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Down-regulation of Tet2 prevents TSDR demethylation in IL2 deficient regulatory T cells

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ABSTRACT

Stable expression of Foxp3 in regulatory T (Treg) cells is dependent on both intrinsic factors like epigenetic changes (demethylation) of Treg cell specific demethylation region (TSDR) and environmental cues like inflammations. Interleukin-2 (IL2) was reported to be one of the cytokines that give signals to Foxp3 stability but the underlying mechanism is still elusive. Here we show that IL2 and epigenetic changes in *foxp3* locus are closely connected through tet methylcytosine dioxygenase 2 (Tet2) and, together help Treg cells to express Foxp3 stably. TSDR in *foxp3* locus was not demethylated and Foxp3 expression was labile in IL2 deficient Treg cells, which was not restored by recombinant IL2, but correlated with the down-regulation of Tet2. Tet2 was up-regulated by TCR signaling and IL2 had a minimal effect. Rather, IL2 seemed to maintain the high level of Tet2 indirectly. Furthermore, over-expression of Tet2 restored TSDR demethylation in IL2 deficient Treg precursors. Collectively, our results suggest that up-regulation of Tet2 is required for Foxp3 stability and IL2 is required to maintain the high level of Tet2 during the thymic Treg development.

1. Introduction

Forkhead box P3$^+$ (Foxp3$^+$) Treg cells are a dedicated cell population that maintains immune tolerance and prevents autoimmune diseases [1,2]. Since Treg cells regulate immune responses usually in a Foxp3-dependent manner [3,4], the stability of Foxp3 is critical. Indeed, T cells whose Foxp3 was shut down have been reported to play roles in the development of some inflammatory diseases like diabetes [5] and lethal infection [6]. Stable Foxp3 expression is related to the epigenetic mechanisms, namely DNA demethylation of the conserved non-coding regions in *foxp3* locus, called Treg cell specific demethylation region (TSDR) [7,8]. In Treg cells with demethylated TSDR, Foxp3 protein binds to the demethylated TSDR and enhances its own expression, which induces the positive feedback loop [9]. However, the detail mechanism of TSDR demethylation is unknown.

Interleukin-2 (IL2) plays dual and often opposing roles in immune responses, contributing to both the generation of effector T cells and the maintenance of Foxp3$^+$ Treg cells [10,11]. Many studies indicate that IL2 provides essential signals for Tregs in at least 3 different levels: thymic development [12,13], competitive fitness [14,15] and Foxp3 stability [16–19]. Here, we studied TSDR demethylation in IL2 deficient Treg cells and found that IL2 and epigenetic changes in *foxp3* locus are closely related, which, as a result, helps Treg cells to express Foxp3 stably.

2. Materials and methods

2.1. Mice

CD45.1 congenic (B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ), IL2 deficient (B6.129P2-*Il2*^{tm1Hor}/J, *Il2*$^{-/-}$), Foxp3-GFP transgenic (Foxp3 bicistronic reporter mice expressing EGFP: B6.Cg-*Foxp3*^{tm2Tch}/J), Bcl2 transgenic (B6.Cg-Tg(BCL2)25Wehi/J, hBcl-2^{TG}), Lck-Cre transgenic (B6.Cg-Tg(Lck-cre)548Jxm/J), and floxed Tet2 transgenic (B6;129S-*Tet2*$^{tm1.1Iaai}$/J, *Tet2*$^{fl/fl}$) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). *Il2*$^{-/-}$ mice were crossed to Foxp3-GFP transgenic mice to identify Treg cells (GFP$^+$) and used at ⩽16 days of age unless specified in the text or figure legends. Floxed Tet2 transgenic mice [20] were crossed with Lck-Cre transgenic mice

Abbreviations: CD4SP, CD4 single positive; Foxp3, forkhead box P3; hBcl-2^{TG}, human Bcl-2 transgene; IL2, interleukin-2; KO, knock-out; mAb, monoclonal antibody; pTreg, peripheral Treg; Tet, tet methylcytosine dioxygenase; Treg cell, regulatory T cell; TSDR, Treg cell specific demethylation region; tTreg, thymic Treg; WT, wild-type.

