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Arginine methylation of FOXP3 is crucial for the suppressive function of regulatory T cells

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ABSTRACT

Forkhead box transcription factor 3 (FOXP3) plays a pivotal role in the suppressive function of regulatory T cells. In addition to mRNA levels, FOXP3 activity can also be controlled by posttranslational mechanisms, which have not been studied in a comprehensive manner. Through extensive screening using selective inhibitors, we demonstrate that the inhibition of type I protein arginine methytransferases (PRMTs) attenuates the suppressive functions of regulatory T cells. FOXP3 undergoes methylation on arginine residues at positions 48 and 51 by interacting with protein arginine methyltransferase 1 (PRMT1). The inhibition of arginine methylation confers gene expression profiles representing type I helper T cells to FOXP3$^+$ T cells, which results in attenuated suppressive activity. A methylation-defective mutant of FOXP3 displays less potent activity to suppress xenogeneic graft-versus-host disease *in vivo*. These results elucidate an important role of arginine methylation to enhance FOXP3 functions and are potentially applicable to modulate regulatory T cell functions.

1. Introduction

Regulatory T (Treg) cells are required for the control of multiple immunological reactions. While Treg cells are essential for the maintenance of self-tolerance and immune homeostasis under normal conditions, they infiltrate into tumor tissues and can hamper antitumor immune responses [1,2]. The forkhead family transcription factor FOXP3 is an indispensable molecule in the development and function of Treg cells. Mice that carry a loss-of-function mutation in the *Foxp3* gene develop a fatal lymphoproliferative disease characterized by hyper-responsive CD4$^+$ T cells [3]. In humans, genetic mutations of FOXP3 induce autoimmune phenotypes known as immune dysfunction, polyendocrinopathy, enteropathy, and X-linked (IPEX) syndromes [4]. In addition, ectopic expression of FOXP3 can confer, at least partly, suppressive phenotypes to conventional CD4$^+$ T cells, further demonstrating its central role in Treg function [5,6].

Stable transcriptional activity of FOXP3 is necessary for functional Treg cells. Constitutive FOXP3 transcription is accomplished by Treg cell-specific DNA demethylation and histone acetylation in the FOXP3 promoter region [7,8]. In addition to mRNA levels, FOXP3 activity is also regulated by posttranslational mechanisms. Posttranslational protein modifications are mediated by “writers” and “erasers”, the enzymes that add or remove specific modifications, respectively. The modified proteins are recognized by proteins containing “reader” domains [9]. Early studies have elucidated that posttranslational modification of histone proteins significantly affects the chromatin accessibility and recruitment of specific transcription factors that recognize the modified histones. Accumulating evidence suggests that these histone modifiers and readers also target non-histone proteins and modulate their functions [10–13]. Recent studies revealed that FOXP3 also undergoes multiple posttranslational modifications [14]. FOXP3 is ubiquitinated at multiple lysine residues, which promotes its degradation [15–17].

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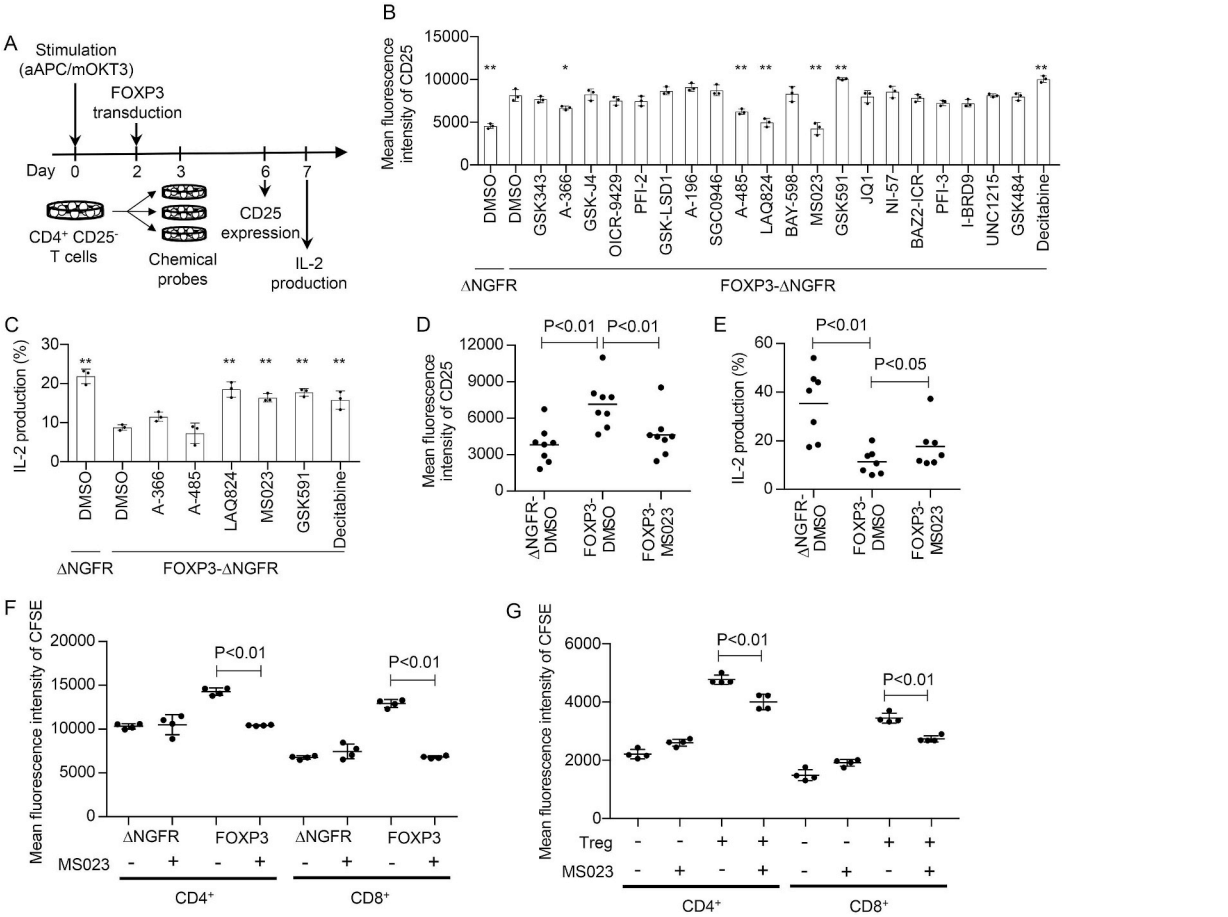

Fig. 1. Exploration of epigenetic/posttranslational targets that modulate FOXP3 function. (A–C) $CD4^+$ $CD25^-$ T cells were stimulated with artificial antigen-presenting cells (aAPC/mOKT3) and transduced with truncated nerve growth factor receptor (ΔNGFR) alone or FOXP3-P2A-ΔNGFR. The FOXP3-transduced T cells were individually treated with chemical probes. The data shown are CD25 expression levels in the $CD4^+$ $\Delta NGFR^+$ T cell population on day 6 (B) and IL-2 production in the $CD4^+$ $\Delta NGFR^+$ T cell population upon restimulation with aAPC/mOKT3 on day 7 (C; n = 3 technical replicates, one-way ANOVA with Tukey's multiple comparisons test). * $P < 0.05$, ** $P < 0.01$ compared with the T cells transduced with FOXP3-ΔNGFR and treated with DMSO. (D and E) $CD4^+$ $CD25^-$ T cells transduced with ΔNGFR or FOXP3-P2A-ΔNGFR were treated with DMSO or MS023. CD25 expression and IL-2 production were analyzed on days 6 and 7, respectively (n = 8 or 7 different donor samples, repeated measures one-way ANOVA with Tukey's multiple comparisons test). (F and G) CFSE-labeled $CD3^+$ T cells were stimulated with aAPC/mOKT3 in the presence of ΔNGFR alone or FOXP3-ΔNGFR-transduced $CD4^+$ T cells (F) or peripheral blood regulatory T cells (G) with or without MS023 (0.25 μM). CFSE dilution in the $CD4^+$ and $CD8^+$ T cell population was analyzed on day 3 (n = 4 cultures, ordinary one-way ANOVA with Tukey's multiple comparisons test).

