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# Vitamin C Facilitates Demethylation of the *Foxp3* Enhancer in a Tet-Dependent Manner

Varun Sasidharan Nair, Mi Hye Song, and Kwon Ik Oh

Demethylation of CpG motifs in the *Foxp3* intronic element, conserved noncoding sequence 2 (CNS2), is indispensable for the stable expression of *Foxp3* in regulatory T cells (Tregs). In this study, we found that vitamin C induces CNS2 demethylation in Tregs in a ten-eleven-translocation 2 (Tet2)-dependent manner. The CpG motifs of CNS2 in Tregs generated in vitro by TGF- $\beta$  (iTregs), which were methylated originally, became demethylated after vitamin C treatment. The conversion of 5-methylcytosine into 5-hydroxymethylcytosine was more efficient, and the methyl group from the CpG motifs of *Foxp3* CNS2 was erased rapidly in iTregs treated with vitamin C. The effect of vitamin C disappeared in *Tet2*<sup>-/-</sup> iTregs. Furthermore, CNS2 in peripheral Tregs in vivo, which were demethylated originally, became methylated after treatment with a sodium-dependent vitamin C transporter inhibitor, sulfinpyrazone. Finally, CNS2 demethylation in thymic Tregs was also impaired in *Tet2*<sup>-/-</sup> mice, but not in wild type mice, when they were treated with sulfinpyrazone. Collectively, vitamin C was required for the CNS2 demethylation mediated by Tet proteins, which was essential for *Foxp3* expression. Our findings indicate that environmental factors, such as nutrients, could bring about changes in immune homeostasis through epigenetic mechanisms. *The Journal of Immunology*, 2016, 196: 2119–2131.

orkhead box protein 3<sup>+</sup> regulatory T cells (Tregs) are a dedicated cell population that maintains immune tolerance and prevents autoimmune diseases (1, 2). It was previously assumed that Tregs arise from the thymus through the interaction between self-reactive TCR and self peptide–MHC complex (3). However, it is now appreciated that Tregs can be generated not only in the thymus but also in the periphery, and even in vitro from naive CD4<sup>+</sup> T cells. Thus, Treg populations can be divided into two groups (4)—namely, thymus-derived Tregs (tTregs) and those that are derived outside the thymus, known as induced Tregs, which can be further classified into two subgroups: Tregs generated in vivo (referred to as peripheral Tregs [pTregs]) (5–8) and Tregs generated in vitro by TGF- $\beta$  (referred to as iTregs hereafter) (9). Although all three Treg populations show suppressive activity (10, 11) and sometimes can be interchangeable in vivo (12), each type of Treg shows unique genetic (13) and epigenetic (14)

profiles, which suggests that each type develops under different environments.

Tregs usually regulate immune responses in a *Foxp3*-dependent manner (15, 16). Therefore, the stable expression of *Foxp3* is critical, and it is still contentious whether *Foxp3* instability is pathologically relevant (17–21). Stable expression of *Foxp3* is accompanied by epigenetic modulation of the CpG-rich conserved noncoding sequence 2 (CNS2) within the first intron of the *Foxp3* locus (22–25). It has been shown that the demethylation of CpG motifs allows critical transcription factors, such as *Foxp3* itself and Runx1–Cbf- $\beta$  complex, to bind to the CNS2 region and keep the transcription of *Foxp3* active in the progeny of dividing Tregs (26–28). Furthermore, CNS2 is demethylated in tTregs expressing *Foxp3* stably, but it is fully methylated in activated T cells or iTregs expressing *Foxp3* transiently (18, 29). CNS2 demethylation has been reported to occur within the thymus during the developmental stages and achieved through an active DNA oxidation process including ten-eleven-translocation (Tet) family (30, 31). However, the underlying mechanism of the epigenetic regulation of CNS2 is still elusive.

Tet proteins (Tet1, Tet2, and Tet3) are Fe<sup>2+</sup>-dependent and 2-oxoglutarate-dependent dioxygenases that oxidize 5-methylcytosines (5mC) into intermediates, such as 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) (32–34), which would eventually be replaced with unmethylated cytosine (35). Recently, we demonstrated that the overexpression of Tet2 restored CNS2 demethylation in *Il2*<sup>-/-</sup> tTregs, implying that the upregulation of Tet2 participates in CNS2 demethylation (36). This report led us to analyze the role of Tet2 in the CNS2 demethylation of iTregs. In this study, we found that the cooperation between Tet2 and its activator, vitamin C, plays an essential role in CNS2 demethylation of iTregs and tTregs.

## Materials and Methods

### Mice

Wild type (WT) C57BL/6 (B6) mice were purchased from Koatech (Pyeongtaek, Gyeonggi-do, Korea). CD45.1 congenic (B6.SJL-*Ptprca*<sup>a</sup> *Peprc*<sup>b</sup>/BoyJ), Rag1-deficient (B6.129S7-*Rag1*<sup>tm1Mom</sup>/J), *Foxp3*-GFP transgenic (*Foxp3* bicistronic reporter mice expressing EGFP: B6.Cg-*Foxp3*<sup>tm2Tch</sup>/J),

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V.S.N. performed experiments, analyzed the data, and prepared the manuscript; M.H.S. helped with the *Dnmt1* knock-down experiments; K.I.O. designed experiments, analyzed the data, and prepared the manuscript.

The microarray data presented in this article have been submitted to the National Center for Biotechnology Information's Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE67653>) under accession number GSE67653.

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The online version of this article contains supplemental material.

Abbreviations used in this article: bwt, body weight; 5caC, 5-carboxylcytosine; CD4SP, CD4 single-positive; CNS2, conserved noncoding sequence 2; CTV, CellTrace Violet; DP, double-positive; 5fC, 5-formylcytosine; 5hmC, 5-hydroxymethylcytosine; iTreg, Treg generated in vitro by TGF- $\beta$ ; KLH, keyhole limpet hemocyanin; LN, lymph node; 5mC, 5-methylcytosine; pTreg, peripheral Treg; qPCR, quantitative PCR; SVCT, sodium-dependent vitamin C transporter; Tet, ten-eleven-translocation; Tfh, T follicular helper; Treg, regulatory T cell; tTreg, thymus-derived Treg; WT, wild type.

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floxed Tet2 transgenic (B6;129S-Tet2<sup>tm1.1laai</sup>/J, Tet2<sup>fl/fl</sup>) (37) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). To generate Tet2-deficient mice, Lck-Cre or CD4-Cre transgenic mice were crossed to floxed Tet2 transgenic mice (Lck-Cre  $\times$  Tet2<sup>fl/fl</sup> and CD4-Cre  $\times$  Tet2<sup>fl/fl</sup>, referred to as Lck-Tet2<sup>-/-</sup> and CD4-Tet2<sup>-/-</sup>, hereafter). The level of Tet2 expression was checked by RT-PCR in double-positive (DP) and CD4 single positive (CD4SP) thymocytes and Tet2 was greatly downregulated in both Lck- and CD4-Tet2<sup>-/-</sup> mice. OT-II mice expressing OVA-specific transgenic TCR were bred to Foxp3-GFP transgenic, Rag1<sup>-/-</sup> or Tet2<sup>-/-</sup> mice to generate OT-II/Foxp3-GFP, OT-II/Rag1<sup>-/-</sup> and OT-II/Tet2<sup>-/-</sup> mice. All animal experimentations were conducted in accordance with guidelines and approval of the International Animal Care and Use Committees (IACUC) of Hallym University (Hallym 2013-109, 2014-74).

### Cell isolation and flow cytometry

To sort peripheral T cell subpopulations from pooled mouse spleens and lymph nodes (LNs), the CD4<sup>+</sup> fraction was first purified on the MACS Cell Separator using anti-mouse CD4 beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The enriched CD4<sup>+</sup> fractions were then separated further into each subpopulation by FACS-sorting using the FACS Aria-II flow cytometer (BD Biosciences, San Jose, CA). To sort naive CD4<sup>+</sup> cells and iTregs, CD44 and GFP were used (naive cells: CD4<sup>+</sup>CD8<sup>-</sup>GFP<sup>-</sup>CD44<sup>low</sup>; Tregs: CD4<sup>+</sup>CD8<sup>-</sup>GFP<sup>+</sup>). The purity for each cell type after sorting was usually >95%. We purchased the following mAbs from BD Biosciences, eBioscience (San Diego, CA), or BioLegend (San Diego, CA) for flow cytometry: R-PE- or PerCP-Cy5.5- or allophycocyanin- or Brilliant Violet 421 (BV421)-labeled anti-CD25 (clones PC61 and 7D4), PerCP-Cy5.5- or allophycocyanin- or BV421-labeled anti-CD4 (clones RM4-5 and GK1.5), PE- or PerCP-Cy5.5-labeled anti-CD44 (clone IM7), PerCP-Cy5.5- or allophycocyanin-labeled anti-CD45.1 (clone A20), PE- or PerCP-Cy5.5- or Brilliant Violet 510 (BV510)-labeled anti-CD45.2 (clone 104), PE-labeled anti-CD62L (clone MEL-14), PE-labeled anti-CD69 (clone H1.2F3), PerCP-Cy5.5- or allophycocyanin- or allophycocyanin-Cy7-labeled anti-CD8 (clone 53-6.7), allophycocyanin-labeled anti-CD86 (clone GL1, Rat IgG2a), PE-labeled anti-CTLA4 (clone UC10-4F10-11), allophycocyanin-labeled anti-DYKDDDDK (anti-FLAG, clone L5, Rat IgG2a), Alexa Fluor 488-labeled anti-Foxp3 (clone FJK-16s), allophycocyanin-labeled anti-IFN- $\gamma$  (clone XMGI.2) and PE- or allophycocyanin-labeled IL17 (clone TC11-18H10.1). Intracellular Foxp3, FLAG, and cytokines were stained using Foxp3 Staining Buffer set (eBioscience). For cytokine analysis, cells were cultured for 4 h in the presence of PMA/ionomycin plus monensin (BD Biosciences) before intracellular cytokine staining. Data were acquired through FACSCalibur or FACSCanto-II (BD Biosciences) and were analyzed with FlowJo software (Tree Star, Ashland, OR).

### Cell culture

FACS-sorted cells were cultured in complete RPMI 1640 medium (WelGENE, Daegu, Korea), supplemented with 10% FBS (WelGENE), penicillin, streptomycin (Sigma-Aldrich, St. Louis, MO), L-glutamine (2 mM; Life Technologies, Carlsbad, CA), sodium pyruvate (2 mM; Sigma-Aldrich), nonessential amino acid (0.1 mM; Sigma-Aldrich), and 2-ME (50  $\mu$ M; Sigma-Aldrich). For iTreg generation, FACS-sorted naive CD4<sup>+</sup> T cells (CD4<sup>+</sup>GFP<sup>-</sup>CD44<sup>low</sup> from mice having Foxp3-GFP knock-in allele or CD4<sup>+</sup>CD25<sup>-</sup>CD44<sup>low</sup> from mice without Foxp3-GFP knock-in allele like Tet2<sup>-/-</sup> mice) were stimulated with plate-bound anti-CD3 (2C11, 1  $\mu$ g/ml; eBioscience) plus CD28 (37.51, 1  $\mu$ g/ml), recombinant murine IL2 (rIL2, 15 ng/ml; Peprotech, Rocky Hill, NJ), and recombinant TGF- $\beta$  (rTGF- $\beta$ , 10 ng/ml; Peprotech) in the presence or absence of vitamin C (10  $\mu$ g/ml was used unless specified otherwise; Sigma-Aldrich) for 5–6 d and then GFP<sup>+</sup>CD25<sup>+</sup> iTregs were resorted.