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to delete *Tet2* in T cells. All animal experimentations were conducted in accordance with guidelines and approval of the International Animal Care and Use Committees (IACUC) of Hallym University (Hallym 2012-61, 2012-61-1, 2013-109).

2.2. Cell isolation and flow cytometry

To sort peripheral T cell subpopulations from pooled mouse spleens and lymph nodes, the $CD4^+$ fraction was first purified on the MACS Cell Separator using anti-mouse CD4 beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The enriched $CD4^+$ fractions were then further separated into each subpopulation by FACS sorting using FACS Aria-II flow cytometer (BD Biosciences, San Jose, CA). To sort wild-type (WT) peripheral Treg (pTreg) cells, CD25 and GFP were used because CD25 is up-regulated in cells which receive IL2 recently. However, we used CD44, instead of CD25, to sort *Il2*$^{-/-}$ pTreg (GFP^+CD44^{high} and GFP^+CD44^{low}) and naïve $CD4^+$ T cells (GFP^-CD44^{low}) because CD25 was down-regulated in *Il2*$^{-/-}$ pTreg cells and some of *Il2*$^{-/-}$ Treg cells showed activated phenotypes ($CD44^{high}$, $CTLA4^{high}$, $Ki67^{high}$ and $Foxp3^{high}$). To sort thymic CD4 single positive (CD4SP, $CD4^+CD8^-$) thymocyte subpopulations, pooled thymocytes were first depleted of $CD8^+$ cells ($CD4^+CD8^+$ (double positive, DP) and $CD4^-CD8^+$ (CD8 single positive, CD8SP) cells) by using anti-mouse CD8 beads and LD columns (Miltenyi Biotec). The resulting $CD8^-$ cells ($CD4^-CD8^-$ (double negative, DN) and $CD4^+CD8^-$ (CD4SP) cells) were further purified into subpopulations by FACS sorting. We used GFP and CD25 (instead of CD44) for WT and *Il2*$^{-/-}$ thymic Treg (tTreg) cells isolation, because TSDR profiles of $CD44^{high}$ and $CD44^{low}$ tTreg cells in *Il2*$^{-/-}$ mice were similar. The post-sort purity for each cell type was usually >95% except Treg precursor (GFP^-CD25^+, 60–85%). $CD4^+CD25^+$ cells were FACS-sorted and used as tTreg cells in LckCre-*Tet2*$^{fl/fl}$ mice. Foxp3 expression in sorted cells was checked by using intracellular staining.

2.3. Cell culture

FACS-sorted cells were resuspended in complete RPMI-1640 medium supplemented with 10% FCS, penicillin, streptomycin, L-glutamine (2 mM; Life Technologies, Carlsbad, CA), sodium pyruvate (2 mM; Sigma–Aldrich, St. Louis, MO), non essential amino acid (0.1 mM; Sigma–Aldrich) and 2-ME (50 μM; Sigma–Aldrich). Cells were then stimulated by recombinant murine IL2 (rIL2, 250 ng/ml for Treg cells, 15 ng/ml for naïve $CD4^+$ T or CD4SP cells; Peprotech, Rocky Hill, NJ) with plate-coated anti-CD3 (2C11, 1 μg/ml; eBioscience) and anti-CD28 (37.51, 1 μg/ml; eBioscience) for 6 days and then re-sorted for TSDR analysis. For inducible Treg cells experiments, FACS-sorted CD4SP (GFP^-CD25^-) cells were cultured in the presence of anti-CD3/CD28 + rIL2 (15 ng/ml) + TGF-β (10 ng/ml; Peprotech) for 6 days and re-sorted for TSDR analysis.

2.4. Transfection

DNA transfection experiments were performed using MACS-purified $CD8^-$ thymocytes including DN ($CD4^-CD8^-$) and CD4SP ($CD4^+CD8^-$) cells. Cells were transfected with plasmids encoding FLAG-tagged mouse Tet2 or empty vector by using the electroporation kit (Amaxa Nucleofector Kit L and Nucleofector Device, Lonza, Walkersville, MD) and the protocol provided by the manufacturer. For TSDR analysis, Tet2-transfected *Il2*$^{-/-}$ $CD8^-$ thymocytes were rested overnight in the presence of rIL2 (200 ng/ml) and sorted next day into Treg ($CD4^+CD8^-GFP^+$), Treg precursors ($CD4^+CD8^-GFP^-CD25^+$), and naïve CD4SP cells ($CD4^+CD8^-GFP^-CD25^-$) by FACS Aria-II cytometer. Transfection efficiency was checked about 5 h after transfection by flow cytometry using anti-FLAG mAb (L5, Biolegend) and >90% were FLAG positive usually.