Acetylation of FOXP3 at these same lysine residues stabilizes FOXP3 protein by blocking ubiquitination [18–23]. Phosphorylation of FOXP3 on 418S plays a crucial role in its repressive activity without affecting the protein level [24]. However, posttranslational regulation of FOXP3 functions has not been studied in a comprehensive manner. Elucidation of the modifications that affect FOXP3 functions would provide a better understanding of how the activity of mature Treg cells is controlled in normal and disease conditions. In this study, we extensively studied posttranslational targets that modulate FOXP3 functions by using an array of selective inhibitors. We demonstrate that the inhibition of type I protein arginine methytransferases (PRMTs) attenuates the transcriptional activity of FOXP3, thereby constricting the suppressive functions of FOXP3-transduced or naturally occurring Treg cells.

2. Materials and methods

2.1. Reagents

The epigenetic probes and doses used in the screening of Fig. 1 are listed in Supplementary Table 1. The treatment dose was based on previous studies or by referring to the cell-based assay data available in the SGC website (http://www.thesgc.org/chemical-probes/epigenetics). The Akt inhibitor VIII was purchased from Cayman Chemical. DMSO was added at a final concentration of 0.01% (volume per volume), including in the control samples.

2.2. Cell lines

The aAPC/mOKT3 is derived from the human erythroleukemia cell line K562 and stably expresses a membranous form of an anti-CD3 mAb (clone OKT3), CD80 and CD83 as previously described [25,26]. The HEK293T cells were directly obtained from the American Type Culture Collection (ATCC) (Manassas, VA). All cells were routinely checked for the presence of mycoplasma contamination using PCR-based technology.

2.3. In vitro culture of human T cells

Human T cells were derived from healthy donor-derived peripheral blood mononuclear cells isolated by Ficoll-Paque PLUS density gradient centrifugation (GE Healthcare). $CD3^+$ or $CD4^+$ T cells were negatively isolated by using a Pan T Cell Isolation Kit (Miltenyi Biotec) or a $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec), respectively. Conventional $CD4^+$ $CD25^-$ T cells were isolated from $CD4^+$ T cells through negative

magnetic selection with CD25 Microbeads II (Miltenyi Biotec). $CD4^+$ $CD25^+$ $CD127^-$ regulatory T cells were sorted by MoFlo Astrios (Beckman Coulter). Isolated T cells were stimulated with aAPC/mOKT3 irradiated with 120 Gy at an effector to target (E:T) ratio of 5:1 or anti-CD3/CD28 Dynabeads (Thermo Fisher Scientific) at a bead/T cell ratio of 1:2. On the following day, 100 IU/ml IL-2 and 10 ng/ml IL-15 (Peprotech) were added to the cultures unless otherwise noted. Culture media were replenished every 2–3 days.

2.4. Suppression assays

$CD3^+$ T cells isolated from PBMC were labeled with 5 μM CFSE (Thermo Fisher Scientific), stimulated with aAPC/mOKT3 in the presence of FOXP3-transduced $CD4^+$ T cells or natural Treg cells isolated from peripheral blood at a ratio of 2:1 and cultured without cytokine administration. When indicated, MS023 and Akt inhibitor were added at 0.25 μM and 2 μM, respectively. CFSE dilution was analyzed by flow cytometry 3 days following the coculture.

2.5. Flow cytometry

The following antibodies were used for flow cytometry: APC-Cy7-anti-CD4 (clone RPA-T4; BioLegend), PE-Cy7-anti-CD8 (clone SFCI21Thy2D3; Beckman Coulter), PE-Cy7-anti-CD25 (clone BC96; BioLegend), PE-anti-CD127 (clone HIL-7R-M21; BD Biosciences), V450-anti-CD271 (clone C40-1457; BD Biosciences), FITC-anti-IL-2 (clone 5344.111; BD Biosciences), PE-anti-TNF-α (clone MAb11; BioLegend), PE-Cy7-anti-IFN-γ (clone B27; BioLegend), and APC-anti-CD45 (clone HI30; BioLegend). The stained cells were analyzed with a FACSCanto II (BD Biosciences). The obtained data were analyzed by using the FlowJo software (version 9.7.6, Tree Star).

2.6. Analysis of cytokine production

For intracellular cytokine staining, T cells were stimulated with aAPC/mOKT3 at an E:T ratio of 1:1 and incubated for 2 hours. The T cells were then treated with Brefeldin A (BioLegend) to prevent cytokine secretion and were further cultured for 4 hours. The cells were stained for surface antigens, fixed and permeabilized by using a Cytofix/Cytoperm kit (BD Biosciences), and stained for intracellular cytokines.

2.7. Retroviral transduction

For retrovirus transduction of T cells, an amphotropic retrovirus was produced by using PG13 packaging cells stably transduced with a retrovirus plasmid. Transduction was performed by using Retronectin (Clontech-Takara Bio Inc.) on day 2 following stimulation with aAPC/mOKT3. The FOXP3 cDNA was linked to the truncated NGFR (ΔNGFR) gene using a Furin-SGSG-P2A sequence and cloned into the pMX retrovirus plasmid. The truncated NGFR (ΔNGFR) gene alone was used as a control.

HEK293T cells were transduced with the indicated genes by using retroviruses produced from 293GPG cells. FOXP3 and PRMT1 cDNAs were cloned into pMX-internal ribosome entry site (IRES)-ΔNGFR and pMX-IRES-puromycin-resistant gene, respectively. A Dominant-negative form of PRMT1 (VLD to AAA at amino acids 74–76) was generated by site-directed mutagenesis [27].

2.8. Microarray data analysis

$CD4^+$ T cells derived from three different donors were stimulated with aAPC/mOKT3, transduced with ΔNGFR alone or FOXP3-ΔNGFR and cultured in the presence of DMSO or MS023 (1 μM). The $\Delta NGFR^+$ cells were isolated on day 7 after stimulation, and RNA was extracted by using an RNeasy Micro Kit (Qiagen). The gene expression profiles were analyzed using the Affymetrix PrimeView™ Human Gene Expression Array by the Centre for Applied Genomics (TCAG) at the Hospital for Sick Children (Toronto, ON). The raw expression data were normalized and annotated by the Affymetrix Expression Console version 1.4.1 (Affymetrix).

For clustering analysis, differentially expressed genes between regulatory and conventional T cells ($P < 0.01$ by unpaired two-sided *t*-test and fold expression change > 1.5) were extracted from the Gene Expression Omnibus (GEO) database (GSE20934) [28]. An unsupervised hierarchical clustering was performed by using the HeatPlus software package from Bioconductor. A principal component analysis was conducted using the function 'prcomp' in the R package 'stats,' and the data were shown on a 2-dimensional plot using the 'ggplot2' package. The differentially expressed genes between the DMSO- and MS023-treated $FOXP3^+$ T cells ($P < 0.01$ by paired two-sided *t*-test) were extracted, and the upregulated and downregulated genes in MS023-treated T cells were individually analyzed by the DAIVD for enriched pathways within the KEGG database. The pathways with P values < 0.0001 and FDR calculated by the Benjamini-Hochberg method < 0.05 were extracted. The Th1 and Th2 signature scores were calculated in individual samples by adding the mean-centered log2-transformed expression values of the genes upregulated in Th1 or Th2 cells and subtracting the values of the downregulated genes in Th1 and Th2 cells, respectively. The genes upregulated or downregulated in Th1 and Th2 cells compared to naïve $CD4^+$ T cells were extracted from the publicly available data (GSE60680, FDR < 0.01 by RankProd analysis) [29]. Gene set enrichment analysis (GSEA) was performed by using the GSEA v2 software (Broad Institute). The differentially expressed pathway genes detected by the DAVID analysis were used as gene sets. Nominal P values were determined by an empirical phenotype-based permutation test.