### Foxp3 demethylation analysis

The genomic DNA was extracted from the FACS-sorted live cells by using the Blood & Tissue Genomic DNA Extraction kit (Qiagen, Valencia, CA). For isolation of DNA from cells fixed and stained with anti-Foxp3 mAb (Fig. 6B), we used the protocol described previously (38, 39). Briefly, sorted cells (CD4<sup>+</sup>CD8<sup>-</sup>TCR $\beta$ <sup>+</sup>Foxp3<sup>+</sup>CD25<sup>+</sup>) were incubated with 300  $\mu$ l lysis buffer (10 mM Tris-HCl, 100 mM NaCl, 50 mM EDTA, 0.5% SDS, 0.1  $\mu$ g/ml proteinase K, and 20  $\mu$ g/ml RNase A) for 24 h at 60°C. Next, DNA was extracted by phenol/chloroform/isoamyl alcohol solution (25:24:1) and precipitated overnight by ethanol. Extracted genomic DNAs were converted by the EpiTect Bisulfite kit (Qiagen) or EZ DNA methylation gold kit (Zymo Research, Irvine, CA). Sense (23) or anti-sense (14) strands of bisulfite-treated DNA were then subjected to PCR

for amplification of CNS2. The PCR products obtained were cloned into the pGemT-easy vector (Promega, Madison, WI) and 10–30 individual clones from each sample were sequenced with M13-reverse primer (GAAACAGCTATGACCATG; Genentech, Daejeon, Korea). Results were not affected by bisulfite treatment methods (EpiTect or EZ Gold Kit) or target strands (sense or anti-sense strands). The sequences of primers are in Supplemental Table I.

### DNA immunoprecipitation

Genomic DNA was prepared using the Blood & Tissue Genomic DNA Extraction Kit (Qiagen) and sonicated to make small DNA fragments ranging from 200 to 500 bp. Sheared DNA (1  $\mu$ g) was immunoprecipitated by anti-5mC (BI-MECY; Eurogentec, Fremont, CA) and anti-5hmC Ab (39769; Active Motif, Carlsbad, CA) independently. Isotype-matched control Ab was used for the negative control. DNA was collected using Dynabeads (Invitrogen) and analyzed by quantitative PCR (qPCR).

The sequences of primers are as below.

*Foxp3* CNS1 forward, 5'-CTTTTCTTGTTGGGGCTTCTG-3'  
*Foxp3* CNS1 reverse, 5'-GACAGTCTGGCTCCCATACC-3'  
*Foxp3* CNS2 forward, 5'-AACCTGGGCCCCCTCTGGCA-3'  
*Foxp3* CNS2 reverse, 5'-GGCCGGATGCATTGGGCTTCA-3'  
*Foxp3* CNS3 forward, 5'-TAAGCAGGGTGGGGTACTTG-3'  
*Foxp3* CNS3 reverse, 5'-CTCTGAAGCCTGGAGAGTGG-3'

### Quantitative RT-PCR

RNA was isolated from FACS-sorted cells using the RNeasy Mini kit (Qiagen) or Trizol (Life Technologies), and reverse-transcribed into cDNA using QuantiTect Reverse Transcription kit (Qiagen). qPCR reactions were performed on RotorGene 6000 system (Qiagen) using AccuPower GreenStar qPCR kit (Bioneer). All data were normalized to actin. Non-specific amplification was checked with the use of melting curve and agarose gel electrophoresis.

The sequences of primers are as follows:

*Tet1* forward, 5'-GAGCCTGTTCCTCGATGTGG-3'  
*Tet1* reverse, 5'-CAAACCCACCTGAGGCTGTT-3'  
*Tet2* forward, 5'-AACCTGGCTACTGTCATTGCTCCA-3'  
*Tet2* reverse, 5'-ATGTTCTGCTGGTCTCTGTGGGAA-3'  
*Tet3* forward, 5'-TCCGGATTGAGAAGGTCATC-3'  
*Tet3* reverse, 5'-CCAGGCCAGGATCAAGATAA-3'  
*Dnmt1* forward, 5'-CCTAGTTCCTGGCTACGAGGAGAA-3'  
*Dnmt1* reverse, 5'-TCTCTCTCTCTGCAGCCGACTCA-3'

### Lentiviral transduction

293T cells were transiently transfected with lentiviral vector (pSicoR-Dnmt1) and packaging vectors using the traditional calcium phosphate method. Supernatant was collected in 3 d, and infected naive CD4<sup>+</sup> T cells were cultured under iTreg conditions. GFP<sup>+</sup> cells were isolated by FACS-sorting and used for DNA demethylation study. pSicoR-Dnmt1 vector (Addgene plasmid No. 12166) was a gift from Tyler Jacks (40).

### Transfection

DNA transfection experiments were performed using MACS-purified CD8<sup>-</sup> thymocytes including CD4<sup>+</sup>CD8<sup>-</sup> (DN) and CD4<sup>+</sup>CD8<sup>-</sup> (CD4SP) cells. Cells were transfected with plasmids encoding FLAG-tagged mouse Tet2 (34) or empty vector by using the electroporation kit (Amaxa Nucleofector Kit L and Nucleofector Device, Lonza, Walkersville, MD). The next day, transfected CD4SP cells were FACS-sorted and subsequently cultured under the iTreg condition for demethylation analysis. Transfection efficiency was checked 5–6 h after transfection by flow cytometry using anti-FLAG mAb (L5, BioLegend) and >90% were FLAG positive.

### In vitro suppression assay

FACS-sorted naive CD4<sup>+</sup> T cells labeled with CellTrace Violet (CTV; Life Technologies) were used as responders. APCs were prepared by depleting CD4<sup>+</sup> and CD8<sup>+</sup> cells from WT B6 splenocytes using flow cytometry. Responder cells (5  $\times$  10<sup>3</sup>) were cultured with APCs (2  $\times$  10<sup>4</sup>) and soluble anti-CD3 mAb (0.3  $\mu$ g/ml) in the absence or presence of various numbers



of Tregs for 3 d. The division of responder cells was assessed by dilution of CTV, and the Foxp3 stability of Tregs was checked with GFP.

#### Adoptive transfer and immunization experiments

PBS- or vitamin C-treated OT-II (or OT-II/*Rag1*<sup>-/-</sup>) iTregs ( $2 \times 10^5$  cells per mouse) were injected i.v. into congenic WT B6 mice. The next day, the recipient mice were s.c. immunized with 100  $\mu$ g of OVA in CFA (Sigma-Aldrich), and sacrificed after 1 wk for flow cytometry.

#### Generation of pTregs and Foxp3 analysis

**Colitis model.** Naive CD4<sup>+</sup> cells (CD4<sup>+</sup>CD8<sup>-</sup>CD44<sup>low</sup>CD25<sup>-</sup>GFP<sup>-</sup> cells from mice having Foxp3-GFP alleles or CD4<sup>+</sup>CD8<sup>-</sup>CD44<sup>low</sup>CD25<sup>-</sup> cells from CD4-*Tet2*<sup>-/-</sup> mice,  $0.7 \times 10^6$  cells per mouse) were isolated from LNs of mice treated with sulfapyrazone or DMSO for 7 d and transferred to *Rag1*<sup>-/-</sup> mice. The recipient mice were treated i.p. with sulfapyrazone (10  $\mu$ g/g bodyweight; Sigma-Aldrich) or DMSO every day. In 14 d, GFP<sup>+</sup> CD25<sup>+</sup> (or Foxp3<sup>+</sup>CD25<sup>+</sup>) pTregs were FACS-sorted from the mesenteric LNs and spleen and then used for demethylation study. Some cells were cultured with PMA plus ionomycin for 4 h and then subjected to cytokine analysis.

**OVA immunization model.** Naive OT-II T cells ( $1 \times 10^6$  cells per mouse) were injected i.v. into congenic WT B6 mice. The next day, the recipient mice were fed with 1% OVA in drinking water (to induce oral tolerance) and injected i.p. with sulfapyrazone (10  $\mu$ g/g body weight) or vehicle (DMSO) every day. In 6 d, OT-II GFP<sup>+</sup>CD25<sup>+</sup> pTregs were FACS-sorted from the mesenteric LNs and subjected to CNS2 demethylation study. For immunization experiments, oral tolerance to OVA was induced as above with or without sulfapyrazone. Immunization with OVA/CFA was then performed 7 d after oral tolerance induction. After immunization, mice were not treated further with sulfapyrazone or DMSO.

#### Foxp3 demethylation analysis in young mice

From 2 d after birth, the mice started to be injected i.p. with DMSO (vehicle), sulfapyrazone (5  $\mu$ g/g, body weight) or vitamin C (10 mg/g, body weight) every other day for 2–4 wks. Next, GFP<sup>+</sup>CD25<sup>+</sup> Tregs were FACS sorted from the thymus (CD8<sup>-</sup> thymocytes, obtained with CD8 MACS) and periphery (LNs and spleens together) and used for CNS2 demethylation study. For the long-term treatment, the dose of sulfapyrazone was doubled from the fourth week (10  $\mu$ g/g, body weight, every other day), and sulfapyrazone treatment was done every day from the sixth week (10  $\mu$ g/g bodyweight, every day).

#### Microarray

Total RNA was extracted using Trizol (Invitrogen), purified using RNeasy columns (Qiagen) according to the manufacturer's protocol. For quality control, RNA purity and integrity were evaluated by denaturing gel electrophoresis, OD 260/280 ratio, and analyzed on Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Total RNA was amplified and purified using the Ambion Illumina RNA Amplification Kit (Ambion, Austin, TX) to yield biotinylated cRNA according to the manufacturer's instructions. Briefly, 550 ng total RNA was reverse-transcribed to cDNA using a T7 oligonucleotide deoxythymine primer. Second-strand cDNA was synthesized, in vitro transcribed, and labeled with biotin-NTP. After purification, the cRNA was quantified using the ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE). Labeled cRNA samples (750 ng) were hybridized to each Mouse Ref-8 expression v.2 bead array for 16–18 h at 58°C, according to the manufacturer's instructions (Illumina, San Diego, CA). Detection of array signal was carried out using Amersham Fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, U.K.) following the bead array manual. Arrays were scanned with an Illumina bead array Reader confocal scanner according to the manufacturer's instructions. Raw data were extracted using the software provided by the manufacturer (Illumina GenomeStudio v2011.1 [Gene Expression Module v1.9.0]). Array probes were transformed by logarithm and normalized by quantile method. Statistical significance of the expression data were determined using Fold change. Microarray data have been deposited at the National Center for Biotechnology Information Gene Expression Omnibus (GSE67653, <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE67653>).

