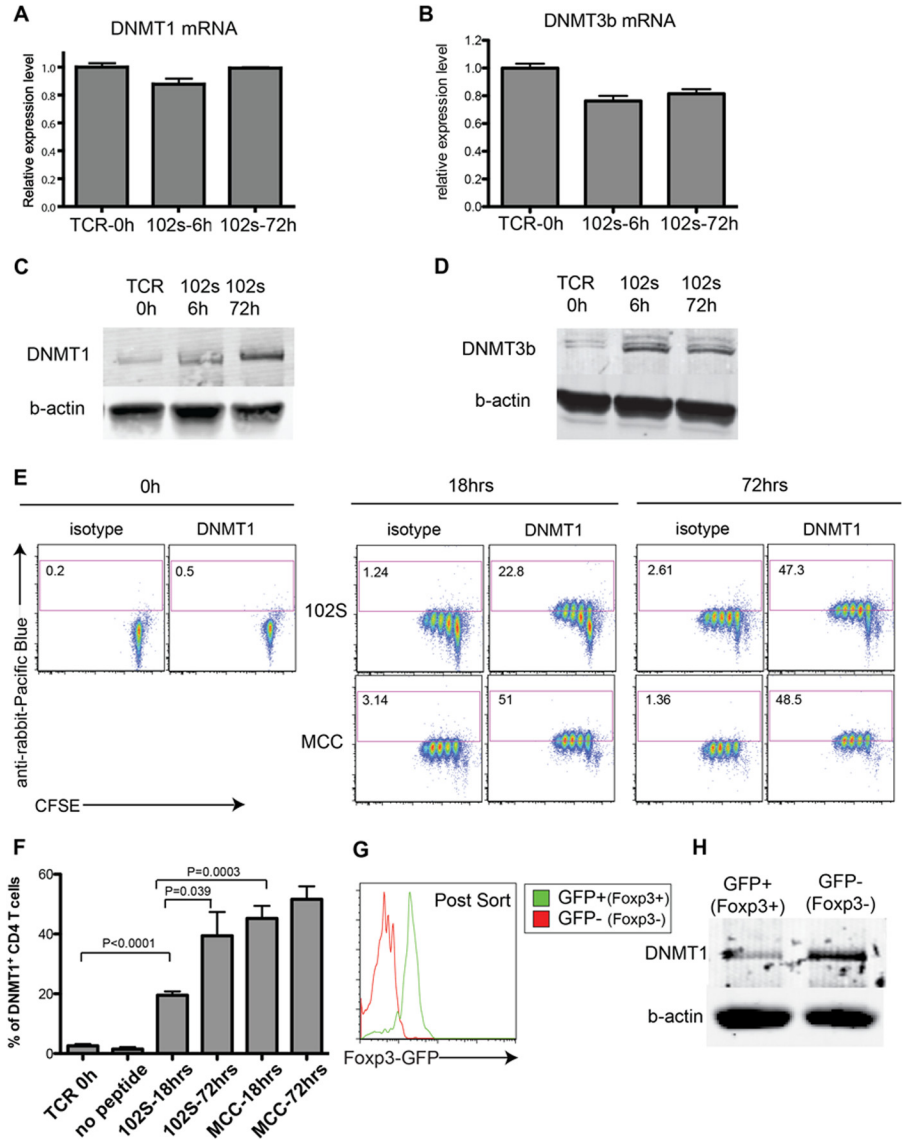

FIGURE 4. **DNMT1 and DNMT3b are posttranscriptionally up-regulated by TCR signaling in a strength- and duration-dependent manner.** *A* and *B*, $CD4^+CD25^-$ T cells from 5C.C7 transgenic mice were left unstimulated (*TCR-0h*) or stimulated with 1 μM 102S for 6 h (*TCR-6h*) or 72 h (*TCR-72h*) and then sorted by FACS. Total RNA and protein were extracted, and relative expression of dnmt1 mRNA (*A*) and dnmt3b mRNA (*B*) and DNMT1 protein (*C*) DNMT3b protein (*D*) were determined by quantitative PCR and Western blot analysis. In *A* and *B*, data show the means ± S.E. from three independent experiments. *E* and *F*, $CD4^+CD25^-$ T cells from 5C.C7 transgenic mice were labeled with CFSE and stimulated as described in Fig. 1*A*. The expression of DNMT1 protein at the single cell level was determined by intracellular staining. *E*, representative FACS plots are shown. *F*, statistical analysis is shown. Data show the means ± S.E. from three independent experiments. *G* and *H*, sorted $CD4^+GFP^-$ T cells from the lymph nodes of Foxp3-GFP-Cre mice were stimulated with 0.1 μg/ml plate-bound anti-CD3 and 1 μg/ml anti-CD28 antibody for 18 h and then maintained without further TCR stimulation for 54 h. GFP^+ and GFP^- CD4 T cells were then sorted (*G*) for examination of DNMT1 protein by Western blot (*H*).