2.9. Immunoprecipitation and immunoblotting

Total cell lysates were extracted with ice-cold Nonidet P-40 (NP-40) lysis buffer (50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl and 1% NP-40). For immunoprecipitation, total cell lysates were incubated with Protein G Dynabeads (Thermo Fisher Scientific) and the antibody of interest at 4 degrees overnight. The beads were washed with NP-40 lysis buffer and wash buffer (50 mM Tris-HCl pH 7.6 and 150 mM NaCl). The immunoprecipitated proteins were eluted with 2× Laemmli Sample Buffer (Bio-Rad) with 2-mercaptoethanol.

For immunoblotting, equal amounts of proteins were separated by SDS-PAGE and transferred to Immobilon-P PVDF membranes (Millipore, Bedford, MA). The membrane was probed with the indicated primary antibodies at 4 °C overnight, washed and incubated with the HRP-conjugated secondary antibodies. The following antibodies were used: anti-FLAG antibody (clone M2, Sigma-Aldrich), anti-mono-methyl arginine antibody (Cell Signaling Technology: #8015), anti-asymmetric di-methyl arginine antibody (Cell Signaling Technology: #13522), anti-HA antibody (clone 6E2, Cell Signaling Technology), anti-HA antibody (clone 12CA5, Roche Applied Science), anti-PRMT1 antibody (clone A33, Cell Signaling Technology), anti-PRMT8 antibody (Abcam: ab168134), anti-phospho-Akt (Thr308) (clone D25E6, Cell Signaling Technology), anti-Akt (clone 40D4, Cell Signaling Technology), anti-β-actin antibody (clone C4, Santa Cruz Biotechnology: sc-47778), HRP-conjugated anti-mouse IgG (Promega: #4021), HRP-conjugated anti-rabbit IgG (Promega: #4011), HRP-conjugated anti-mouse IgG Veriblot (Abcam: ab131368), and HRP-conjugated anti-rabbit IgG True Blot (Rockland: #18-8816-33). The protein levels of phospho-Akt relative to total Akt levels were quantified with ImageJ software.

2.10. Immunofluorescence microscopy

For immunofluorescence staining, $3–5 \times 10^4$ FOXP3-transduced $CD4^+$ T cells were mounted on glass slides. The cells were fixed with

3.7% formaldehyde in PBS for 20 minutes, permeabilized by treatment with 0.2% Triton X in PBS for 10 minutes and blocked with 1% BSA in PBS for 45 minutes. The specimens were then incubated with mouse anti-FOXP3 antibody (Abcam: ab22510; 1:1000 dilution) overnight at 4 °C followed by Alexa Fluor 555 goat anti-mouse IgG (1:250 dilution; Cell Signaling Technologies) for 60 minutes and DAPI (1 μg/ml; Cell Signaling Technologies) for 5 minutes. After washing with PBS, the cells were covered with ProLong Gold Antifade Mountant (Thermo Fisher Scientific). Immunofluorescence was analyzed by using an Olympus FluoView confocal microscope. Nuclear localization of FOXP3 was analyzed as previously described [30]. Briefly, the mean fluorescence intensity of FOXP3 in the nucleus and cytoplasm was measured in individual cells with the FV10-ASW software, and the intensity ratio of nucleus/cytoplasm was calculated. More than 50 cells were analyzed in each specimen.

2.11. Quantitative real-time PCR

RNA was extracted with TRIzol reagent (Thermo Fisher Scientific) and reverse transcribed using Superscript III (Thermo Fisher Scientific). Quantitative real-time PCR was performed on the CFX96 real-time PCR detection system (BioRad) using SYBR Select Master Mix (Thermo Fisher Scientific). The results were normalized to *UBC*, and relative expression levels were calculated using the 2-ΔΔCT method. The following primers were used: *IL2* forward, AAGTTTTACATGCCCAAGA AGG, and reverse, AAGTGAAAGTTTTTGCTTTGAGCTA; *IFNG* forward, GGAAAGAGGAGAGTGACAGAAAA, and reverse, TTGGATGCTCTGGT CATCTTTA; *TNF* forward, CGCTCCCCAAGAAGACAG, and reverse, AGAGGCTGAGGAACAAGCAC; *TBX21* forward, CTACCGAGGCCAGGA GGT, and reverse, GCCCATCTTGGGAGGGTA; *IRF8* forward, ACGCTG TGCTTTGAATAAGAGC, and reverse, TCCTCAGGAACAATTCGGTA AAC; and *UBC* forward, ATTTGGGTCGCGGTTCTTG, and reverse, TGC CTTGACATTCTCGATGGT.

2.12. Mouse experiments

In the mouse experiments, 6- to 12-week-old male NSG mice (The Jackson Laboratory) were used. To evaluate the development of xenogeneic GVHD by cultured T cells, the mice were irradiated (1.6 Gy) and infused with 6 million cultured $CD3^+$ T cells with or without 3 million FOXP3-transduced $CD4^+$ T cells on the following day. The mice were monitored for health and body weight at least three times per week and were euthanized when they exhibited one of the following symptoms: more than 20% loss of initial body weight, pronounced lethargy, hunched posture, severe diarrhea, or severe dermatitis. Human T cells in peripheral blood were discriminated as $CD45^+$ $CD4/CD8^+$ cells. Mice were randomly assigned to each treatment group. The investigators were not blinded to allocation during animal experiments and outcome assessment.

2.13. Statistics

The significance of the differences between two groups was assessed with a two-sided paired or unpaired *t*-test. Comparisons between more than two groups were performed by paired or unpaired analysis of variance (ANOVA) and multiple comparison tests. The relative mRNA expression levels of MS023-treated T cells compared to DMSO-treated T cells were analyzed with a one-sample *t*-test. Differences were considered significant at a P value of less than 0.05. In the mouse xenogeneic GVHD experiments, the weight loss-free survival and overall survival of the mice transplanted with T cells was depicted with a Kaplan-Meier curve, and the survival difference between groups was compared with the log-rank test. The P-values were adjusted according to the number of comparisons with the Bonferroni method. All statistical analyses were performed using GraphPad Prism 7.

2.14. Study approval

This study was performed to identify novel posttranslational mechanisms to regulate human Treg cell functions. The study was performed in accordance with the Helsinki Declaration and approved by the Research Ethics Board of the University Health Network, Toronto, Canada. Written informed consent was obtained from all the donors. All mouse experiments were approved by the Ontario Cancer Institute/Princess Margaret Cancer Centre Animal Care Committee at the University Health Network.

2.15. Data availability

The microarray data have been deposited in the Gene Expression Omnibus (GEO) under the accession number GSE112269.

3. Results

3.1. Screening of posttranscriptional modification of FOXP3

To identify potential modulators of FOXP3 function, we performed a screening experiment using chemical probes with various defined targets against enzymes or effector molecules associated with epigenetic or posttranslational modifications (Supplementary Table 1). $CD4^+$ $CD25^-$ conventional human T cells were retrovirally transduced with FOXP3 and individually treated with each chemical probe (Fig. 1A). CD25 upregulation and repression of IL-2 are robust readouts to evaluate FOXP3 transcriptional activity [31]. The probe-treated T cells were first analyzed for CD25 expression, and the probes that significantly altered CD25 expression were further assessed for their effects on IL-2 production by the $FOXP3^+$ $CD4^+$ T cells. As shown in Fig. 1B and C and Supplementary Figs. 1A and 1B, several chemical probes significantly affected CD25 expression and/or IL-2 production. Treatment with MS023 (type I protein arginine methyltransferase (PRMT) inhibitor) [32] or LAQ824 (histone deacetylase (HDAC) inhibitor) significantly downregulated CD25 expression and elevated IL-2 production in $FOXP3^+$ T cells, suggesting that these probes reduced FOXP3 transcriptional activity. Although both A-366 and A-485 downregulated CD25 (Fig. 1B), they failed to increase IL-2 production in $FOXP3^+$ T cells (Fig. 1C). This suggests that these drugs might not work through the modulation of FOXP3 transcriptional activity. It has been reported that multiple types of HDACs interact with FOXP3 and support its repressive activity for *IL2* expression [33,34]. On the other hand, the role of type I PRMT enzymes in regulatory T cells has not been investigated. The effects of MS023 treatment on CD25 expression and IL-2 production were further confirmed in different donor samples (Fig. 1D and E). Importantly, MS023 treatment significantly attenuated the suppressive activity of both FOXP3-transduced T cells and naturally occurring Treg (nTreg) cells *in vitro* (Fig. 1F and G; Supplementary Figs. 2 and 3). These results suggest that type I arginine methyltransferases positively regulate the suppressive functions of FOXP3-expressing T cells. We therefore investigated how type I PRMTs modulate FOXP3 activity in more detail.