2.5. Quantitative RT-PCR

RNA was isolated from FACS-sorted cells using the RNeasy Mini kit (Qiagen, Valencia, CA) or Trizol (Life Technologies), and reverse-transcribed into cDNA using QuantiTect Reverse Transcription kit (Qiagen). PCR reactions were performed on RotorGene 6000 system (Qiagen) using Power SYBR Green PCR Master (Life Technologies) or AccuPower GreenStar qPCR kit (Bioneer, Seoul, Korea). All data were normalized to actin. Non-specific amplification was checked by the use of melting curve and agarose gel electrophoresis. The sequences of primers are as below.

Mouse *Tet2*-Forward: 5′-AACCTGGCTACTGTCATTGCTCCA-3′
Mouse *Tet2*-Reverse: 5′-ATGTTCTGCTGGTCTCTGTGGGAA-3′ [21]
Mouse *Tet3*-Forward: 5′-TCCGGATTGAGAAGGTCATC-3′
Mouse *Tet3*-Reverse: 5′-CCAGGCCAGGATCAAGATAA-3′
Mouse *Actin*-Forward: 5′-CATCCGTAAAGACCTCTATGCCAAC-3′
Mouse *Actin*-Reverse: 5′-ATGGAGCCACCGATCCACA-3′

2.6. Foxp3 demethylation analysis

The genomic DNA was extracted from the FACS-sorted cells by using the Blood & Tissue Genomic DNA Extraction kit (Qiagen) and converted by the EpiTect Bisulfite kit (Qiagen). Bisulfite-treated DNA was then subjected to PCR for amplification of TSDR (TaKaRa Taq Hot Start Version, Takara Bio, Shiga, Japan). The PCR products obtained were cloned into the pGemT-easy vector (Promega, Madison, WI) and 10–20 individual clones from each sample were sequenced with M13-reverse primer (GAAACAGCTATGACCATG, Genotech, Daejon, Korea). The following nested primers were used for TSDR amplification [8].

TSDR Outer-Forward: 5′-TATTTTTTTGGGTTTTGGGATATTA-3′
TSDR Outer-Reverse: 5′-AACCAACCAACTTCCTACACTATCTAT-3′
TSDR Inner-Forward: 5′-TTTTGGGTTTTTTTGGTATTTAAGA-3′
TSDR Inner-Reverse: 5′-TTAACCAAATTTTTCTACCATTAAC-3′

2.7. Statistical analyses

A two-tailed, unpaired, Student's *t*-test was used to calculate the statistical significance of differences between groups. P values are represented as follows: $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$, whereas NS, not significant, is used to denote *P* values >0.05. Error bars indicate s.e.m.

3. Results and discussion

3.1. IL2 plays essential roles in TSDR demethylation

While the suppressive function of *Il2*$^{-/-}$ peripheral Treg (pTreg) cells [22] was examined, we accidentally found that many *Il2*$^{-/-}$ pTreg cells lost Foxp3 expression (data not shown). These results led us to study the role of IL2 in Foxp3 stability and TSDR demethylation. WT pTreg cells (GFP^+CD25^+ and GFP^+CD25^-) and naïve T cells (GFP^-CD25^-) were FACS-sorted and used for the analysis of TSDR. The purified cells were also cultured with recombinant IL2 (rIL2) and anti-CD3 plus anti-CD28 mAbs (anti-CD3/CD28) for 6 days and then Foxp3 expression was checked. Since some *Il2*$^{-/-}$ pTreg cells and conventional $CD4^+$ T cells (GFP^-) showed activated phenotypes, CD44 was used for FACS-sorting (pTreg cells, GFP^+CD44^{high} or GFP^+CD44^{low}; naïve T cells, GFP^-CD44^{low}, See Section 2) in *Il2*$^{-/-}$ mice. TSDR and Foxp3 expression were checked as in WT. As expected, TSDR of naïve $CD4^+$ T cells (GFP^-CD25^-) was fully methylated but that of pTreg cells was demethylated in WT. The demethylation profiles of each population were correlated with the Foxp3 expression in cells cultured *in vitro* (Supplemental