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. 6*A*). Reciprocally, whereas blocking TCR engagement, PI3K activation, or PLC activity at the18 h point led to a substantial Foxp3 induction, the addition of GSK3β inhibitor could partially diminish this effect (Fig. 6*B*). 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

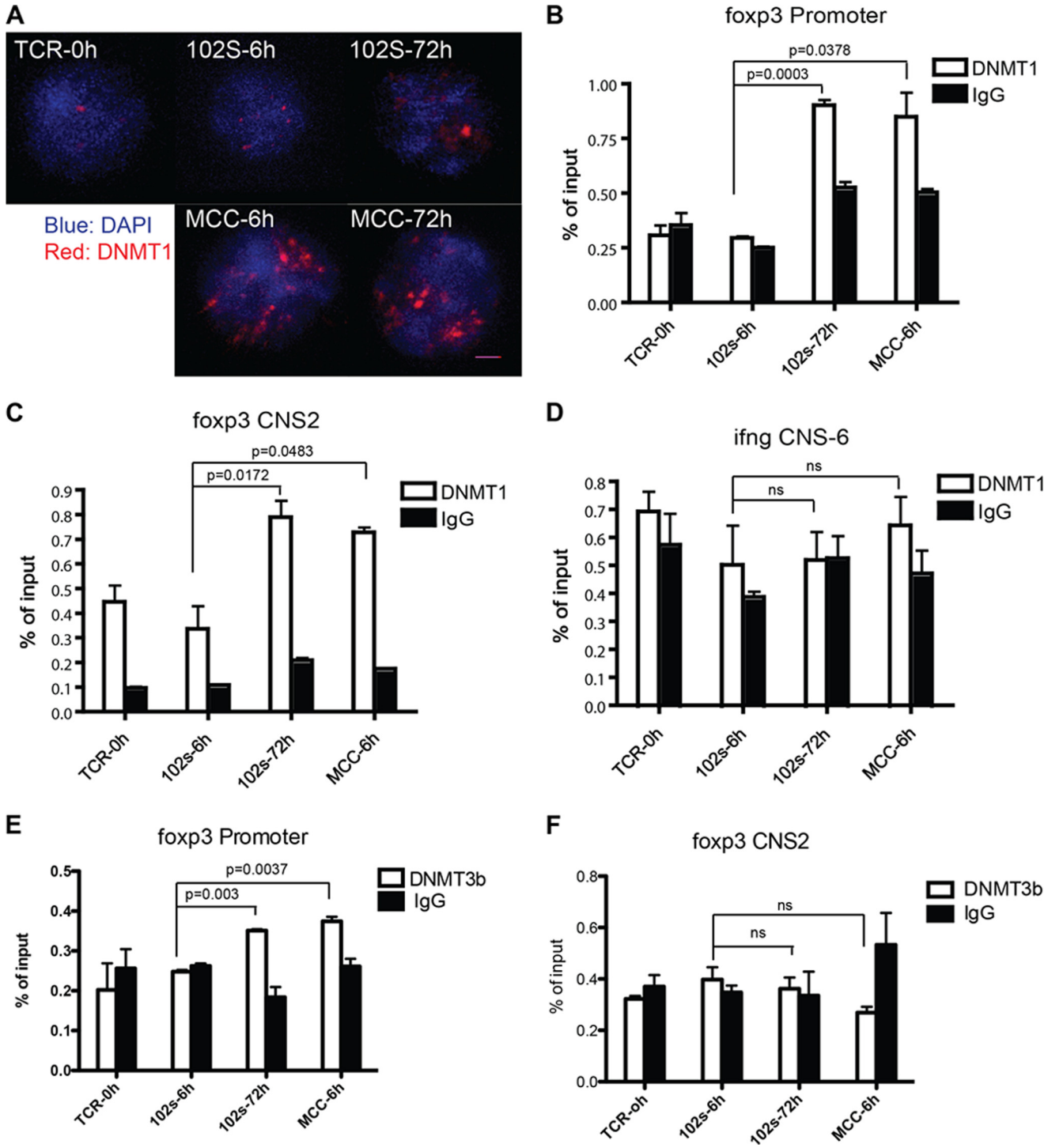

FIGURE 5. **Strong TCR signaling causes enhanced enrichment of DNMT1 and DNMT3b at the *foxp3* locus.** *A*, representative images show the nuclear localization of DNMT1. $CD4^+CD25^-$ T cells from 5C.C7 transgenic mice were stimulated as described in Fig. 1*A*. The cells were then fixed with 4% paraformaldehyde 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 DNMT1or 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.