3.2. Arginine methyltransferase PRMT1 interacts with and methylates arginine residues of FOXP3

Immunoblotting analysis of methyl-arginine antibodies showed that FOXP3 undergoes mono-methylation of arginine (MMA) and asymmetric-di-methylation of arginine (ADMA) (Fig. 2A). Although previous studies have shown that inhibition of type I PRMT enzymes increased global levels of MMA [32,35], MS023 treatment decreased both MMA and ADMA levels in the FOXP3 protein. Since arginine methylation of FOXP3 was similarly detected in HEK293T cells retrovirally transduced with FOXP3, we used this platform for subsequent methylation assays (Supplementary Fig. 4). Type I PRMTs include five proteins, PRMT1,

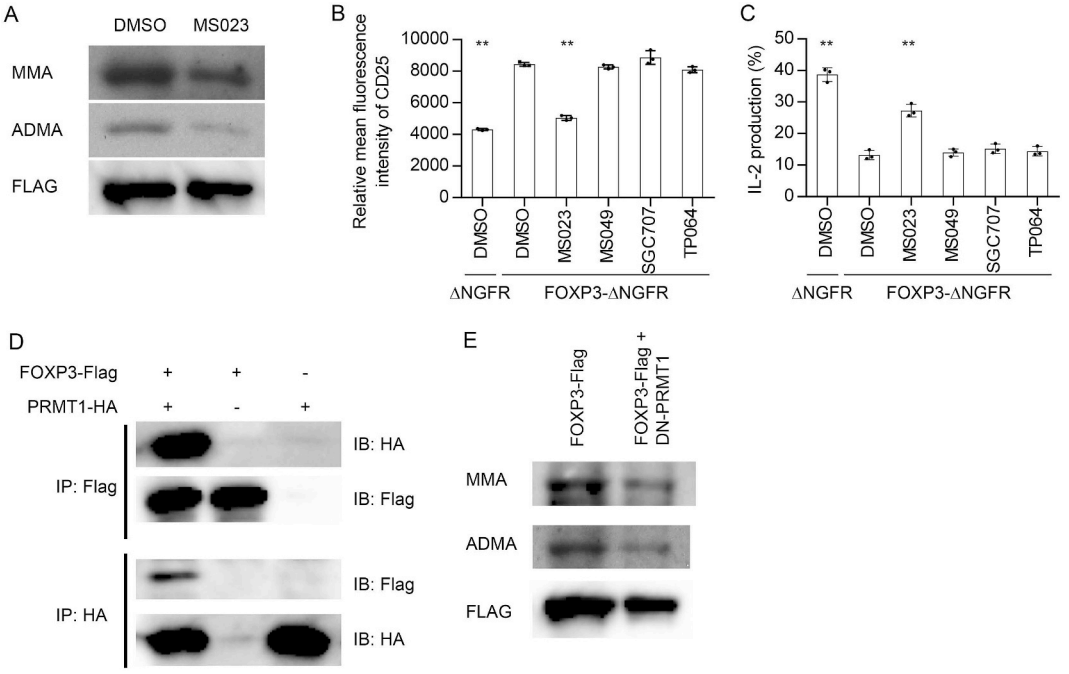

Fig. 2. FOXP3 is arginine-methylated by protein arginine methyltransferase 1 (PRMT1). (A) $CD4^+$ T cells were transduced with FOXP3 N-terminally tagged with 3xFlag and treated with DMSO or MS023. The cell lysates were immunoprecipitated with an anti-FLAG antibody and analyzed with immunoblotting using anti-MMA and anti-ADMA antibodies. (B and C) $CD4^+$ $CD25^-$ T cells were transduced with ΔNGFR alone or FOXP3-P2A-ΔNGFR and individually treated with the indicated chemical probes targeting type I PRMT enzyme(s). The data shown are CD25 expression (B) and IL-2 production upon restimulation with aAPC/mOKT3 (C) in the $CD4^+$ $ΔNGFR^+$ T cell population (n = 3 samples, one-way ANOVA with Tukey's multiple comparisons test). ** $P < 0.01$ compared with the T cells transduced with FOXP3-ΔNGFR and treated with DMSO. (D) Immunoblotting analysis of proteins immunoprecipitated with an anti-FLAG or anti-HA antibody in HEK293T cells transduced with FOXP3-FLAG and/or PRMT1-HA. (E) Immunoblotting analysis of proteins immunoprecipitated with an anti-FLAG antibody in FOXP3-FLAG-transduced HEK293T cells with or without ectopic expression of a dominant-negative form of PRMT1 (DN-PRMT1).

PRMT3, CARM1, PRMT6 and PRMT8, which have non-redundant functions in normal and malignant cells [36,37]. To explore which methyltransferases play a predominant role in the modification of FOXP3 functions, FOXP3-transduced $CD4^+$ T cells were treated with chemical probes with selective or narrow-target specificity against type I PRMTs (Supplementary Table 1). As shown in Fig. 2B and C, treatment with MS049 (CARM1 and PRMT6), SGC707 (PRMT3) or TP064 (CARM1) did not alter CD25 expression or IL-2 production by the $FOXP3^+$ T cells, suggesting that these enzymes do not play an essential role in FOXP3 transcriptional activity. While PRMT1 is ubiquitously expressed in various cell types, PRMT8 expression is limited to the brain among neural tissues, and its aberrant expression is detected in cancer cells [38,39]. In fact, PRMT1, but not PRMT8, was easily detected at endogenous levels in human T cells (Supplementary Figs. 5A and 5B). We confirmed a physical interaction between retrovirally transduced FOXP3 and PRMT1 (Fig. 2D). Moreover, ectopic expression of a dominant-negative form of PRMT1 significantly reduced arginine methylation levels in FOXP3 (Fig. 2E). These results suggest that PRMT1 is predominantly associated with the arginine methylation of FOXP3.

3.3. FOXP3 undergoes arginine methylation on R48 and R51

We then explored which arginine residues are methylated in the FOXP3 protein. We used PRmePRed, a recently developed tool for the prediction of arginine methylation sites, to retrieve candidate methylation sites [40]. Interestingly, all of the putative methylation residues (cut-off prediction score > 0.95) were within the N-terminal proline-rich repressor domain (Supplementary Table 2). Indeed, deletion of the N-terminal, but not the C-terminal, region abrogated the arginine methylation of FOXP3 (Fig. 3A and B). The protein with the FOXP3 N-terminal domain only was arginine-methylated as efficiently as the native FOXP3 (Supplementary Fig. 6). We further investigated the position(s) of methylated arginine by using a deletion mutant approach (Fig. 3C). As shown in Fig. 3D, the FOXP3 protein with a deletion of amino acids 1–40 underwent arginine methylation similar to the full-length FOXP3, while the deletion of amino acids 1–59 or 1–121 abolished the arginine methylation of FOXP3. Based on these results, we mutated R48 and/or R51 to alanine (R48A, R51A and R48/51A) and analyzed the arginine methylation levels in FOXP3. Neither MMA or ADMA expression level was largely affected by single mutations on R48 or R51. However, both expression levels were greatly diminished when dual mutations were introduced into the two residues, suggesting that FOXP3 is arginine-methylated predominantly on R48 and R51 (Fig. 3E). Similar results were obtained in FOXP3-transduced T cells (Fig. 3F). We then investigated the functions of mutant FOXP3 in comparison with wild-type FOXP3. When transduced into $CD4^+$ T cells, the FOXP3 R48/51A mutant increased CD25 expression and suppressed IL-2 production to a lesser extent than the wild-type FOXP3, suggesting an attenuated transcriptional activity of FOXP3 (Fig. 3G and H). The MS023 treatment of the FOXP3 R48/51A-transduced T cells further downregulated CD25 expression and increased IL-2 production, suggesting that MS023 affected these parameters in a mechanism other than the direct methylation of FOXP3 (Fig. 3I and J). Importantly, the FOXP3 R48/51A-transduced $CD4^+$ T cells showed reduced suppressive activities on T cell proliferation at similar levels as the MS023-treated T cells with the wild-type or R48/51A FOXP3 (Fig. 3K). Since MS023 treatment did not show an additive effect on the suppressive activity of FOXP3 R48/51A-T cells, PRMT enzymes contribute to suppressive functions of $FOXP3^+$ T cells predominantly through direct methylation of FOXP3. Although previous studies reported that the N-terminal 51 amino acids (aa1-51) of FOXP3 are associated with its nuclear localization [41], we did not observe a significant change in the subcellular localization of FOXP3 by