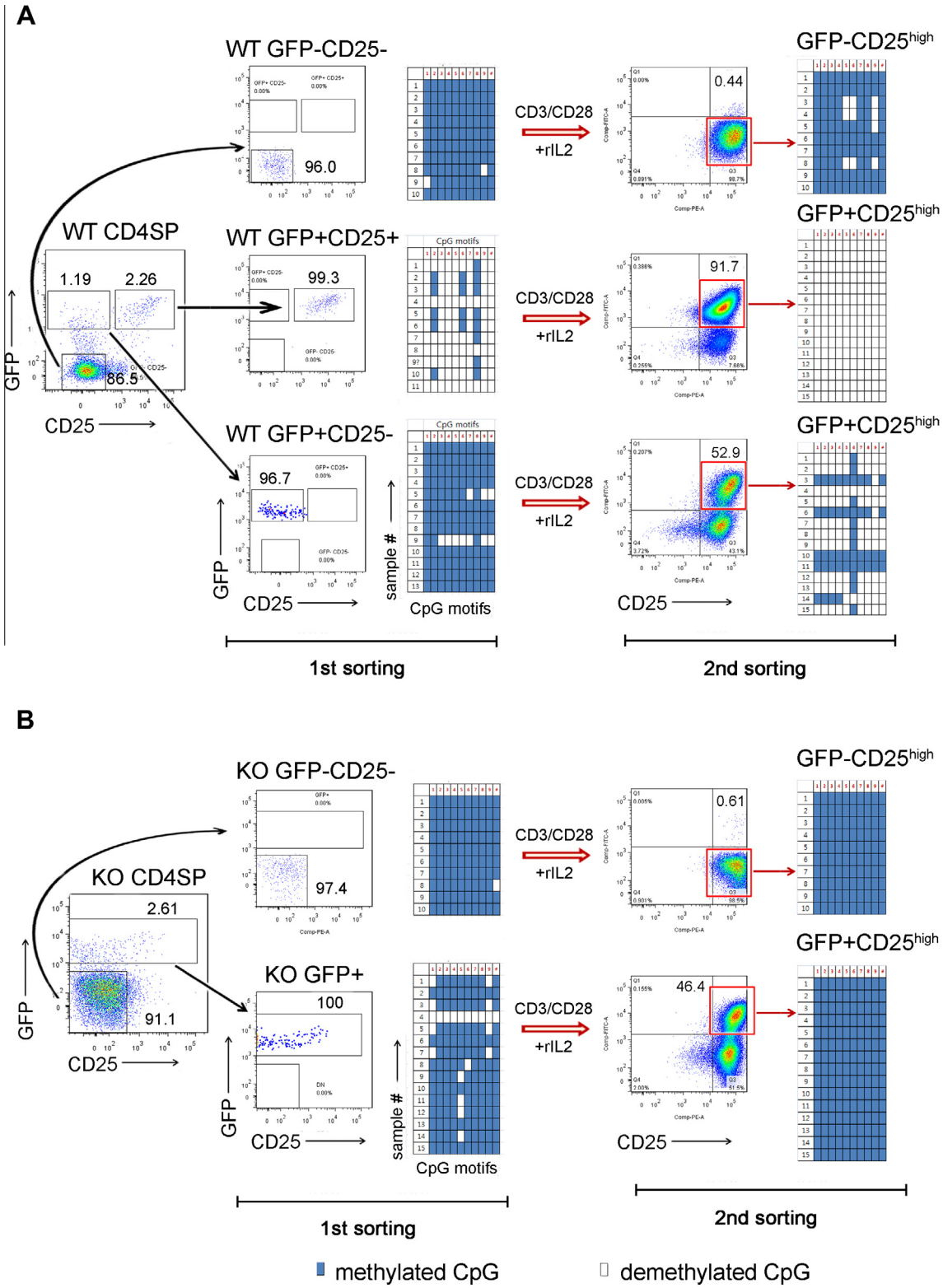

Fig. 1. TSDR demethylation is not initiated in IL2 deficient tTreg cells. (A) Conventional CD4SP thymocytes ($GFP^{-}CD25^{-}$), $CD25^{+}$ ($GFP^{+}CD25^{+}$) and $CD25^{-}$ ($GFP^{+}CD25^{-}$) Treg cells were FACS-sorted from WT CD4SP cells and TSDR demethylation was analyzed by bisulfite sequencing on genomic DNA of each subpopulation (1st sorting). Remaining cells were cultured for 6 days, subjected to second FACS-sorting and subsequent second TSDR analysis (2nd sorting). Foxp3 expression was also checked at the same time. (B) Conventional CD4SP thymocytes ($GFP^{-}CD25^{-}$), total Treg cells (GFP^{+}) were FACS-sorted from IL2 deficient (knock-out, KO) CD4SP cells. TSDR demethylation and culture study were performed as in (A). Numbers in the FACS plots of (A) and (B) refer to the percentage of each subset. Methylation status of individual CpG motif was shown by white (demethylation) or blue (methylation) colors. Data are representative of at least 3 independent experiments. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$; NS, not significant. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