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. S3*A*). Upon TCR engagement, expression levels of all three miRNAs within this family, miR-148a, miR-148b, and miR-152, were

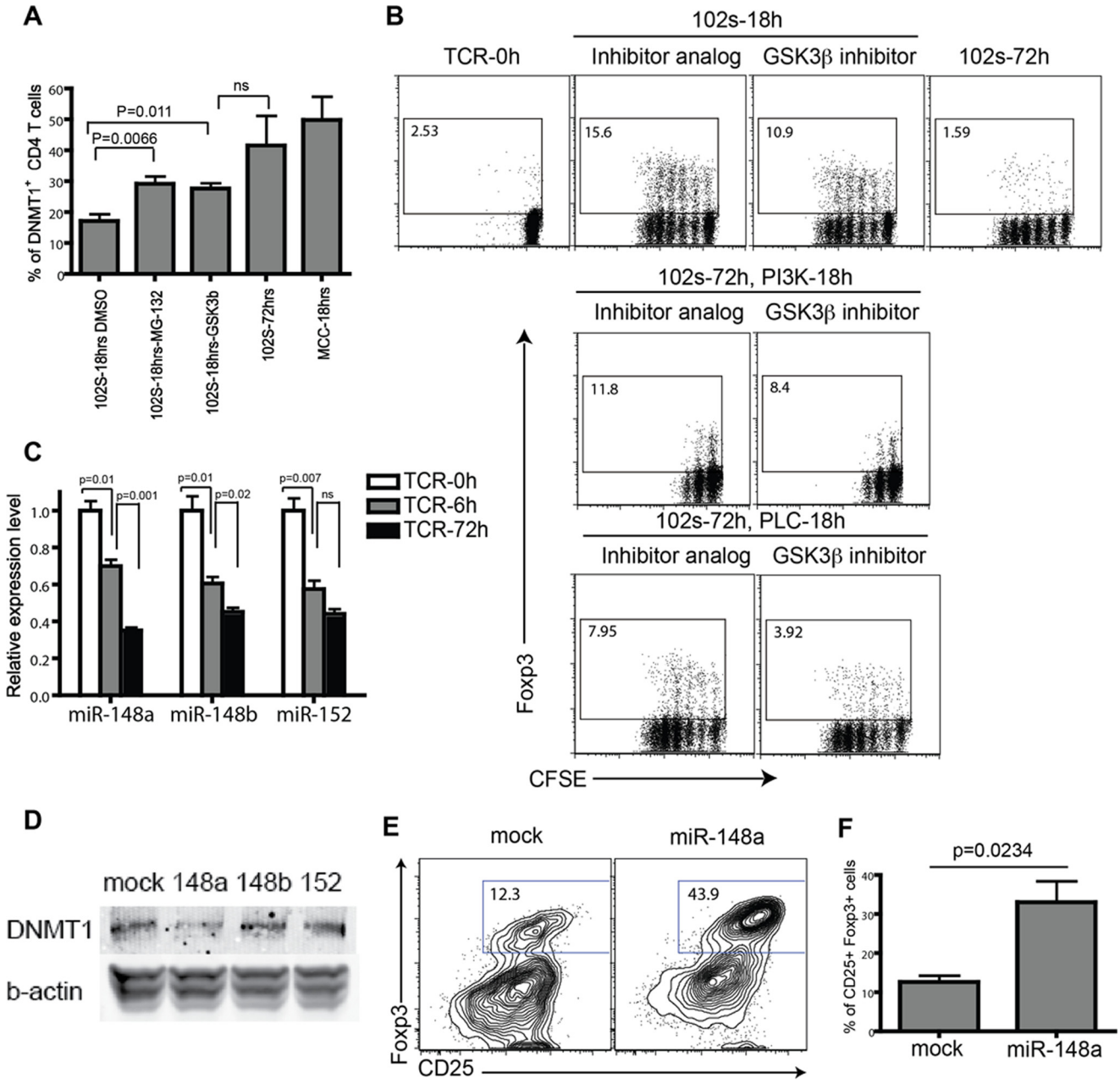

FIGURE 6. **TCR signaling stabilizes DNMT1 by inhibiting GSK3-β-induced proteasomal degradation of DNMT1 and repressing miR-148a-mediated inhibition of DNMT1 translation.** *A*, $CD4^+CD25^-$ T cells from 5C.C7 transgenic mice were stimulated with 1 μM 102S for 18 h and treated with 0.4 μM MG-132 or 1 μM GSK3-β inhibitor SB-216763 at 18 h post-TCR stimulation. The expression of DNMT1 protein at 72 h was quantified by intracellular staining of DNMT1 followed by flow cytometry analysis. Cells that were activated with 1 μM 102S for 72 h or 1 μM MCC for 18 h without other treatment were used as controls. Data show the means ± S.E. from three independent experiments. *ns*, not significant. *B*, $CD4^+CD25^-$ T cells from 5C.C7 transgenic mice were labeled with CFSE and stimulated with 1 μM 102S for the indicated durations. 1 μM GSK3-β inhibitor or its non-functional analog were added at 18 h together with either 100 nM PIK-75 (PI3K-18h) or 1 μM U-73122 (PLC-18h). The percentages of $CD4^+Foxp3^+$ T cells were analyzed by flow cytometry at 72 h after TCR activation. Data represent three independent experiments. *C*, 5C.C7 $CD4^+CD25^-$ T cells were stimulated with 1 μM 102S for the indicated durations. The CD4 