#### Statistical analyses

A two-tailed, unpaired, Student *t* test was used to calculate the statistical significance of differences between groups unless specified. The *p* values were represented as follows: \*\*\**p* < 0.001, \*\**p* < 0.01, \**p* < 0.05. A *p* value > 0.05 was NS. Error bars in the figures indicate SEM.

## Results

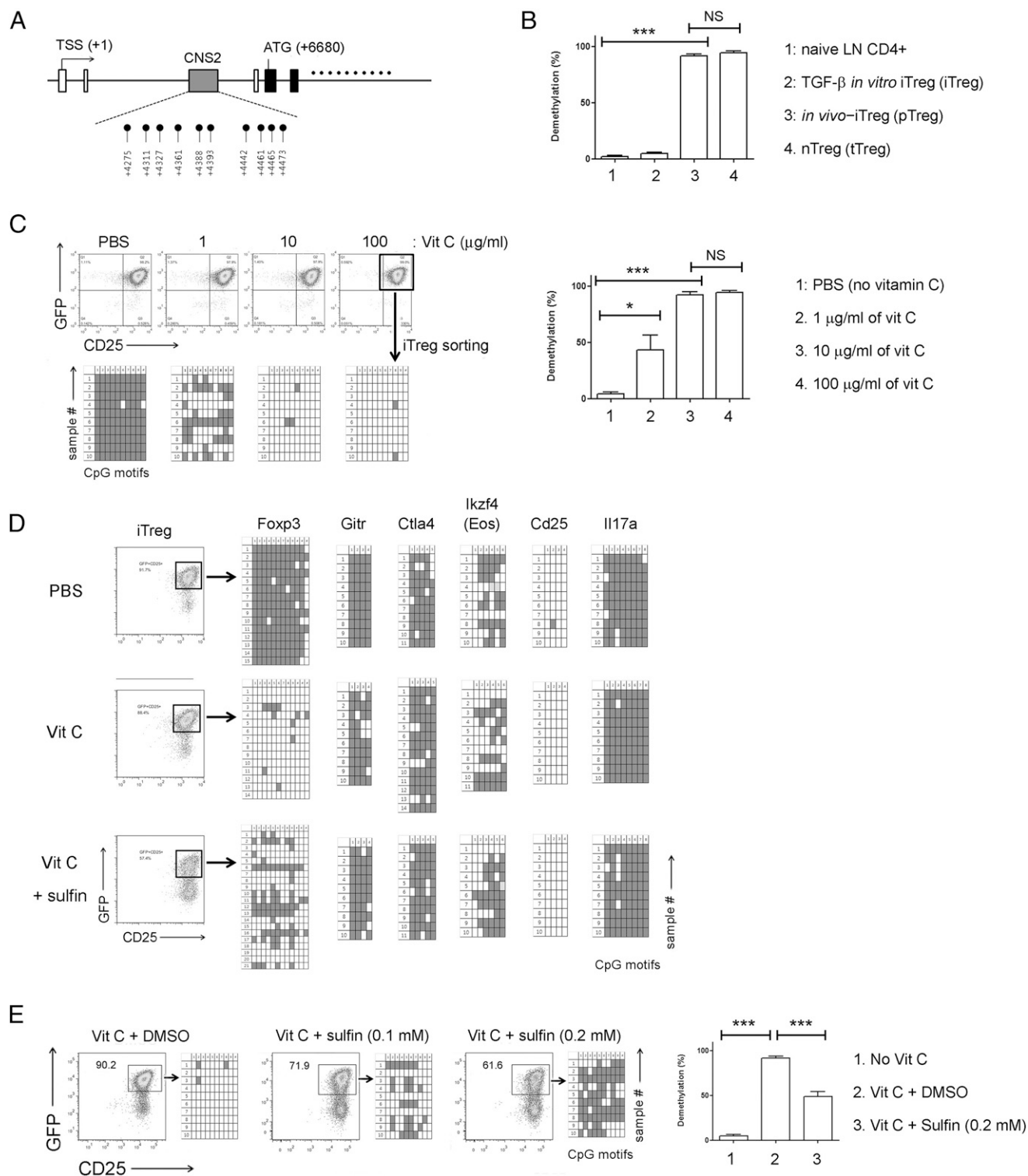
### Vitamin C induced CNS2 demethylation in iTregs

The Foxp3 CNS2 region contains 14 CpG motifs. We analyzed 10 CpG motifs in the sense (Fig. 1A) or 12 CpG motifs in the antisense strands of various Tregs, such as iTregs, pTregs, and tTregs. To get pTregs, naive CD4<sup>+</sup> T cells (CD4<sup>+</sup>CD8<sup>-</sup>GFP<sup>-</sup>CD44<sup>low</sup>) were FACS-sorted from the LNs and spleens of OT-II male mice having a Foxp3-GFP allele and injected i.v. into congenic WT mice fed with OVA. In 6 d, GFP-expressing donor T cells were sorted from the mesenteric LNs and used as pTregs. CD4<sup>+</sup>CD8<sup>-</sup>GFP<sup>+</sup> cells were FACS-sorted from the LNs and spleens of WT male mice having a Foxp3-GFP allele and were used as tTregs. The CpG motifs of CNS2 in pTreg and tTregs were demethylated as reported previously (14, 29). In the case of iTregs, the methylation pattern was opposite (Fig. 1B). These results prompted us to search for unknown factors leading to the CNS2 demethylation of pTregs, and we tested vitamin C on the basis of reports that had showed a relationship between vitamin C and DNA demethylation (41–44). Because 40–60  $\mu$ M (7.04–10.56  $\mu$ g/ml) vitamin C is detected in serum and RBCs (45, 46), we cultured naive CD4<sup>+</sup> T cells under iTreg conditions (anti-CD3/CD28, rIL2 and rTGF- $\beta$ ) with various doses of vitamin C (1, 10, and 100  $\mu$ g/ml). GFP<sup>+</sup> cells were FACS-sorted and subjected to CNS2 demethylation analysis. CNS2 in iTregs treated with vitamin C ( $\geq 10$   $\mu$ g/ml) was almost completely demethylated (Fig. 1C), suggesting that vitamin C works in a dose-dependent manner, and its effect reaches a plateau in 10  $\mu$ g/ml. To exclude the possibility that vitamin C works nonspecifically, like 5-azacytidine (23), we checked the methylation status of other Treg signature genes such as *Tnfrsf18* (Gitr), *Ctla4*, *Irf4* (Eos), *Cd25* (14), and *Il17a* (a negative control) (47). We found that vitamin C works specifically in the Foxp3 CNS2 region (Fig. 1D). We confirmed the effect of vitamin C once again using a sodium-dependent vitamin C transporter (SVCT) inhibitor, sulfapyrazone (44). Treatment with sulfapyrazone did not alter the expression level of Foxp3 on a single-cell basis, but it partially hindered CNS2 demethylation in vitamin C-treated iTregs in vitro (Fig. 1E).

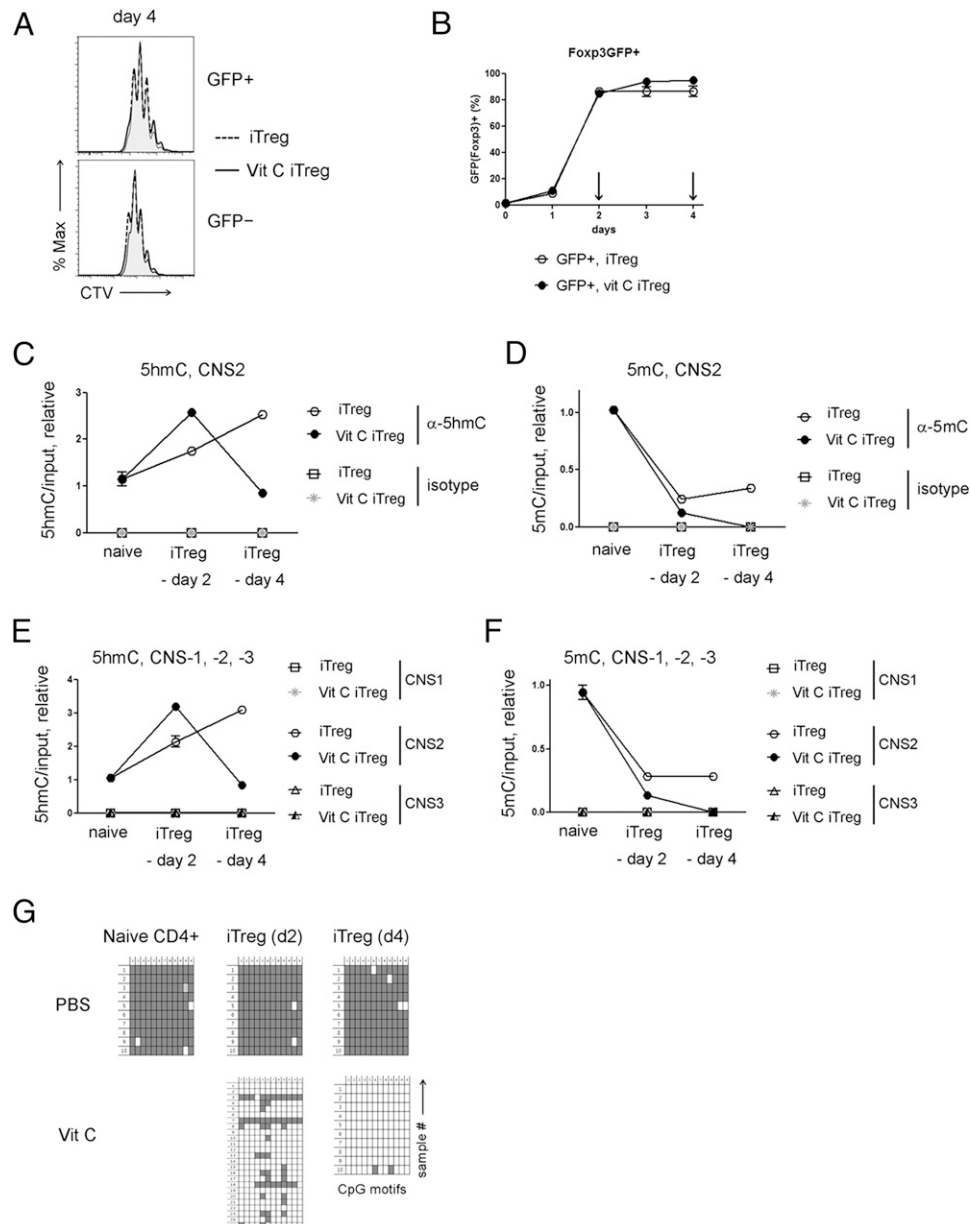
### Vitamin C accelerated active demethylation in iTregs

DNA demethylation occurs in two different manners: passive and active. Passive demethylation is caused by impaired transfer of CH<sub>3</sub><sup>-</sup> groups to a nascent DNA strand during cell division. Active DNA demethylation involves oxidation reactions of 5mC into oxidized methylcytosines, such as 5hmC, 5fC and 5caC. Eventually, the oxidized methylcytosines are replaced with unmodified cytosine in various ways, including passive dilution and base excision repair (35, 48, 49).