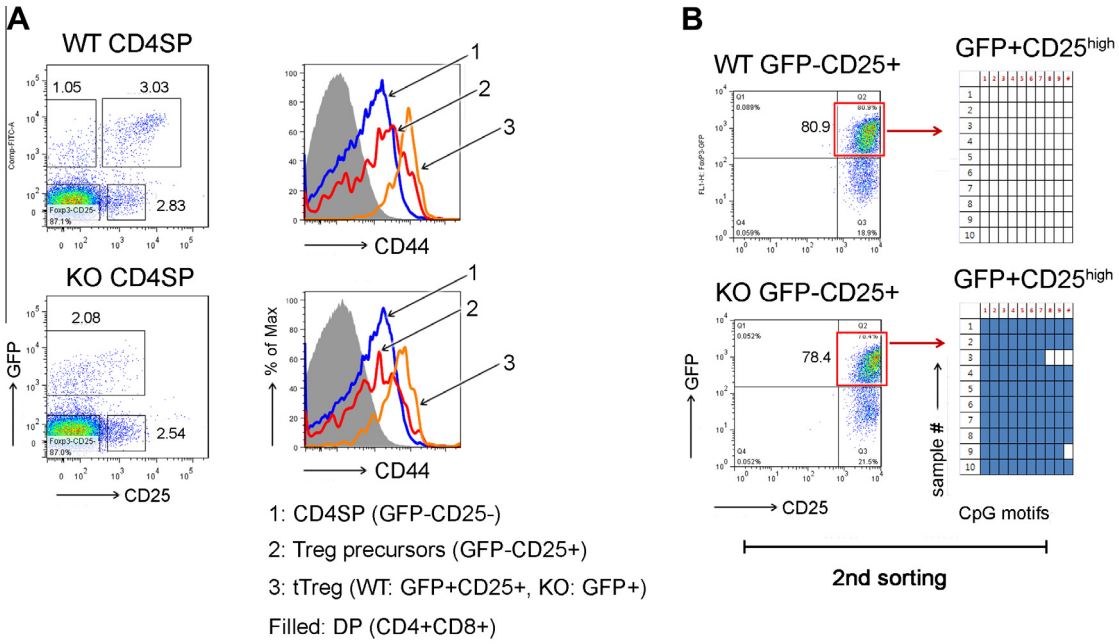

Fig. 2. IL2 deficient Treg precursors are not ready for TSDR demethylation. (A) The frequencies of conventional CD4SP thymocytes, Treg and Treg precursors were shown in the left. CD44 expression levels were compared in the right. (B) WT and IL2 deficient Treg precursors (GFP^-CD25^+, FACS-sorted) were treated with anti-CD3/CD28 + rIL2 for 6 days and then, TSDR was analyzed using the second FACS-sorted GFP^+CD25^{high} cells. Numbers in FACS plots of (A) and (B) refer to the percentage of each subset. Methylation status of individual CpG motif was shown by white (demethylation) or blue (methylation) colors. Data are representative of at least 3 independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 1A). In contrast to WT, however, TSDR of *Il2*$^{-/-}$ pTreg cells was much less demethylated and Foxp3 was also unstable (Supplemental Fig. 1B).