MS023 treatment or the R48/51A mutation (Supplementary Figs. 7A and 7B). These results indicate that FOXP3 undergoes arginine methylation on R48 and R51, which helps to enhance the suppressive functions of FOXP3$^+$ T cells. Since single mutations of R48 or R51 to alanine did not significantly alter the FOXP3 activity, methylation of either residue is considered to be sufficient to support FOXP3 activity.

3.4. MS023 treatment confers Th1-like gene expression profiles to FOXP3-expressing T cells

We further investigated how arginine methylation modulates FOXP3 functions. MS023 treatment or a methylation-defective mutation (R48/51A) did not alter the protein levels of FOXP3, suggesting that arginine methylation does not affect the stability of FOXP3

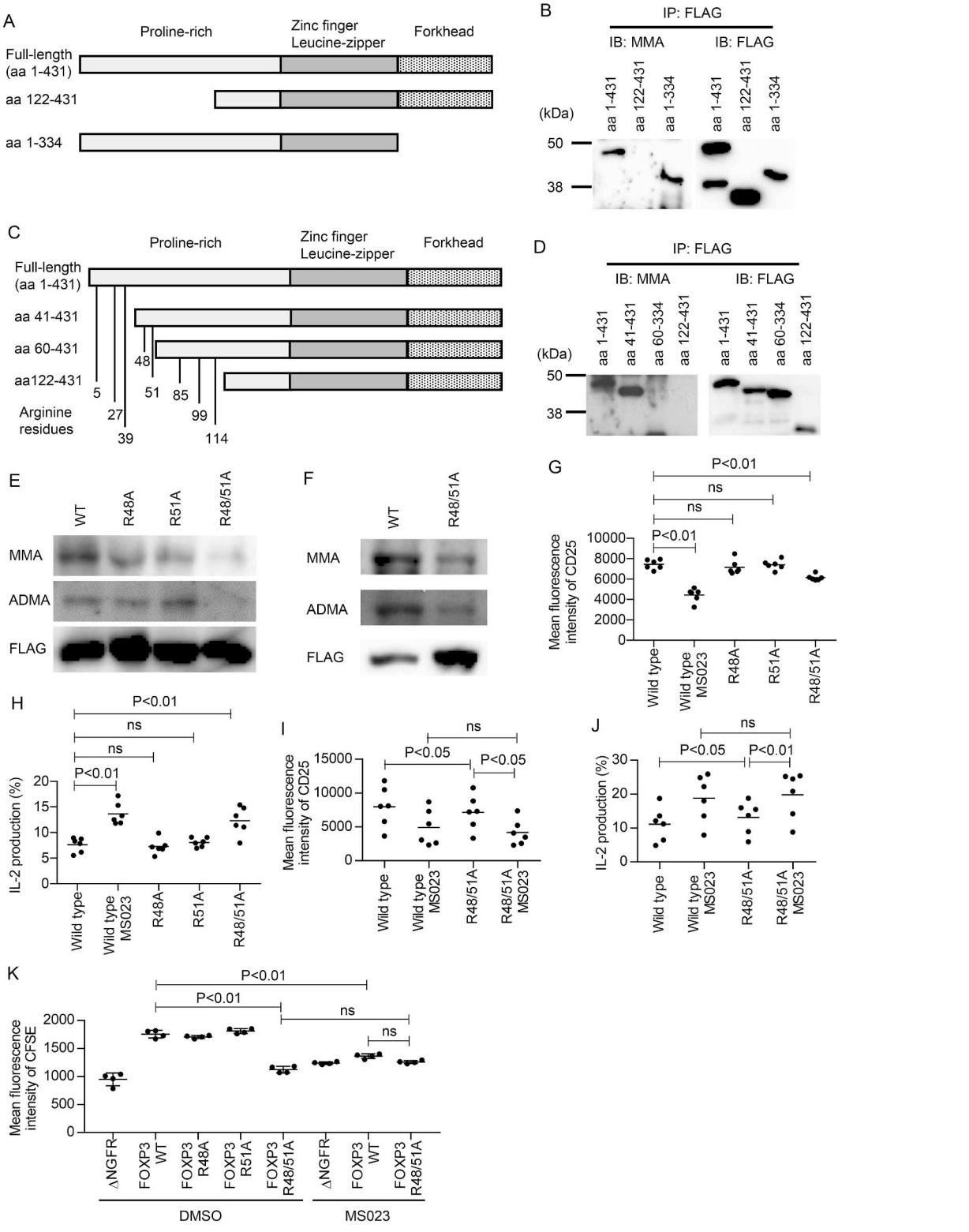

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Fig. 3. FOXP3 is arginine-methylated on R48 and R51. (A) Schematic diagram for FOXP3 with deletion of the N- or C-terminal region. (B) Anti-FLAG immunoprecipitated lysates from HEK293T cells expressing the full-length or truncated FOXP3-FLAG were analyzed by immunoblotting using an anti-MMA antibody. (C) The N-terminally truncated FOXP3 protein of different lengths. The positions of arginine residues are indicated. (D) Immunoblotting analysis by an anti-MMA antibody of anti-FLAG immunoprecipitated lysates from HEK293T cells expressing the full-length or N-terminally truncated FOXP3-FLAG. (E, F) Immunoblotting analysis by anti-MMA and anti-ADMA antibodies of anti-FLAG immunoprecipitated lysates from HEK293T cells (E) or $CD4^+$ T cells (F) expressing the wild-type or mutant FOXP3 tagged with FLAG peptide. (G and H) $CD4^+$ $CD25^-$ T cells were transduced with the wild-type or mutant FOXP3 and analyzed for CD25 expression (G) and IL-2 production (H) (n = 6 different donor samples, repeated measures one-way ANOVA with Tukey's multiple comparisons test). (I and J) $CD4^+$ $CD25^-$ T cells were transduced with the wild-type or R48/51A mutant FOXP3 and analyzed for CD25 expression (I) and IL-2 production (J) in the presence or absence of MS023 (n = 6 different donor samples, repeated measures one-way ANOVA with Tukey's multiple comparisons test). (K) CFSE-labeled $CD3^+$ T cells were stimulated with aAPC/mOKT3 in the presence of $CD4^+$ T cells transduced with ΔNGFR alone, wild-type or mutant FOXP3 with or without MS023. The mean fluorescence intensity of CFSE was analyzed on day 3 (n = 4 cultures for each condition, one-way ANOVA with Tukey's multiple comparisons test). Representative data of two experiments. ns, not significant.