T cells were then FACS-sorted, and total RNA was extracted. The relative expression of miR-148a, miR-148b, and miR-152 transcript was quantified by quantitative PCR analysis. Data show the means ± S.E. from three independent experiments. *D*, 5C.C7 $CD4^+CD25^-$ T cells were activated with 1 μM 102S and transduced with retrovirus that encodes GFP only (*mock*), miR-148a together with GFP (*miR-148a*), miR-148b together with GFP (*miR-148b*), and miR-152 together with GFP (*miR-152*). Three days after transduction, $CD4^+GFP^+$ T cells were sorted and extracted for total protein. DNMT1 protein level was quantified by Western blot analysis. Data represent three independent experiments. *E* and *F*, 5C.C7 $CD4^+CD25^-$ T cells were primed and transduced with mock virus or miR-148a as described and then cultured in the presence of 50 units/ml IL-2 and 2 ng/ml TGF-β for 4 days. The percentages of $CD25^+Foxp3^+$ T cells were analyzed by flow cytometry. *E*, a representative FACS plot is shown. *F*, statistical analysis is shown. Data show the means ± S.E. from three independent experiments.

suppressed (Fig. 5*C*). When ectopically expressed using a retroviral tool during T cell activation, all three failed to suppress DNMT1 mRNA levels (supplemental Fig. S3*B*). However, one of the three, miR-148a, significantly suppressed DNMT1 expression at the protein level (Fig. 6*D*). 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