To investigate the underlying mechanisms of CNS2 demethylation induced by vitamin C, we checked cell proliferation by using flow cytometry. Naive CD4<sup>+</sup> T cells labeled with cell-division dye (CTV) were cultured under iTreg conditions for 4 d. The extent of dilution of CTV was similar in both the untreated and vitamin C-treated GFP<sup>+</sup> cells (iTregs) (Fig. 2A), suggesting the minor roles of passive demethylation. Vitamin C treatment did not change the proliferation of GFP<sup>+</sup> cells either. Next, the levels of 5hmC in the Foxp3 CNS2 region were investigated using DNA immunoprecipitation. Because GFP expression (Foxp3 transcription) started 1 d after iTreg culture and reached a plateau in 2 d (Fig. 2B), the enrichment of 5hmC was checked on day 0 (naive T cells), day 2 (early iTreg), and day 4 (late iTreg). As expected, the level of 5hmC in the CNS2 region was low in naive T cells (Fig. 2C), but reciprocally, the level of 5mC was the highest (Fig. 2D), suggesting that originally CpG motifs in CNS2 of naive T cells were methylated. As time passed, 5hmC was enriched in untreated and



**FIGURE 1.** CpG motifs of CNS2 are demethylated in iTreg cells treated with vitamin C. **(A)** A schematic of the first part of the *Foxp3* gene locus. White boxes indicate untranslated regions. ATG is located 6680 bases downstream of TSS (transcription start site, +1). CNS2 is indicated in gray, and the locations of 10 individual CpG motifs within CNS2 are also shown. **(B)** The extent of CNS2 demethylation in (1) naive CD4<sup>+</sup> T cells isolated from LNs, (2) iTreg, (3) pTreg, and (4) tTreg ( $n = 2$ ). **(C)** WT naive CD4<sup>+</sup> T cells were cultured under the iTreg condition in the presence of various doses of vitamin C (Vit C). GFP<sup>+</sup> CD25<sup>+</sup> iTreg cells were sorted and subjected to bisulfite sequencing to analyze the demethylation pattern of CNS2. Methylation status of individual 10 CpG motifs in the sense strands was shown by white (demethylation) or gray (methylation) colors. Results of statistical analysis are shown at right ( $n = 3$ ). **(D)** WT naive CD4<sup>+</sup> T cells were cultured under the iTreg condition in the presence of vitamin C (Vit C, 10  $\mu$ g/ml) with or without an SVCT inhibitor (sulfapyrazone, sulfin, 0.2 mM). GFP<sup>+</sup>CD25<sup>+</sup> iTregs were sorted and subjected to bisulfite sequencing to analyze the demethylation patterns of *Foxp3* CNS2, *Gitr*, *Ctla4*, *Ikzf4* (Eos), *Cd25*, and *Il17a*. Methylation status of individual CpG motif was shown by white (demethylation) or gray (methylation) colors. **(E)** WT naive CD4<sup>+</sup> T cells were cultured under the iTreg condition in the presence of vitamin C (Vit C, 10  $\mu$ g/ml) with or without an SVCT inhibitor (sulfapyrazone, sulfin). GFP<sup>+</sup>CD25<sup>+</sup> iTreg cells were sorted, and CNS2 demethylation was analyzed. Results of statistical analysis are shown at right ( $n = 2$ ). Numbers in the indicated area in FACS plots refer to the percentage of each subset. Data are representative of two (B and D) and three (C and E) independent experiments. \* $p < 0.05$ , \*\*\* $p < 0.001$ .



**FIGURE 2.** Vitamin C accelerated active demethylation of CNS2 in iTreg cells. WT naive CD4<sup>+</sup> T cells were cultured under the iTreg condition with or without vitamin C (10  $\mu$ g/ml). **(A)** CVT-labeled naive CD4<sup>+</sup> T cells were cultured under iTreg conditions with or without vitamin C. Dilution of CVT was analyzed after 4 d. **(B)** Changes in the frequency of GFP<sup>+</sup> iTregs over time. DNA was extracted on day 2 and day 4 (arrows) for DNA immunoprecipitation and CNS2 demethylation analysis. **(C)** Genomic DNA extracted from naive CD4<sup>+</sup>, iTreg, and vitamin C-treated iTregs on days 2 and 4 was immunoprecipitated with anti-5hmC ( $\alpha$ -5hmC) or isotype control Ab. qPCR was performed on precipitated DNA with CNS2 specific primers. **(D)** Genomic DNA was precipitated and analyzed as above (C) using anti-5mC ( $\alpha$ -5mC) or isotype control mAb. **(E)** Genomic DNA was precipitated by using anti-5hmC and subjected to qPCR with CNS1-, CNS2-, and CNS3-specific primers each. **(F)** Genomic DNA was precipitated using anti-5mC and subjected to qPCR with CNS1, CNS2, and CNS3 specific primers each. **(G)** Genomic DNA extracted from naive CD4<sup>+</sup>, iTreg, and vitamin C-treated iTreg cells on days 2 and 4 was subjected to bisulfite sequencing to analyze the demethylation pattern of *Foxp3* CNS2. Methylation status of 12 individual CpG motifs in the anti-sense strands is shown with white (demethylation) or gray (methylation) colors. Numbers in indicated area in FACS plots refer to the percentage of each subset. Data are representative of two independent experiments [(C–G) mean  $\pm$  SEM of triplicates].

vitamin C-treated iTregs with different kinetics. Although 5hmC was gradually enriched in the untreated iTregs, it was enriched and then disappeared abruptly in the vitamin C-treated iTregs (Fig. 2C). 5mC was decreased in both iTregs; however, in contrast to the untreated iTregs, where some 5mC remained, 5mC was not detected at all in the late vitamin C-treated iTregs (Fig. 2D). These findings suggest that 5mC was maintained, albeit at a low level and supplied continuously as a substrate for the production of 5hm in iTregs. Neither 5hmC (Fig. 2E) nor 5mC (Fig. 2F) was detected in the CNS1 and CNS3 regions. Finally, the methylation

status of individual CpG motifs was checked using bisulfite sequencing, and we found that CNS2 demethylation started to be achieved in early (day 2) vitamin C-treated iTregs (Fig. 2G). These findings indicate that vitamin C helps to complete the active demethylation of CNS2 more quickly, which otherwise would proceed slowly and inefficiently.

#### *Vitamin C works in a Tet2-dependent manner*

Because 5mC is known to be oxidized into 5hmC by Tet enzymes, we checked whether Tet enzymes are involved in vitamin C-induced



CNS2 demethylation. The expression pattern of each Tet was different from others during iTreg development and not affected by vitamin C treatment. In contrast to Tet1 and Tet3, Tet2 was upregulated, implying that the effects of vitamin C might be related to Tet2. Interestingly, the level of Dnmt1 increased substantially in both iTregs (Fig. 3A). These findings led us to study the role of Tet2 and Dnmt1 in iTregs. We crossed *Tet2<sup>fl/fl</sup>* mice with Lck-Cre transgenic mice to delete Tet2 in T cells (referred to as Lck-*Tet2<sup>-/-</sup>*) and found that *Tet2*, but not *Tet1* or *Tet3*, mRNA was greatly downregulated in DP and CD4SP thymocytes of Lck-*Tet2<sup>-/-</sup>* mice (Fig. 3B). Naive CD4<sup>+</sup> cells were FACS-sorted from WT (Lck-Cre × *Tet2<sup>+/+</sup>*) and Lck-*Tet2<sup>-/-</sup>* (Lck-Cre × *Tet2<sup>fl/fl</sup>*) mice, cultured under iTreg conditions with or without vitamin C. CNS2 demethylation was analyzed as before. CNS2 was more methylated in Lck-*Tet2<sup>-/-</sup>* iTregs in the presence of vitamin C (Fig. 3C). We also performed the same experiments using CD4-*Tet2<sup>-/-</sup>* (CD4-Cre × *Tet2<sup>fl/fl</sup>*) mice and obtained similar results (Supplemental Fig. 1). However, a high dose (100 μg/ml) of vitamin C induced CNS2 demethylation in *Tet2<sup>-/-</sup>* and in WT iTregs (data not shown), implying that other Tet proteins, such as Tet1 (31) and Tet3 (50), could be substituted for Tet2 in vitamin C-rich environments. These results led us to hypothesize that the overexpression of Tet2 could make vitamin C redundant and induce CNS2 demethylation without vitamin C. We introduced the plasmid encoding FLAG-tagged mouse *Tet2* into WT CD8<sup>+</sup> thymocytes (DN and CD4SP), sorted GFP<sup>+</sup>CD25<sup>+</sup>CD4SP cells, and then cultured under the iTreg condition for 5 d. GFP<sup>+</sup>CD25<sup>+</sup> iTregs were sorted again and used for the demethylation study. CNS2 in vector-transfected iTregs was methylated; however, *Tet2*-transfected iTregs showed fully demethylated CNS2 (Fig. 3D) and maintained Foxp3 expression pretty well, even without TGF-β (data not shown). In addition, it was notable that CNS2 in GFP<sup>+</sup>FLAG<sup>+</sup> cells cultured under iTreg condition was methylated like that in vector-transfected iTregs, suggesting that the factors required for Foxp3 transcription are also essential for CNS2 demethylation. Together, these findings indicate that vitamin C promotes CNS2 demethylation in a Tet2-dependent fashion, but it could be dispensable in cells with a high level of Tet2, such as thymic Tregs (fig. 6 in Ref. 30) and vice versa. Next, we tested the role of Dnmt1 in CNS2 demethylation using RNA interference. Naive CD4<sup>+</sup> T cells cultured under iTreg condition were infected by lentivirus coexpressing *Dnmt1* small-hairpin RNA (shRNA) and GFP, and then GFP<sup>+</sup> cells were FACS-sorted for CNS2 demethylation study. Depletion of Dnmt1 significantly enhanced CNS2 demethylation in iTregs (Fig. 3E).