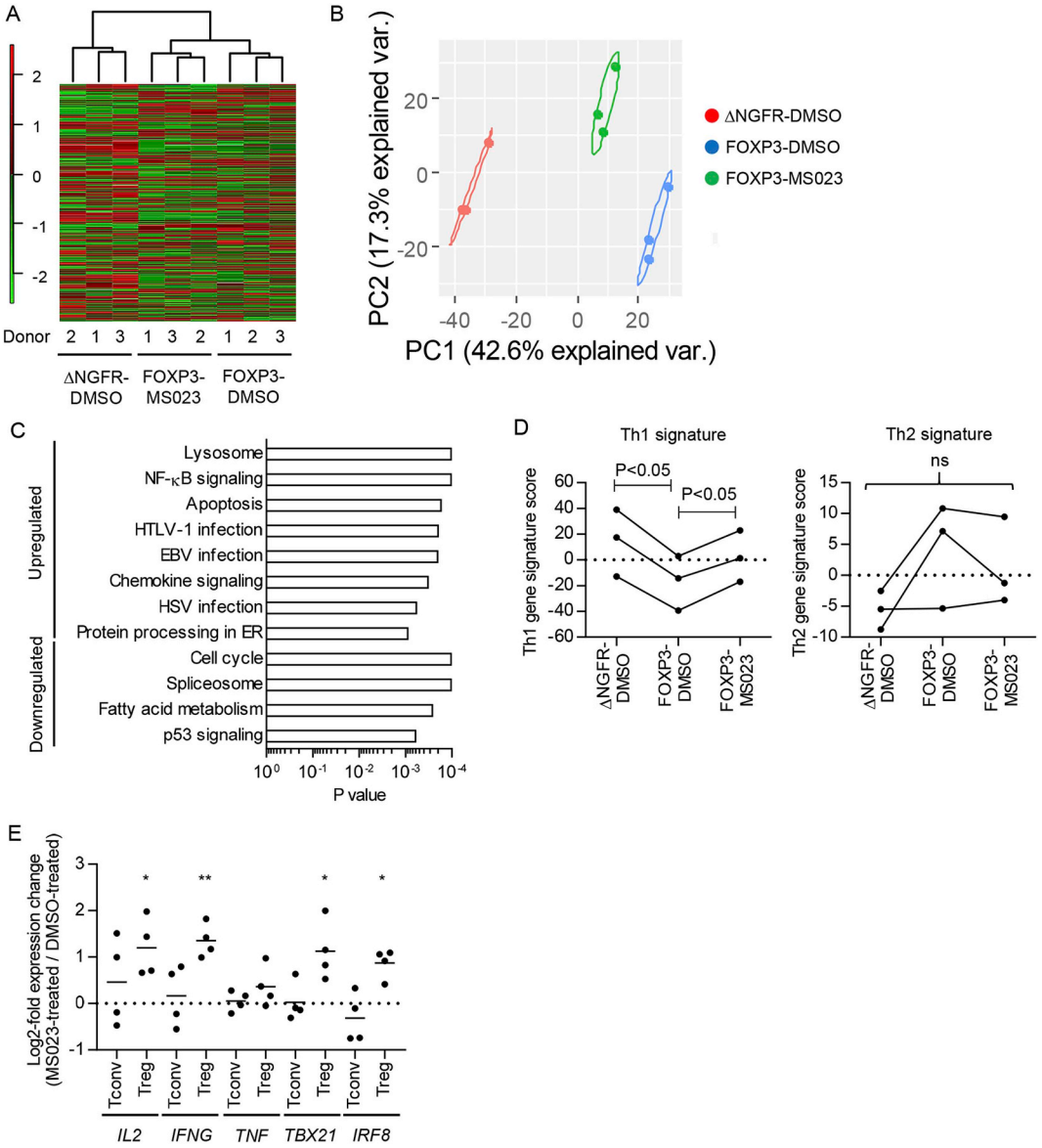

Fig. 4. Gene expression profiles of MS023-treated $FOXP3^+$ T cells. (A and B) $CD4^+$ $CD25^-$ T cells were stimulated with aAPC/mOKT3 and transduced with ΔNGFR alone or FOXP3-P2A-ΔNGFR and treated with DMSO or MS023. The $\Delta NGFR^+$ T cells were isolated on day 7, and gene expression profiles were compared by microarray analysis (n = 3 different donor samples). Unsupervised hierarchical clustering (A) and principal component analysis (B) of genes that are differentially expressed between conventional and regulatory T cells retrieved from the previous data. (C) Differentially expressed genes between the DMSO- and MS023-treated $FOXP3^+$ T cells were analyzed for pathway enrichment by the Database for Annotation, Visualization and Integrated Discovery (DAVID) functional annotation tool. Raw P-values for the indicated pathways were shown. (D) Gene expression signature scores representing T helper type 1 (Th1) and type 2 (Th2) cells were calculated in each sample (n = 3 different donor samples, repeated-measures one-way ANOVA with Tukey's multiple comparisons test). (E) Expression levels of the indicated genes in conventional or regulatory $CD4^+$ T cells treated with MS023 relative to those in the DMSO-treated controls were analyzed by qPCR at 48 hours after stimulation by anti-CD3/CD28 Dynabeads (n = 4 samples, one-sample *t*-test). * $P < 0.05$, ** $P < 0.01$. ns, not significant.

(Supplementary Fig. 8). To interrogate how MS023 treatment modulates the attributes of FOXP3$^+$ T cells, we analyzed gene expression profiles of the control truncated nerve growth factor receptor (ΔNGFR) or FOXP3-transduced CD4$^+$ T cells with or without treatment with MS023. To compare the transcriptional activity of FOXP3, we retrieved genes that are differentially expressed between regulatory and conventional CD4$^+$ T cells from the publicly available data (GSE20934; Supplementary Table 3) [28]. Hierarchical clustering and principal component analysis of these genes demonstrated that although DMSO-treated and MS023-treated FOXP3$^+$ T cells showed more similar gene expression profiles than the control T cells, they formed independent clusters regardless of the donor types (Fig. 4A and B). To explore what types of genes are specifically affected in terms of expression levels by MS023 treatment, we analyzed a set of differentially expressed genes between the DMSO- and MS023-treated FOXP3$^+$ T cells by the Database for Annotation, Visualization and Integrated Discovery (DAVID) functional annotation tool (Supplementary Table 4). Interestingly, the genes upregulated by MS023 treatment were significantly enriched with the genes associated with inflammatory T cell responses, such as NF-κB signaling pathway genes and the genes induced by viral infections (Fig. 4C, raw P values < 0.0001 and false discovery rate (FDR) < 0.05). These pathway genes also tended to be enriched upon MS023 treatment when analyzed in gene set enrichment analysis (Supplementary Fig. 9). Although Treg cells play a suppressive role against inflammatory responses, they also acquire type 1 helper T (Th1) cell-like phenotypes under proinflammatory conditions, which compromises their immunosuppressive functions [42,43]. Indeed, the MS023-treated FOXP3$^+$ T cells displayed Th1-like gene expression signatures compared with the control FOXP3$^+$ T cells (Fig. 4D). We also tested whether these gene expression profiles are induced by MS023 treatment in nTreg cells. As shown in Fig. 4E, MS023-treated nTreg cells showed increased levels of multiple Th1-related genes encoding cytokines (*IL2*, *IFNG*, *TNF*) and transcription factors essential for Th1 functions (*TBX21*, *IRF8*) compared with the DMSO-treated control nTreg cells. Importantly, these phenotypes were not evident in conventional T cells, suggesting that the effect of MS023 on the upregulation of Th1 genes is unique to FOXP3$^+$ T cells.

We then examined whether the repression of a Th1 phenotype restores suppressive functions of arginine methylation-inhibited FOXP3$^+$ T cells. Previous studies showed that PI3K/AKT signaling pathway is downregulated in Treg cells, and the pathway activation is involved in the induction of Th1-like Treg cells in autoimmune diseases [44,45]. In fact, FOXP3-transduced CD4$^+$ T cells showed attenuated phosphorylation of Akt compared with the T cells transduced with ΔNGFR alone. Intriguingly, both MS023-treated wild-type FOXP3$^+$ T cells and DMSO-treated FOXP3 R48/51A$^+$ T cells showed significantly enhanced Akt phosphorylation compared with DMSO-treated wild-type FOXP3$^+$ T cells (Fig. 5A and B). Consistent with these results, we confirmed that pharmacologic inhibition of the Akt pathway significantly attenuated Th1 cytokine production by both MS023-treated FOXP3 and FOXP3 R48/51A-T cells (Fig. 5C). We also analyzed suppressive functions of arginine methylation-inhibited FOXP3$^+$ T cells upon Akt inhibition. The treatment of the stimulated T cells with an Akt inhibitor at 2 μM did not substantially affect cellular division in the absence of FOXP3$^+$ T cells (Fig. 5D). However, Akt inhibition significantly decreased T cell proliferation when they were cocultured with the MS023-treated wild-type FOXP3 or FOXP3 R48/51A-transduced T cells. These results suggest that repressing a Th1 phenotype abrogates the effect of arginine methylation inhibition on the suppressive activity of FOXP3$^+$ T cells.