We next checked the thymus. Since CD44 did not separate *Il2*$^{-/-}$ thymic Treg (tTreg) cells clearly and demethylation profiles of TSDR were similar in $CD44^{high}$ and $CD44^{low}$ *Il2*$^{-/-}$ tTreg cells (data not shown), we used CD25 for gating. As reported [23], TSDR in WT $CD25^+$ tTreg cells was partially demethylated. In contrast, WT $CD25^-$ tTreg cells showed methylated TSDR and lost Foxp3 in *in vitro* culture (Fig. 1A). *Il2*$^{-/-}$ tTreg cells were analyzed in the same way and looked similar to WT $CD25^-$ tTreg cells (Fig. 1B). These results led us to ask basic questions: whether both WT $CD25^-$ and *Il2*$^{-/-}$ tTreg cells would return to normal by rIL2, or they have intrinsic defects to become stable $Foxp3^+$ Treg cells, which cannot be corrected by exogenous IL2. To answer, we cultured the sorted cells in the presence of anti-CD3/CD28 + rIL2 for 6 days and TSDR was analyzed again using re-sorted GFP^+CD25^{high} cells (2nd sorting in Fig. 1). TSDR in the GFP^+CD25^{high} cells which were originally WT tTreg cells (both $CD25^+$ and $CD25^-$) were greatly demethylated after IL2 treatment (Fig. 1A), suggesting that

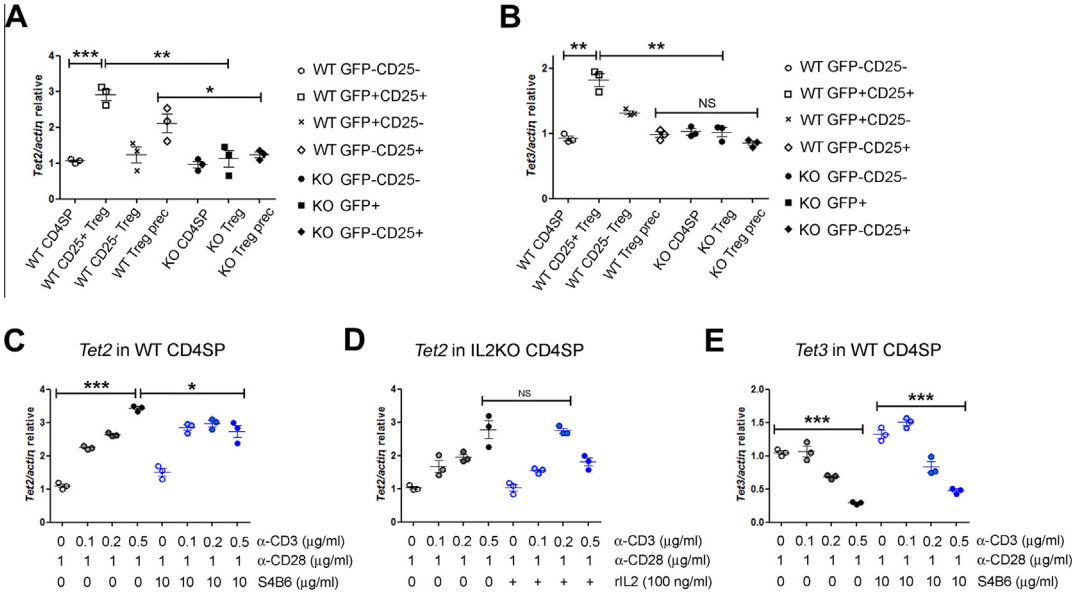

Fig. 3. Down-regulation of Tet2 prevents the TSDR demethylation in IL2 deficient tTreg cells. (A and B) Quantitative RT-PCR was performed to assess the expression levels of *Tet2* (A) and *Tet3* (B) of the indicated cell populations in the mRNA levels. (C) Purified WT CD4SP thymocytes were cultured in the presence of anti-CD3/CD28 ± IL2-blocking mAb (S4B6) for 1 day and the expression of *Tet2* was checked by quantitative RT-PCR. (D) Purified IL2 deficient CD4SP thymocytes were cultured in the presence of anti-CD3/CD28 ± rIL2 for 1 day and the expression of *Tet2* was checked by quantitative RT-PCR. (E) *Tet3* mRNA levels were checked as shown in (C). Data are representative of 3 independent experiments. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$; NS, not significant.