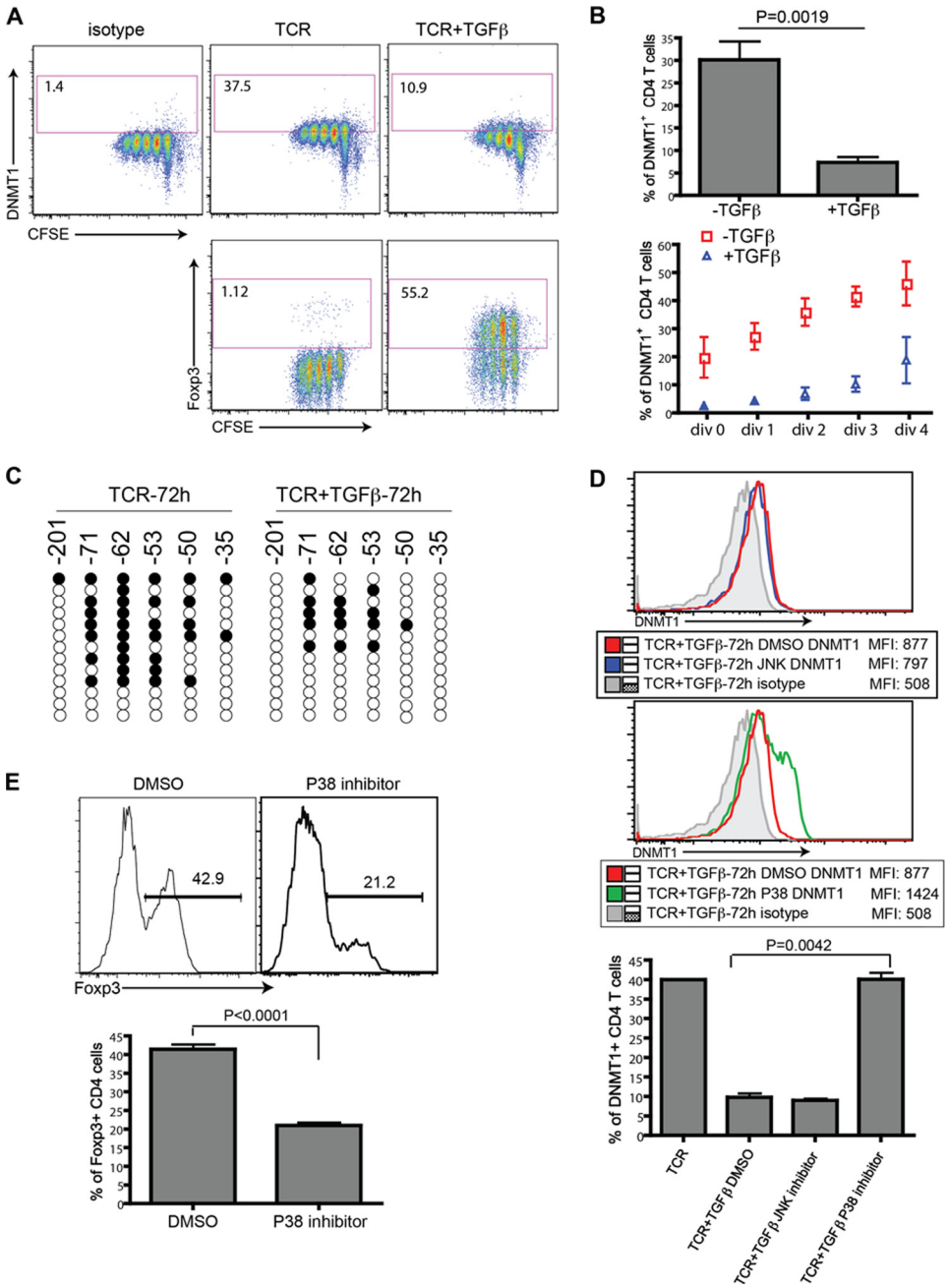

FIGURE 7. **TGF-β signaling antagonizes TCR-signal-mediated DNMT1 stabilization via the p38 pathway.** *A* and *B*, $CD4^+CD25^-$ T cells from 5C.C7 transgenic mice were labeled with CFSE and stimulated with 1 μM 102S in the absence (*TCR*) or presence of 5 ng/ml TGF-β (*TCR+TGFb*) for 72 h. The expression of DNMT1 and Foxp3 at the single cell level was quantified by intracellular staining. *A*, representative FACS plots are shown. *B*, statistical analysis is shown. Data show the means ± S.E. from three independent experiments. *C*, the methylation status of CpG islands in these cell *foxp3* promoters was determined by bisulfite sequencing analysis. Data represent three independent experiments. *D*, $CD4^+CD25^-$ T cells from 5C.C7 transgenic mice were stimulated with 1 μM 102S and 5 ng/ml TGF-β in the presence of 1 μM JNK inhibitor II (SP600125) or 10 μM p38 MAP kinase inhibitor III (ML3403) for 72 h. The expression of DNMT1 was determined by intracellular staining. *MFI*, mean fluorescence intensity. *Top*, representative FACS plot. *Bottom*, statistical analysis. Data show the means ± S.E. from three independent experiments. *E*, 5C.C7 transgenic $CD4^+CD25^-$ T cells were stimulated with 1 μM 102S and 5 ng/ml TGF-β in the presence of 10 μM p38 MAP kinase inhibitor III (ML3403) or DMSO for 72 h. The percentages of $CD4^+Foxp3^+$ T cells were analyzed by flow cytometry. *Top*, representative FACS plot. *Bottom*, Statistical analysis. Data show the means ± S.E. from four independent experiments.

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. 7*A*). 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. 7*B*). 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. S4*A*). 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. 7*C*) and CNS2 region of *foxp3* (supplemental Fig. S4*B*).

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. S5*A*). 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. S5*A*). In agreement with this, when same concentrations of TGF-β were supplemented, MCC induced significantly less Foxp3$^+$ cells (supplemental Fig. S5*B*). 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-MKK3–6-p38 pathways (46). During TCR stimulation of naïve T cells, ERK activation is inhibited by TGF-β treatment (47), and this curtailed-ERK signaling failed to increase *foxp3* expression (Fig. 2*A*). 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. 7*D*). 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. 7*E*). 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 naïve 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 short 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. 5*E*). 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. 4*C* and 7*B*) and a corresponding reduction of *foxp3* expression in T cells from late generations (supplemental Fig. S1*B*). 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. S1*B*). 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 pathogenic 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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