3.5. CD4$^+$ T cells expressing the arginine methylation-defective mutant FOXP3 display attenuated suppressive activity in vivo

Finally, we investigated a role of arginine methylation in the immunoregulatory functions of FOXP3$^+$ T cells *in vivo*. Peripheral blood CD4$^+$ T cells were transduced with wild-type FOXP3 or FOXP3 R48/51A and adoptively transferred together with untransduced CD3$^+$ T cells into irradiated NSG mice. The development of xenogeneic graft-versus-host disease (GVHD) was analyzed by sequentially monitoring the body weights of the mice and expansion of the infused T cells (Fig. 6A). Both the wild-type and R48/51A FOXP3$^+$ T cells significantly delayed the onset of weight loss compared with the control mice transplanted with the untransduced T cells alone (Fig. 6B). However, the majority of the mice infused with mutant FOXP3$^+$ T cells eventually developed fatal weight loss, while the mice with wild-type FOXP3$^+$ T cells survived without developing signs of GVHD (Fig. 6C and D). Consistent with this result, wild-type FOXP3-T cells suppressed expansion of the coinfused T cells more efficiently than the FOXP3 R48/51A-transduced T cells (Fig. 6E). These results substantiate that arginine methylation of FOXP3 on R48 and 51 enhances the suppressive functions of FOXP3-transduced T cells *in vivo*.

4. Discussion

Arginine methylation of non-histone proteins is emerging as a crucial regulator of protein functions [46]. The methylated residues have a significant impact on protein-protein interactions, DNA-binding capacity and subcellular localization of the protein [47–49]. Arginine methylation sites have been identified within more than 3000 proteins in HEK293 cells by mass spectrometry using stable isotope labeling with amino acids in cell culture (SILAC) quantification [50]. Geoghegan et al. also attempted to perform comprehensive analysis for protein arginine methylation in primary T cells by using the SILAC coupled with mass spectrometry-based approach and identified arginine methylation sites from 1257 proteins, including FOXP3 on R51 [51]. Our study suggests that R48 as well as R51 is arginine-methylated. Dual, but not single, mutations of these residues to alanine impaired FOXP3 functions, suggesting that inhibiting the methylation of both residues is required to reduce the suppressive activity of FOXP3-expressing T cells. Although we studied the effect of direct methylation of FOXP3 on its suppressive functions, inhibition of PRMT enzymes may also affect Treg cell activity through modulating the functions of other proteins that interact with FOXP3. Several molecules that have an important role in Treg cell functions, such as RUNX1 and FOXO1, are known to be arginine-methylated by type I PRMT enzymes [27,31,52,53]. Further studies are required to comprehensively elucidate how the inhibition of arginine methylation influences Treg cell functions. In addition to PRMT1, a recent study in mouse T cells suggested that the inhibition of PRMT5, a type II PRMT enzyme, augmented suppressive functions of Treg cells by elevating their frequency *in vivo* [54]. Although we did not detect a symmetric di-methylation of human FOXP3, the treatment of FOXP3$^+$ T cells with a PRMT5 inhibitor GSK591 increased both CD25 expression and IL-2 secretion. It is possible that PRMT5 modifies Treg cell activity in an indirect manner through epigenetic mechanisms or modifying other proteins associated with Treg stability/homeostasis.

Attenuated suppressive functions in the MS023-treated FOXP3$^+$ T cells were at least partly due to the acquisition of Th1-like gene expression profiles. Treg cells differentiate into Th1 effector-like cells in the face of inflammatory conditions [42,43,45,55,56]. Recent studies showed that activation of the Akt signaling in Treg cells promoted the acquisition of Th1 phenotypes and negatively affected Treg cell functions, and conversely, the inhibition of Akt restored their suppressive activity [45] [57]. Interestingly, PRMT1 is known to be upregulated by proinflammatory conditions [58,59]. Arginine methylation of FOXP3 may function as a safeguard to prevent Treg cells from skewing towards Th1-like cells during inflammatory responses. The physiological significance of arginine methylation of FOXP3 in normal and disease conditions should be addressed in *in vivo* studies.

Our findings have potential clinical relevance in cancer immunotherapy. Compelling evidence suggests that Treg cells infiltrate into neoplastic lesions and thereby suppress antitumor immunity [60–64]. As shown in the clinical responses observed by blockade of