#### Vitamin C-treated iTregs maintain Foxp3 in vitro and in vivo

Although CNS2 is required for protecting the Foxp3 expression of tTregs from inflammatory conditions (27, 28), the relationship between Foxp3 stability and CNS2 demethylation in iTregs is still unclear. Because iTregs having methylated and demethylated CNS2 were available, we decided to investigate the role of CNS2 demethylation in the Foxp3 stability of iTregs. FACS-sorted iTregs generated in the presence of vitamin C (0–100 μg/ml) were cultured with anti-CD3/CD28 plus rIL2 (without vitamin C and TGF-β), and Foxp3 expression was checked. Foxp3 expression in iTregs treated with ≥10 μg/ml of vitamin C was maintained stably even in 2 wk (Fig. 4A), which was consistent with the CNS2 demethylation results (Fig. 1C). Next, the suppressive activity of iTregs was checked by an in vitro suppression test. CTV-labeled CD4<sup>+</sup> responder cells (CD45.1<sup>+</sup>) were cocultured with APCs, Tregs (CD45.2<sup>+</sup>), and anti-CD3 mAb. In 3 d, the extent of proliferation of the responders and the Foxp3 expression of the Tregs were estimated with flow cytometry. The vitamin C-treated iTregs showed superior

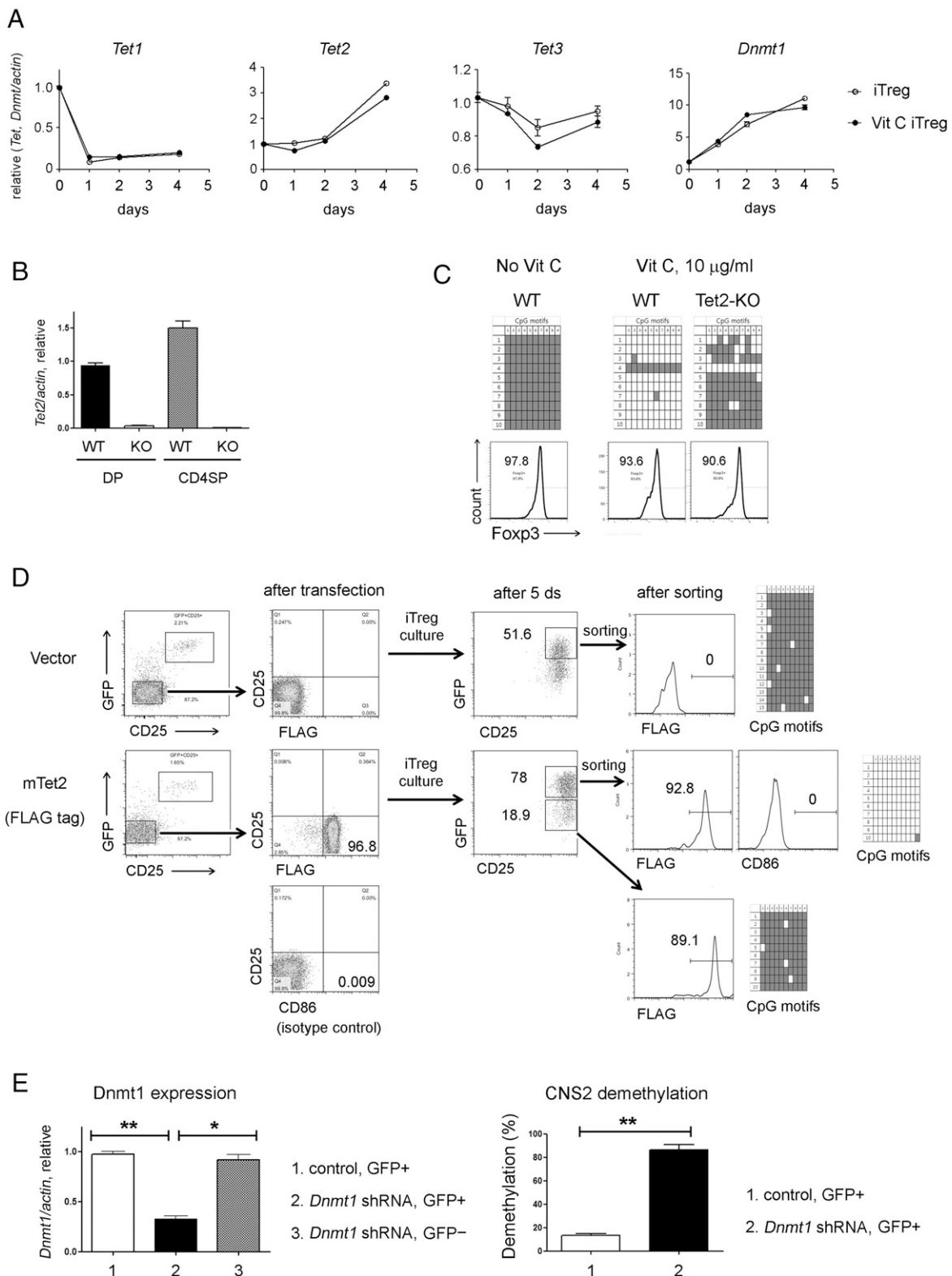
suppressive activity to iTregs (Fig. 4B, upper). Foxp3 was unstable in iTregs, especially with a higher responder to Treg ratio (Fig. 4B, lower). Because the environment of a higher responder-to-Treg ratio favors the division of Tregs, this finding led us to analyze the relationship between Foxp3 stability and cell division. CTV-labeled iTregs were cultured with rIL2 plus anti-CD3/CD28. In 3 d, Foxp3 was downregulated in iTregs, and the extent of downregulation was greater in cells under division (Fig. 4C), indicating that CNS2 demethylation is required to sustain the Foxp3 expression of dividing iTregs.

Next, we checked Foxp3 stability in vivo. OT-II iTregs were generated from naive OT-II CD4<sup>+</sup> T cells (which express OVA-specific TCR) in the presence or absence of vitamin C and injected i.v. into congenically marked WT B6 mice. The recipient mice were immunized with keyhole limpet hemocyanin (KLH) or OVA in CFA the next day and analyzed in 6–8 d by using flow cytometry. In case of KLH (irrelevant Ag), both OT-II iTregs in draining LNs maintained Foxp3 (Fig. 4D, left) and did not differentiate into T follicular regulatory cells expressing both CXCR5 and PD-1 (data not shown). This result implies that Foxp3 can be maintained in iTregs having methylated CNS2 even under inflammatory conditions unless there are relevant Ags. After immunization with OVA (cognate Ag) in CFA, approximately half of the OT-II iTregs, but not the vitamin C-treated OT-II iTregs, lost Foxp3 in draining LNs (Fig. 4D, right). The fate of the OT-II iTregs that lost Foxp3 (herein called ex-Foxp3 cells) was investigated. Interestingly, ex-Foxp3 cells generated from either iTreg or vitamin C-treated iTregs acquired T follicular helper (Tfh) phenotypes (Fig. 4E). Because this finding could be due to the difference of TCR repertoires (TCRs with a high affinity to OVA could allow iTregs to proliferate more vigorously and differentiate into the ex-Foxp3 cells with the Tfh phenotypes), we repeated the experiments using iTregs generated from OT-II TCR transgenic mice in a Rag-1-deficient background (OT-II/*Rag1<sup>-/-</sup>*) and obtained similar results on Foxp3 stability (Fig. 4F, left), cell proliferation (Fig. 4F, right), and the fate of ex-Foxp3 cells (Fig. 4G). Given that the IL2-CD25-STAT5 pathway is known to inhibit Tfh differentiation programs with the help of Blimp-1 (51–53), and CD25 was downregulated in ex-Foxp3 cells (data not shown), Foxp3 might prevent iTregs from getting Tfh phenotypes indirectly by enhancing IL2 signals.

Finally, to determine the effects of vitamin C on gene expression profiles of iTregs, we performed microarray experiments using naive CD4<sup>+</sup> T, iTreg, vitamin C-treated iTreg, and tTregs. Unexpectedly, only 50 probes were changed by more than 2-fold in vitamin C-treated iTregs, compared with iTregs, and 36 probes (72%) were upregulated. In gene-enrichment and functional annotation analysis, 15 genes (35.7%) were categorized into the plasma membrane term (GO: 0005886) and 9 of 15 were upregulated. In addition, five genes (11.9%, *CDKN1A*, *C3*, *TBX21*, *FCER1G*, *LAG3*) were related to ‘positive regulation of immune system process’ (GO: 0002684) and four of five genes were upregulated. Given that 1670 (or 1817) probes were changed by more than 2-fold between (vitamin C-treated) iTregs and tTregs, vitamin C did not seem to alter the gene expression profiles significantly in iTregs (Fig. 4H).

#### Vitamin C is essential for CNS2 demethylation in pTregs

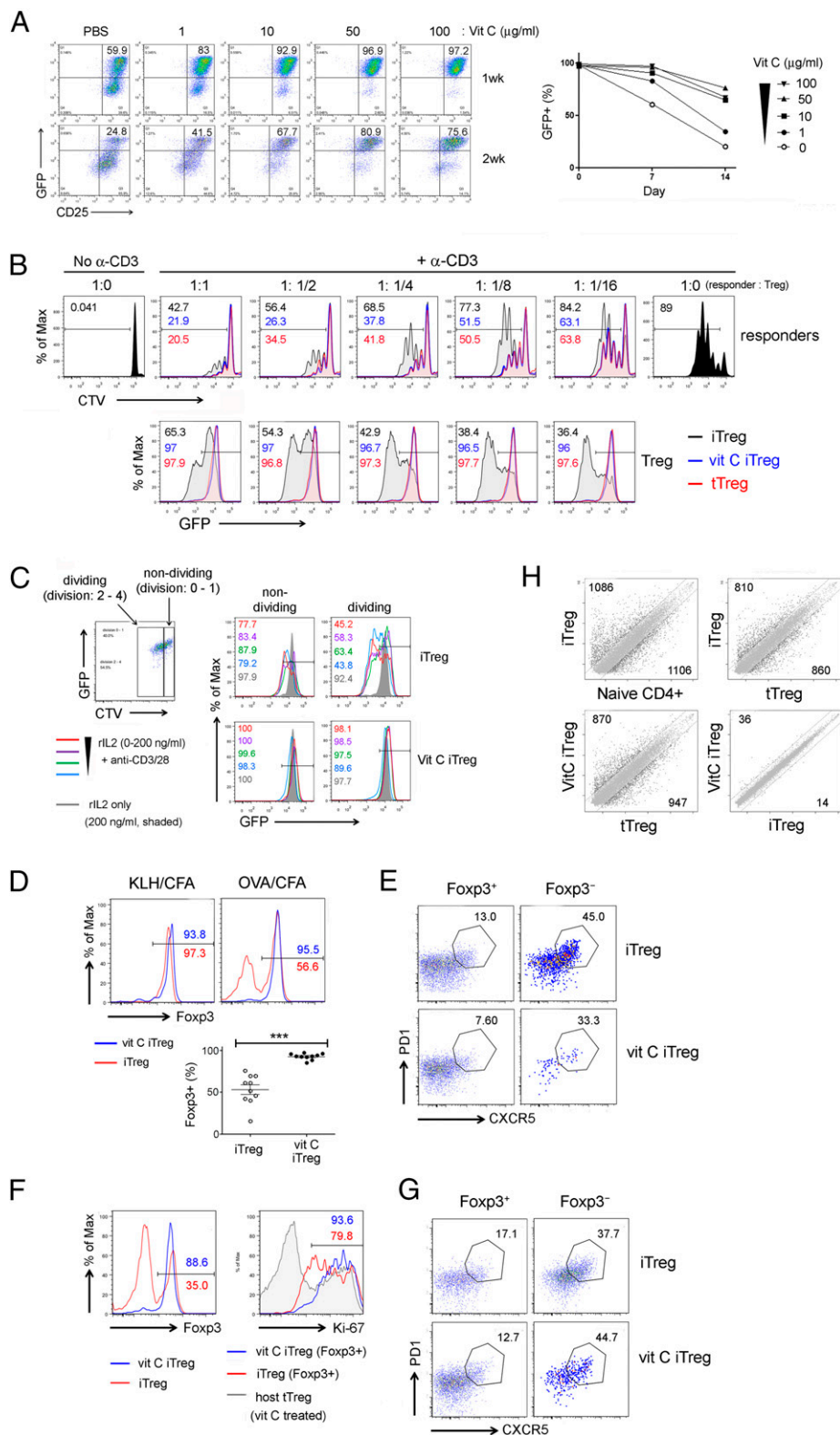
We sought to investigate the role of vitamin C in pTregs by using an SVCT inhibitor, sulfapyrazone (Fig. 1E), and the colitis model. Colitis was induced in *Rag1<sup>-/-</sup>* mice with the i.v. injection of  $0.7 \times 10^6$  naive CD4<sup>+</sup> T cells. The recipient mice were then injected i.p. with DMSO or sulfapyrazone every day and sacrificed after 14 d (Fig. 5A). Neither diarrhea nor weight loss was observed in