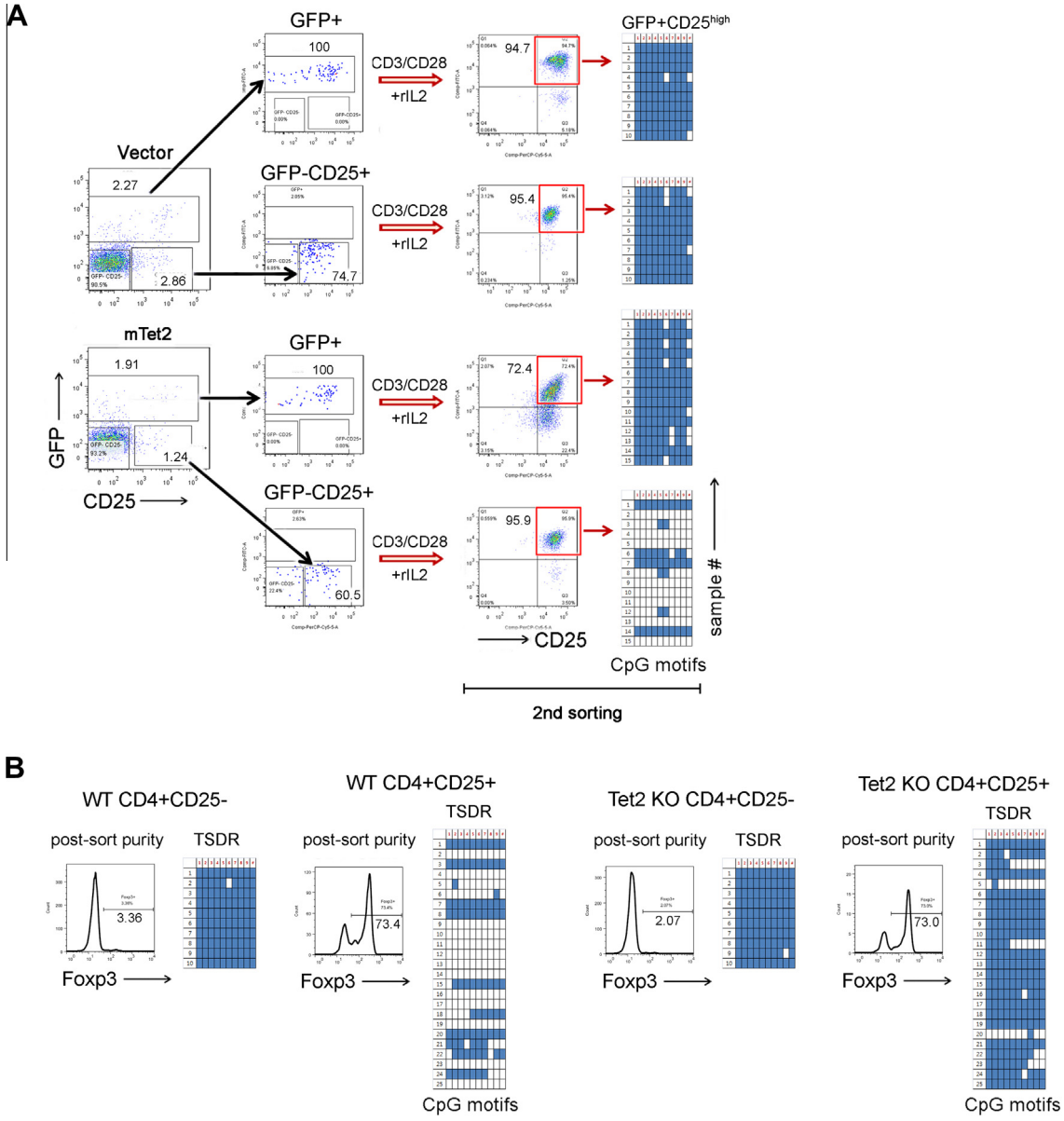

Fig. 4. Over-expression of Tet2 restored TSDR demethylation. (A) Partially purified IL2 deficient thymocytes were transfected with vector or mTet2 cDNA, cultured in the presence of rIL2 overnight to prevent apoptosis (CD25 was up-regulated in some Treg cells) and subjected to FACS-sorting next day. Sorted Treg and Treg precursors were incubated with anti-CD3/CD28 + rIL2 for 6 days. TSDR analysis was performed using the second FACS-sorted GFP$^+$CD25^{high} cells. (B) Conventional CD4SP (CD4$^+$CD25$^-$) and Treg cells (CD4$^+$CD25$^+$) were FACS-sorted from WT and Tet2 deficient (Tet2 KO) CD4SP thymocytes each and TSDR was analyzed by bisulfite sequencing on genomic DNA of each subpopulation. Foxp3 expression was also checked at the same time by intracellular staining. Methylation status of individual CpG motif was shown by white (demethylation) or blue (methylation) colors. Data are representative of 3 (A) and 2 (B) independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

WT CD25$^-$ tTreg cells were primed for TSDR demethylation but seemed to be unstable because IL2 was limited at that time. However, *Il2*$^{-/-}$ tTreg cells showed different responses: TSDR remained completely methylated even after IL2 treatment (Fig. 1B). These results showed that *Il2*$^{-/-}$ Treg cells have intrinsic defects in TSDR demethylation and failed to be committed to a stable Treg lineage in the thymus.

3.2. IL2 deficient Treg precursors are not primed for TSDR demethylation

It was reported that the precursors of Treg cells were enriched in Foxp3$^-$CD25$^+$ population of CD4 single positive (CD4SP) thymocytes and IL2 was essential for their further development [24]. Since it was unclear whether IL2 was necessary for the generation of Treg precursors, we analyzed the phenotypes of WT and *Il2*$^{-/-}$ Treg precursors by using flow cytometry and found no difference in the frequency of Treg precursors and the expression of activation markers like CD44 (Fig. 2A). All CpG motifs in