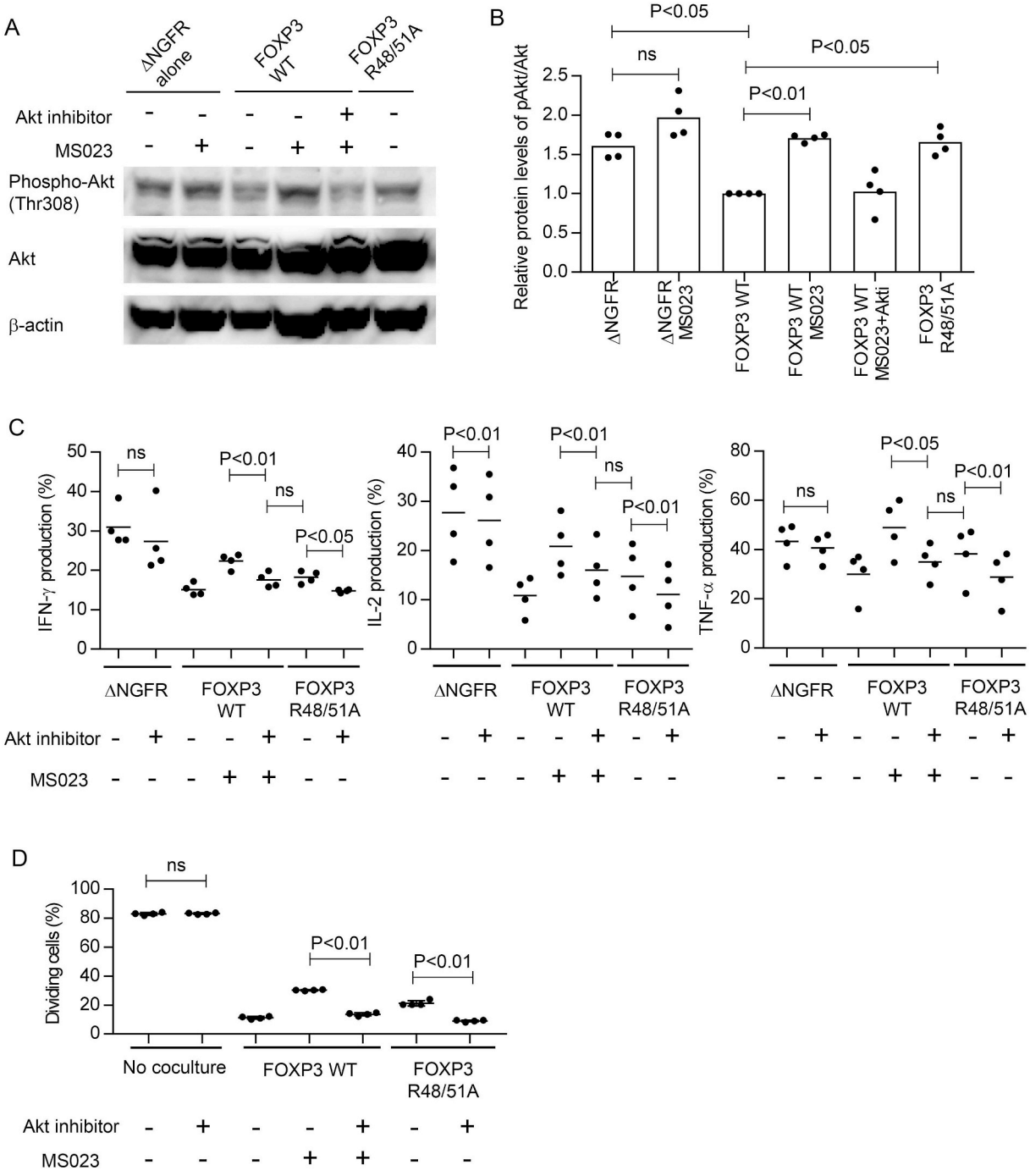

Fig. 5. Suppressive functions of arginine methylation-inhibited FOXP3$^+$ T cells are restored by Akt inhibition. (A, B) Akt phosphorylation levels were analyzed by immunoblotting in CD4$^+$ T cells transduced with ΔNGFR alone, wild-type FOXP3 or FOXP3 R48/51A upon stimulation with anti-CD3 mAb in the presence or absence of MS023 or Akt inhibitor. Representative blots and the quantified protein levels of phospho-Akt relative to total Akt were shown (n = 4 experiments, repeated measures one-way ANOVA with Tukey's multiple comparisons test). (C) Production of IL-2, IFN-γ, and TNF-α by T cells transduced with ΔNGFR, wild-type FOXP3 or FOXP3 R48/51A upon restimulation with aAPC/mOKT3 was analyzed by intracellular flow cytometry. The T cells were treated with MS023 (1 μM) or Akt inhibitor (2 μM) when indicated (n = 4 different samples; repeated-measures one-way ANOVA with Tukey's multiple comparisons test for a comparison among the wild-type FOXP3 group and paired two-sided *t*-test for the other comparisons). (D) CFSE-labeled CD3$^+$ T cells were stimulated with aAPC/mOKT3 in the presence or absence of wild-type or R48/51A mutant FOXP3-transduced T cells and cultured for 3 days with or without MS023 (0.25 μM) and/or Akt inhibitor (2 μM). The frequency of dividing cells in the CD8$^+$ T cell population was analyzed by flow cytometry (n = 4 cultures for each condition, unpaired two-sided *t*-test for 'No coculture' and 'FOXP3 R48/51A' groups and one-way ANOVA with Tukey's multiple comparisons test for 'wild-type FOXP3' groups). ns, not significant.

CTLA4 or CD25, which is constitutively expressed in Treg cells, targeting Treg cells is able to reinvigorate host antitumor immune responses [63,65]. The inhibition of arginine methyltransferases may be applicable to attenuate suppressive activity of tumor-infiltrating Treg cells and boost antitumor immune responses.

5. Conclusions

In this study, we identified arginine methylation as a novel post-translational modification of FOXP3. FOXP3 underwent methylation on arginine residues 48 and 51 by interacting with PRMT1. Inhibition of

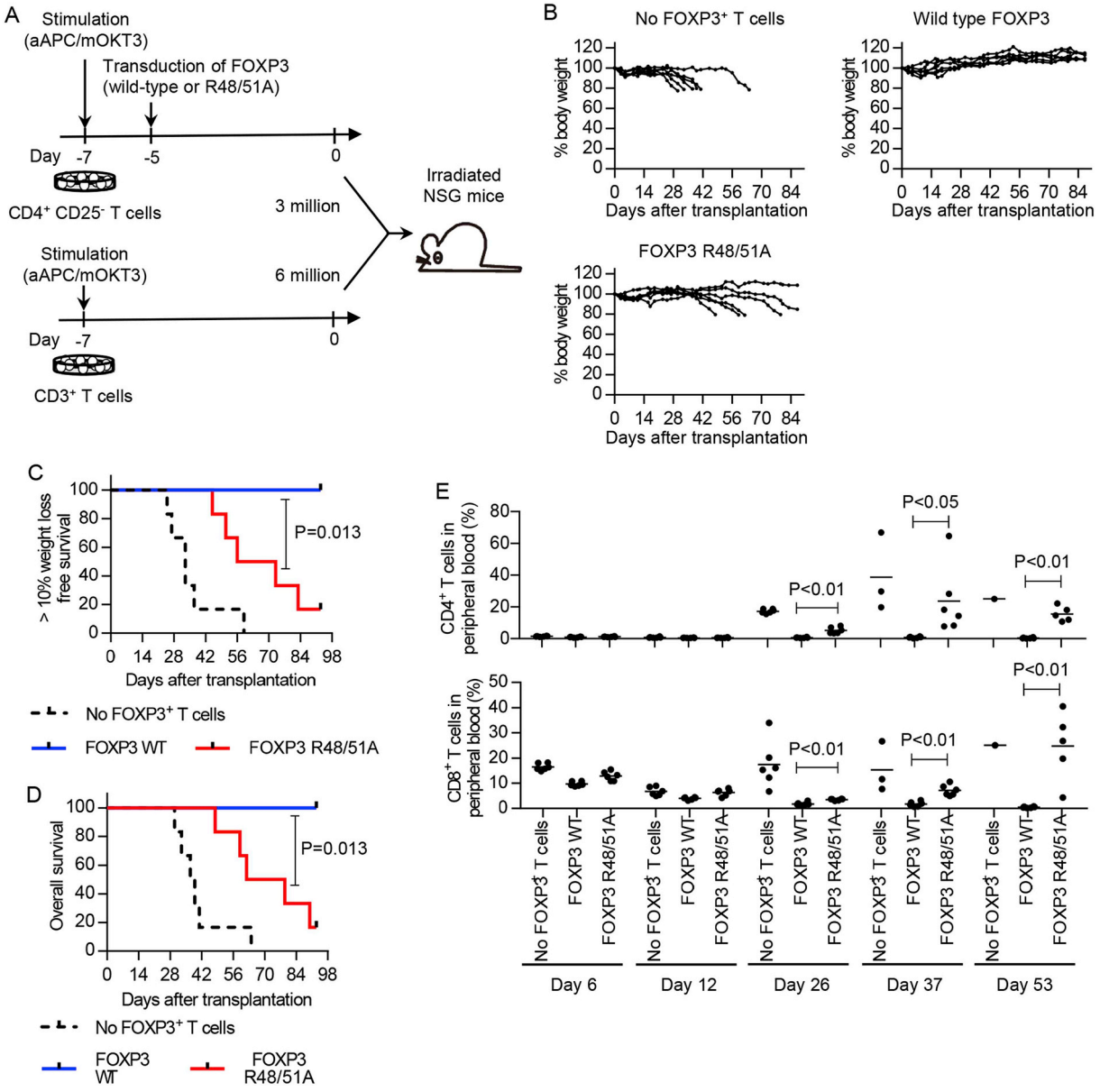

Fig. 6. FOXP3 R48/51A-transduced T cells display impaired suppressive activity for the development of xenogeneic GVHD. (A) Irradiated NSG mice were transplanted with 6 million $CD3^+$ T cells together with 3 million $CD4^+$ T cells transduced with wild type or R48/51A mutant FOXP3-P2A-ΔNGFR (n = 6 mice for each group). (B) Serial monitoring of body weight relative to the weight on day 0. (C and D) Kaplan-Meier analysis for more than 10% weight-loss-free survival (C) and overall survival (D, n = 6 mice for each group, log-rank test with Bonferroni correction). Representative data of two experiments. (E) Frequency of $CD4^+$/$CD8^+$ $\Delta NGFR^-$ T cells in peripheral blood at the indicated time points (unpaired two-sided *t*-test for each time point).

arginine methylation in FOXP3-transduced or naturally occurring Treg cells attenuated their suppressive functions both *in vitro* and *in vivo*.

Author contributions

Conceptualization, Y.K. and N.H.; Methodology, Y.K. and N.H.; Investigation, Y.K., H.S., Y.M., T.G., K.S., M.A., C.-H.W., K.S. and K.M.; Writing - Original Draft, Y.K. and N.H.; Writing - Review & Editing, Y.K., M.O.B., C.H.A. and N.H.; Funding Acquisition, N.H.; Resources, M.O.B., C.H.A. and N.H.; Supervision, N.H.

Declaration of interests

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2018.09.011.

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