**FIGURE 3.** Vitamin C-induced CNS2 demethylation is mediated by Tet2. **(A)** qPCR was performed to assess the expression levels of *Tet1*, *Tet2*, *Tet3*, and *Dnmt1* transcripts in iTregs (0 d means naive CD4<sup>+</sup> T cells, mean  $\pm$  SEM of triplicates). **(B)** qPCR was performed to assess the expression levels of *Tet2* transcripts in DP and CD4SP thymocytes purified from WT and *Tet2*<sup>-/-</sup> thymi. **(C)** Naive CD4<sup>+</sup> T cells isolated from WT (Lck-Cre  $\times$  *Tet2*<sup>+/+</sup>) and *Tet2*<sup>-/-</sup> (Lck-Cre  $\times$  *Tet2*<sup>fl/fl</sup>, Tet2-KO) mice were cultured under the iTreg condition with or without vitamin C (10  $\mu$ g/ml). Because mice without Foxp3-GFP allele were used, all cultured cells were used without sorting, and Foxp3 expression was checked by intracellular staining separately (lower). **(D)** WT CD8<sup>+</sup> thymocytes were transfected with plasmids encoding vector or FLAG-tagged mouse *Tet2*. GFP<sup>+</sup>CD25<sup>+</sup>CD4SP cells were then sorted by FACS and cultured under iTreg conditions. After 5 d, GFP<sup>+</sup>CD25<sup>+</sup> iTregs were sorted and subjected to bisulfite sequencing to analyze CNS2 demethylation. FLAG expression was checked by intracellular staining, and anti-CD86 mAb was used as an isotype control for anti-FLAG mAb. **(E)** Naive CD4<sup>+</sup> T cells isolated WT mice without Foxp3-GFP allele were transduced with lentivirus containing GFP and scrambled (control) or *Dnmt1* shRNA, and cultured under iTreg conditions. GFP<sup>+</sup>CD25<sup>+</sup> cells were sorted and subjected to bisulfite sequencing to analyze CNS2 demethylation (right). qPCR (Figure legend continues)



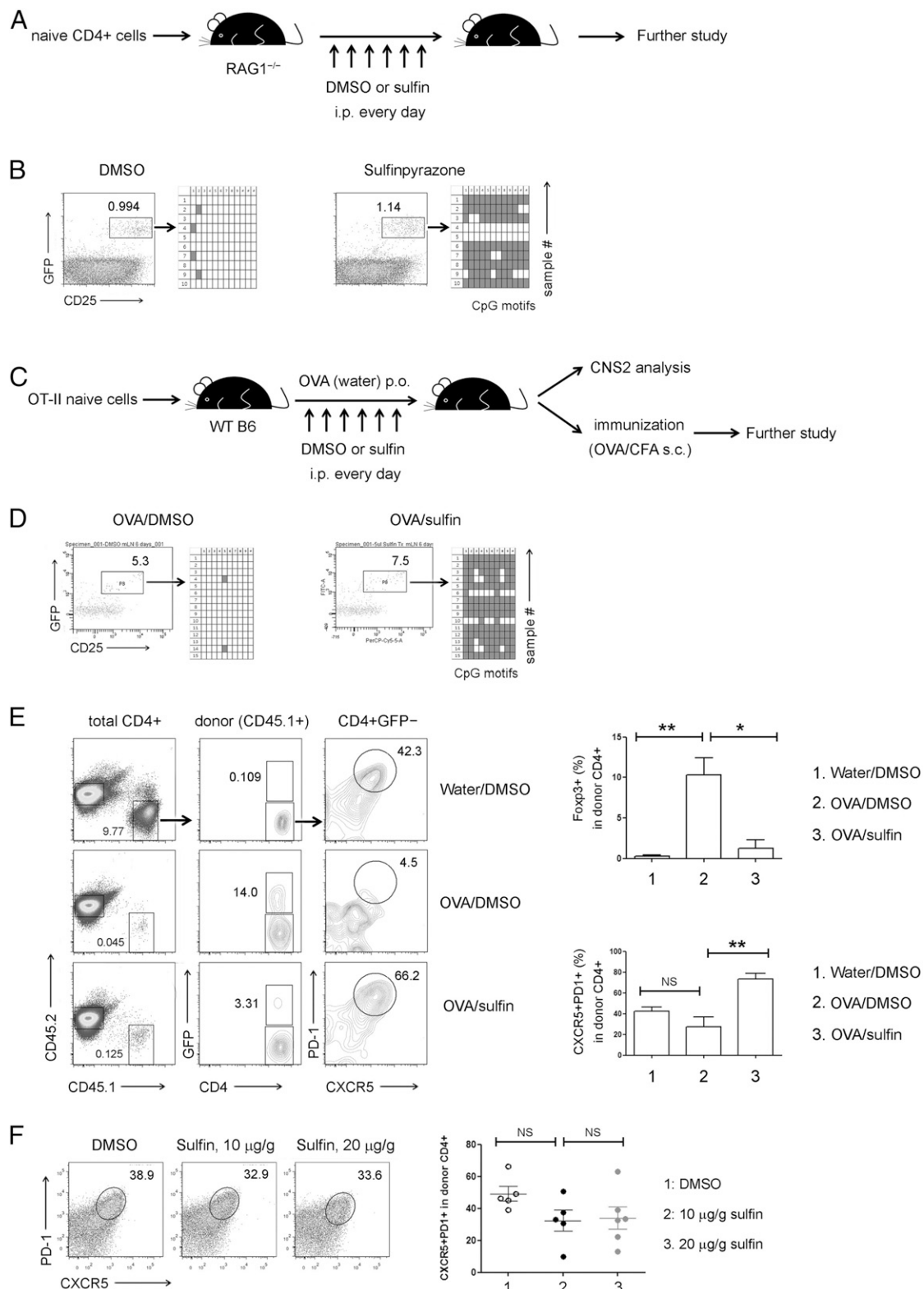
**FIGURE 4.** Vitamin C-treated iTreg cells maintain Foxp3. **(A)** FACS-sorted GFP<sup>+</sup>CD25<sup>+</sup> iTregs generated in the presence of various doses of vitamin C (Vit C, 0–100  $\mu$ g/ml) were cultured in the presence of anti-CD3/CD28 plus rIL2 (200 ng/ml) and subjected to FACS analysis in 1 and 2 wk. The frequencies of GFP<sup>+</sup> cells are shown on the right (mean  $\pm$  SEM of triplicates). **(B)** CTV-labeled naive CD4<sup>+</sup> T cells were cultured in the presence of congenically labeled iTregs, vitamin C-treated iTregs, or tTregs. The levels of CTV (upper) and GFP (lower) were checked in 3 d. **(C)** CTV-labeled iTregs or vitamin C-treated iTregs were cultured in the presence of anti-CD3/CD28 plus various doses (0–200 ng/ml) of rIL2. iTregs were separated by the extent of the dilution of CTV (dividing versus nondividing, left) and then GFP expression was checked in nondividing and dividing cells separately. **(D–G)** Vitamin C-treated and untreated iTregs generated from naive OT-II (D and E) or OT-II/*Rag1*<sup>-/-</sup> T cells (F and G) were injected into congenically marked WT B6 mice. Recipient mice were immunized with KLH or OVA emulsified with CFA the next day. On day 6–8, draining LNs were analyzed using flow cytometry. **(D)** The frequencies of Foxp3<sup>+</sup> cells in OT-II iTregs. Each circle in the graph (lower) indicates the result from an individual mouse; small horizontal lines indicate the mean ( $\pm$  SEM). **(E)** The expression patterns of CXCR5 and PD1 were analyzed in Foxp3<sup>+</sup> and Foxp3<sup>-</sup> populations of OT-II iTregs separately. **(F)** The frequencies of Foxp3<sup>+</sup> (left) or Ki-67<sup>+</sup> (right) cells in OT-II/*Rag1*<sup>-/-</sup> iTreg cells. **(G)** The expression patterns of CXCR5 and PD1 were analyzed in Foxp3<sup>+</sup> and Foxp3<sup>-</sup> populations of OT-II/*Rag1*<sup>-/-</sup> iTreg cells separately. **(H)** Comparison plots between naive CD4<sup>+</sup> T cells, ex vivo tTregs (tTreg), iTregs, and vitamin C-treated iTregs (VitC iTreg). Numbers indicate the number of probes whose expression differed by more than 2-fold. Numbers in indicated area in FACS plots refer to the percentage of each subset. Data are representative of two (A–G) or one (H) independent experiments. \*\*\**p* < 0.001.



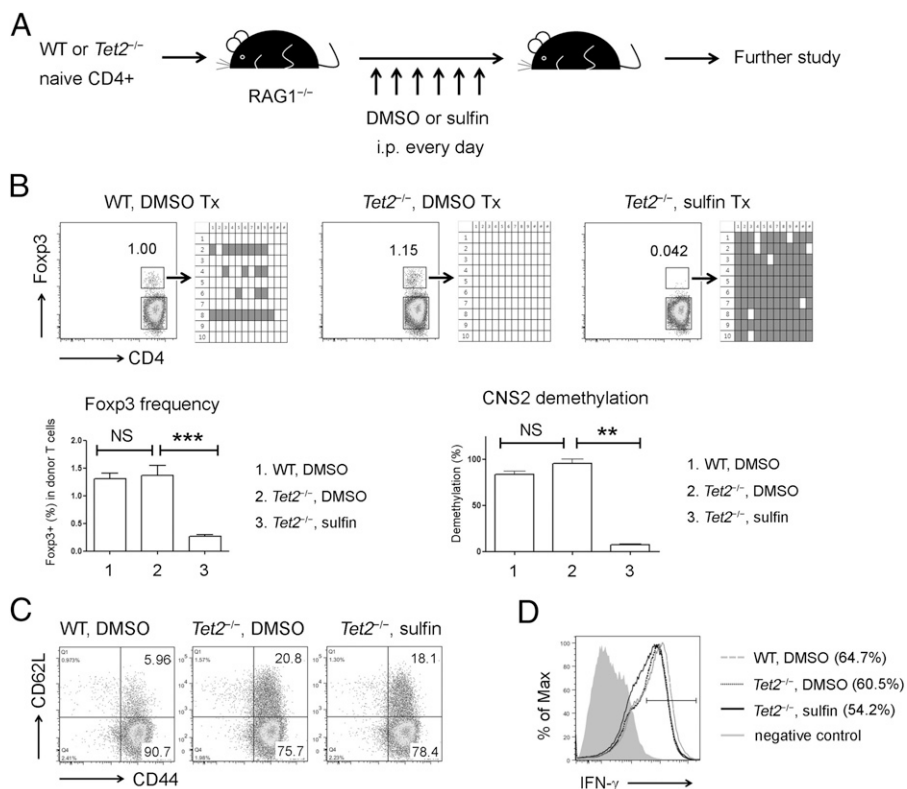
any mice (data not shown) during the period of study. FACS analysis of the transferred T cells revealed that the frequency of GFP<sup>+</sup> T cells was not changed by sulfinpyrazone treatment, reflecting that the generation of pTregs was not dependent on

vitamin C. GFP<sup>+</sup> pTregs were FACS-sorted and subjected to CNS demethylation analysis. Although CNS2 of pTregs treated with DMSO was demethylated, as reported previously (14), sulfinpyrazone treatment inhibited CNS2 demethylation (Fig. 5B).

was performed to analyze the mRNA levels of *Dnmt1* in the sorted cells (left, mean  $\pm$  SEM of duplicates). Methylation status of individual CpG motifs was shown by white (demethylation) or gray (methylation) colors (C and D). Numbers in indicated area in FACS plots refer to the percentage of each subset. Data are representatives of two independent experiments. \**p* < 0.05, \*\*\**p* < 0.01.



**FIGURE 5.** CNS2 demethylation in pTreg cells is impaired by an SVCT inhibitor. **(A)** Experiment scheme. Freshly isolated naive CD4<sup>+</sup> T cells were injected i.v. into Rag1<sup>-/-</sup> mice. The recipient mice were treated with sulfinpyrazone (sulfin) every day (arrows). On day 14, pTregs (GFP<sup>+</sup>CD25<sup>+</sup>) were sorted from the mesenteric LNs and spleen to analyze CNS2 demethylation. **(B)** GFP<sup>+</sup>CD25<sup>+</sup> pTregs were sorted and subjected to CNS2 demethylation analysis. **(C)** Experiment scheme. Freshly isolated naive OT-II T cells were injected i.v. into congenically labeled WT B6 mice. The recipient mice were fed with OVA containing water and treated with sulfinpyrazone (sulfin) every day. On day 6, pTregs were sorted for CNS2 demethylation assay or mice were immunized with OVA/CFA for further study. **(D)** GFP<sup>+</sup>CD25<sup>+</sup> OT-II pTreg cells of donor origin were sorted from the mesenteric LNs and subjected to CNS2 demethylation analysis. **(E)** On day 6, the recipient mice were immunized with OVA in CFA. After 6–8 d, the frequencies of Tregs (middle plots) and Tfh cells (right plots) of the donor origin (CD45.1<sup>+</sup>CD45.2<sup>-</sup>) were analyzed. Statistical results (n = 3) are shown at right. **(F)** Freshly isolated naive OT-II T cells were injected i.v. into congenically labeled WT B6 mice. Recipient mice were immunized with OVA/CFA and simultaneously treated with DMSO or sulfinpyrazone (sulfin) every day. On day 6, the frequencies of Tfh cells of the donor origin were analyzed. Statistical results (Figure legend continues)



**FIGURE 6.** SVCT inhibitor treatment significantly prevented the generation of *Tet2*<sup>-/-</sup> pTreg cells in the colitis model. **(A and B)** Naive CD4<sup>+</sup> T cells isolated from WT or CD4-*Tet2*<sup>-/-</sup> mice were injected i.v. into *Rag1*<sup>-/-</sup> mice. Recipient mice were treated with DMSO or sulfinpyrazone (sulfin) every day. On day 14, pTregs (CD4<sup>+</sup> Foxp3<sup>+</sup>) were sorted from the mesenteric LNs and spleen for CNS2 demethylation analysis. The frequency of Foxp3<sup>+</sup> cells and their CNS demethylation patterns are shown ( $n = 2$ ). **(C)** CD44 and CD62L expression of CD4<sup>+</sup>Foxp3<sup>+</sup> cells of donor origin. **(D)** Intracellular staining for IFN- $\gamma$  in Foxp3<sup>+</sup> cells of donor origin (CD4<sup>+</sup>TCR $\beta$ <sup>+</sup>Foxp3<sup>+</sup>) after PMA/ionomycin stimulation. Numbers in FACS plots refer to the percentage of each subset. Data are representative of two independent experiments. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

To confirm the above results, we repeated the CNS2 demethylation study using the oral tolerance model (5, 54). FACS-sorted naive OT-II T cells were transferred into congenically marked WT B6 mice followed by the administration of OVA to induce pTregs. Simultaneously, sulfinpyrazone was injected i.p. every day to prevent the entry of vitamin C into the cells (Fig 5C). GFP<sup>+</sup>CD25<sup>+</sup> OT-II pTregs were generated at similar rates in both DMSO- and sulfinpyrazone-treated mice. CNS2 in pTregs treated with DMSO was demethylated; however, sulfinpyrazone prevented CNS2 demethylation as shown in the colitis model (Fig. 5D). To address the functional implications, we also immunized the mice with OVA plus CFA and checked Foxp3 expression and Tfh cell differentiation. As expected, the frequency of Foxp3<sup>+</sup> pTregs was significantly lower in mice treated with OVA/sulfinpyrazone than in mice treated with OVA/DMSO (Fig. 5E). In mice treated with DMSO without OVA, few transferred OT-II T cells were converted into Foxp3<sup>+</sup> pTregs. Next, we analyzed the phenotypes of donor GFP<sup>+</sup> cells and found more Tfh cells in mice treated with OVA/sulfinpyrazone than in mice treated with OVA/DMSO (Fig. 5E). To exclude the possibility that sulfinpyrazone directly promotes Tfh responses, we transferred naive OT-II T cells into WT mice, which were immunized with OVA plus CFA and simultaneously treated with DMSO or sulfinpyrazone every day. The frequency of Tfh cells did not increase in mice treated with sulfinpyrazone (Fig. 5F), suggesting that sulfinpyrazone did not directly promote the development of Tfh cells. Collectively, these findings imply that vitamin C is required for CNS2 demethylation of pTregs; otherwise, the expression of Foxp3 could be unstable in the subsequent encounter with the cognate Ags.

Next, we undertook colitis experiments using naive CD4<sup>+</sup> T cells isolated from CD4-*Tet2*<sup>-/-</sup> mice to investigate whether *Tet2* deficiency could synergize with sulfinpyrazone. In CD4-*Tet2*<sup>-/-</sup>

mice, the transcripts of the *Tet2* gene were reduced in DP thymocytes and not detectable in CD4SP thymocytes (data not shown), showing that Cre-mediated ablation of the expression of *Tet2* was efficient and began in the thymic stage of T cell development. Naive CD4<sup>+</sup> T cells isolated from WT or CD4-*Tet2*<sup>-/-</sup> mice were transferred to *Rag1*<sup>-/-</sup> mice, which were treated with DMSO or sulfinpyrazone every day for 14 d (Fig. 6A). Neither colitis symptoms, such as diarrhea and weight loss, nor histologic changes were observed in any mice (data not shown). The frequency of Foxp3<sup>+</sup> pTregs was similar in WT and *Tet2*<sup>-/-</sup> cells in case of DMSO treatment. However, sulfinpyrazone greatly restricted the pTreg differentiation of *Tet2*<sup>-/-</sup> naive CD4<sup>+</sup> T cells (Fig. 6B). Furthermore, CNS2 was methylated in *Tet2*<sup>-/-</sup> pTregs treated with sulfinpyrazone (Fig. 6B). In contrast to the difference in pTregs, WT and *Tet2*<sup>-/-</sup> conventional CD4<sup>+</sup> T cells showed similar phenotypes in cell surface molecules such as CD44, CD62L (Fig. 6C), and CD25 (data not shown), and cytokines such as IFN- $\gamma$  (Fig. 6D) and IL17 (data not shown).

#### Redundant functions of vitamin C and *Tet2* in CNS2 demethylation of tTregs

The findings obtained from the above study led us to test the role of vitamin C in tTreg development. WT newborn (2 d old) mice were treated with DMSO, sulfinpyrazone, or vitamin C for 14 d, and CNS2 demethylation was analyzed using Tregs in the thymus and periphery. In contrast to results with iTregs, neither vitamin C nor sulfinpyrazone made any significant change in the methylation status of WT tTregs (Fig. 7A). Next, we performed a CNS2 demethylation study using CD4-*Tet2*<sup>-/-</sup> mice. After 14 d, no significant abnormality in general appearance, the size of lymphoid organs (the thymus, LN, and spleen), or the frequency of

( $n = 5-6$ ) are shown at right. Methylation status of individual CpG motifs was shown by white (demethylation) or gray (methylation) colors. Numbers in indicated area in FACS plots refer to the percentage of each subset. Data are representative of two (B, D, and E) or one (F) independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ . OVA/sulfin, OVA/sulfinpyrazone; Water/DMSO, DMSO without OVA.



tTregs was found in the  $CD4\text{-}Tet2^{-/-}$  mice treated with DMSO or sulfinpyrazone (data not shown), suggesting that Tet2 and vitamin C do not have a profound influence on T cell development. Next, we isolated tTregs from the thymus, LNs, and spleens and analyzed them for CNS2 demethylation. Deletion of *Tet2* partially reduced the CNS2 demethylation of tTregs in the thymus (36) and synergized with sulfinpyrazone, preventing CNS2 demethylation efficiently (Fig. 7B). We next analyzed CpG demethylation of other Treg signature genes, such as *Ctla4*, *Irf4* (Eos), and *Cd25*. Consistent with the results from iTreg study (Fig. 1D), CpG demethylation patterns of *Ctla4*, *Irf4* (Eos) and *Cd25* were not significantly different between WT and  $Tet2^{-/-}$  tTregs treated with DMSO or sulfinpyrazone (Fig. 7C).

## Discussion

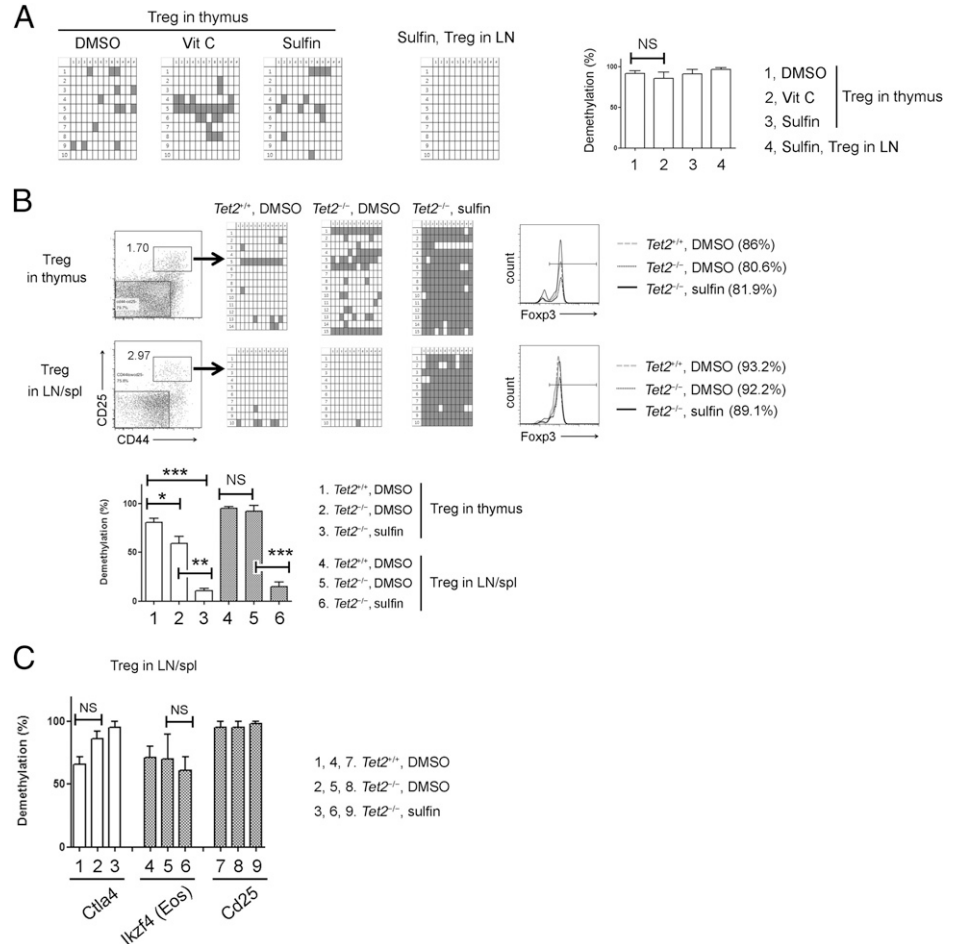
In this study, we showed that vitamin C promotes Tet-mediated active DNA demethylation of *Foxp3* CNS2 region in Tregs. 5mC in *Foxp3* CNS2 was converted into 5hmC in iTregs. Because it proceeded slowly and incompletely, however, 5hmC failed to be oxidized iteratively and replaced with unmethylated cytosines. Vitamin C greatly accelerated these reactions and converted 5mC into unmethylated cytosine efficiently (Fig. 2) in a Tet2-dependent manner (Fig. 3C). This finding suggests that it is the low activity of Tet2 that leads to CNS2 methylation in iTregs, which led us to hypothesize that CNS2 might be demethylated in iTregs expressing a higher level of Tet2. Indeed, CNS2 became demethylated in iTregs overexpressing Tet2, even without vitamin C (Fig. 3D). We next extended the study to pTregs and found that the CNS2 methylation status of pTregs was also dependent on vitamin C. The only difference between iTregs and pTregs was that the

role of Tet2 was somewhat redundant in pTregs. Given that the CNS2 of  $Tet2^{-/-}$  iTregs was demethylated in the presence of a high level (100  $\mu\text{g/ml}$ ) of vitamin C in vitro, these results suggest that vitamin C is readily available in vivo, which allowed *Tet1* and *Tet3* to be substituted for *Tet2*. Indeed, SVCT inhibitor treatment significantly prevented CNS2 demethylation and downregulated *Foxp3* expression in  $Tet2^{-/-}$  pTregs (Fig. 6B). Altogether, our study showed that the cooperation of Tet2 and vitamin C is essential for CNS2 demethylation and the level of *Foxp3* expression.

Vitamin C is known to be not only a general anti-oxidant, but also a cofactor for a large family of enzymes known as iron- and 2-oxoglutarate-dependent dioxygenases (55). A typical example is the collagen prolyl-4-hydroxylase that is involved in collagen maturation and scurvy. In the absence of vitamin C, the initial hydroxylation catalyzed by the collagen prolyl-4-hydroxylase proceeds, albeit less efficiently. However, the catalytically inactive oxidized iron species accumulates soon and lowers the activity of collagen prolyl-4-hydroxylase, leading to an incomplete hydroxylation of residues in collagen (56, 57). These studies reveal that vitamin C plays a role in reducing inactive iron and maintaining continued enzyme cycling. Because Tet dioxygenases are also dependent on iron and 2-oxoglutarate (32, 58), and the catalytic cycle of the iron- and 2-oxoglutarate-dependent dioxygenase is highly conserved (59), it is tempting to speculate that vitamin C might help Tet enzymes like collagen prolyl-4-hydroxylase enzymes. Indeed, although the hydroxylation of 5mC into 5hmC was initiated and 5hmC was enriched in the *Foxp3* CNS2 regions of iTregs, further oxidation reaction was not completed (Fig. 2); it was restored by adding vitamin C (Fig. 2, Supplemental Fig. 2). This idea also led us to hypothesize that

**FIGURE 7.** Vitamin C is required for CNS2 demethylation in  $Tet2^{-/-}$  tTregs.

(A) WT newborn mice were treated i.p. with DMSO, sulfinpyrazone, or vitamin C for 14 d. Next, GFP<sup>+</sup>CD25<sup>+</sup> tTregs were FACS-sorted from the thymus and periphery (LNs and spleens) and CNS2 demethylation was investigated. Results of statistical analysis are shown at right ( $n = 3$ ). (B) WT and  $CD4\text{-}Tet2^{-/-}$  newborn mice were treated as in (A). Next,  $CD4^+CD25^+$  tTregs were FACS-sorted from the thymus and periphery (LNs and spleens) and *Foxp3* CNS2 demethylation was investigated. Because mice without a *Foxp3*-GFP allele were used, *Foxp3* expression was checked by intracellular staining separately (right). Results of statistical analysis are shown (lower,  $n = 2$ ). (C) The demethylation patterns of *Ctla4*, *Irf4* (Eos), and *Cd25* were analyzed in WT and  $CD4\text{-}Tet2^{-/-}$  tTregs, and results of statistical analysis were demonstrated ( $n = 2$ ). Methylation status of individual CpG motif is shown in white (demethylation) or gray (methylation). Data are representative of one (A) or two (B and C) independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .





vitamin C could be redundant if Tet proteins are expressed so highly that newly generated Tet proteins could be substituted for inactivated used ones. Indeed, iTregs overexpressing Tet2 showed fully demethylated CNS2 (Fig. 3D).

It was previously shown that CNS2 is essential for the stable expression of Foxp3 in tTregs, and methylated CpG motifs prevented critical transcription factors from binding to CNS2 regions (26). However, the direct comparison between iTregs having methylated and demethylated CNS2 had not yet been performed, probably because of the lack of appropriate models. In this study, we were able to compare the effect of CNS2 demethylation on the expression of Foxp3 by using vitamin C-treated iTregs. As expected, Foxp3 expression was stable in vitamin C-treated iTregs in vitro and in vivo. Next, we compared the gene expression profiles of iTreg and vitamin C-treated iTregs and failed to find a significant difference between them (Fig. 4H). Fifty probes were changed by more than 2-fold, and only five genes were associated with positive regulation of immune system process GO terms (Fig. 2D). These results demonstrate that changes in DNA demethylation per se do not always lead to changes in gene expression, at least in iTregs, probably because of the availability of transcription factors and other required epigenetic processes (e.g., histone modifications); it is CNS2 demethylation itself, not indirect pathway regulated by vitamin C, that endows iTregs with Foxp3 stability.

Vitamin C is known to enter and accumulate within cells through SVCTs and treatment with sulfapyrazone, an SVCT inhibitor, was reported to prevent the effect of vitamin C on 5hmC in vitro (44). To verify the roles of SVCTs in CNS2 demethylation, iTregs were generated in the presence of an SVCT inhibitor, sulfapyrazone, and subjected to CNS2 demethylation study. The results showed that sulfapyrazone reduced the effect of vitamin C on CNS2 demethylation in both iTregs (Fig. 1E) and pTregs (Figs. 5–6), suggesting that the uptake of vitamin C through SVCTs is critical in the Tet-mediated CNS2 demethylations. Exceptionally, thymically derived Tregs were resistant to sulfapyrazone treatment. All CpG motifs in the Foxp3 CNS2 locus were demethylated in tTregs, irrespective of sulfapyrazone and vitamin C treatment (Fig. 7A). These results led us to hypothesize that a lack of sensitivity of tTregs to vitamin C might be caused by the high levels of Tet expression (30). Indeed, Foxp3 CNS2 in Tet2<sup>-/-</sup> tTregs was methylated after sulfapyrazone treatment. We expected that Tet2<sup>-/-</sup> mice treated with sulfapyrazone would develop inflammatory diseases; however, autoimmune diseases characterized by tissue inflammation and immune cell activation did not develop spontaneously at least until the mice were 8 wk old (data not shown). These issues are actively under investigation.

It was reported that the loss of Tet1 or Tet2 led to hematologic cancers (37, 60, 61), which are associated with low levels of 5hmC in genomic DNA (62). These results suggest that the defect in vitamin C metabolism might also be involved in cancer development or exacerbate the progression of cancers having Tet mutations. Indeed, gene polymorphisms in SVCTs have been reported to be associated with lymphoma (63), leukemia (the enhanced expression of truncated SVCT mRNAs) (64), and gastric cancer (65). Although the functional consequences of these variants remain largely unclear, these studies indicate that SVCTs and vitamin C might be associated with abnormalities of 5hmC in cancer cells.

Although it has been reported that Tregs could be used as novel immune suppressants in various settings, including transplantation, iTregs have never been considered as a source of Tregs for therapeutic purposes because of the issues related to CNS2 demethylation and Foxp3 stability (66). Because vitamin C-treated iTregs have demethylated CNS2 and express Foxp3 stably, we checked the immune suppressive potential of vitamin C-treated iTregs by using

the allogeneic skin graft model. WT B6 mice were grafted with the tail skin of BALB/c mice immediately after administration of vitamin C-treated iTregs induced by BALB/c dendritic cells, and monitored for 2 wks. Unfortunately, vitamin C-treated iTregs failed to delay graft rejection probably because niches for the survival of transferred iTregs are limited in lympho-replete mice (V.S. Nair and K.I. Oh, unpublished observations). Further studies on the immunoregulatory function of allospecific vitamin C-treated iTregs in diverse transplantation models seem to be warranted.

In conclusion, our study shows that vitamin C is required during the development of various Tregs, and environmental factors such as nutrients could influence immune homeostasis through epigenetic mechanisms.

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## Disclosures

The authors have no financial conflicts of interest.

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