TSDR were also methylated in both Treg precursors (data not shown). These results looked consistent with the current view that TCR stimulation might be responsible for the generation of Treg precursors [1,24] and led us to hypothesize that IL2 induces TSDR demethylation in the transition stage from Treg precursors to Foxp3$^+$ Treg cells. To induce Foxp3 expression, WT and *Il2*$^{-/-}$ Treg precursors were incubated with anti-CD3/CD28 + rIL2 for 6 days and GFP expression was checked by flow cytometry. About 80% of WT Treg precursors became GFP$^+$ and TSDR in sorted WT GFP$^+$CD25^{high} cells was fully demethylated (Fig. 2B, *top*). *Il2*$^{-/-}$ Treg precursors also developed into GFP$^+$ cells at the same rate but yet their TSDR was still

methylated (Fig. 2B, *bottom*). These results clearly showed that IL2 provides essential signals for TSDR demethylation at the early stage of thymic Treg development and prompted us to check the molecules related to DNA demethylation in *Il2*$^{-/-}$ tTreg and Treg precursors.

3.3. Tet2 is down-regulated in IL2 deficient Treg cells

DNA can be demethylated via a Tet-mediated active process [25,26] and both Tet2 and Tet3 were reported to be up-regulated in Treg cells [23]. These reports imply that Tet2 and Tet3 might be involved in TSDR demethylation and led us to check their expression in WT and *Il2*$^{-/-}$ tTreg cells. The levels of Tet2 and Tet3 were elevated in WT Treg precursors (Tet2) and CD25^{+} tTregs (Tet2 and Tet3). However, neither Tet2 (Fig. 3A) nor Tet3 (Fig. 3B) were up-regulated in *Il2*$^{-/-}$ tTreg or Treg precursors. To investigate whether IL2 increased the levels of Tet2 and Tet3, WT CD4SP thymocytes were treated with anti-CD3/CD28 ± IL2-blocking mAb and the level of Tet2 was checked. Tet2 was up-regulated by TCR (anti-CD3/CD28) signaling, but the effect of IL2-blocking mAb was minimal (Fig. 3C). We repeated the same experiment using *Il2*$^{-/-}$ CD4SP thymocytes cultured with/without rIL2 and found the similar results (Fig. 3D), suggesting the role of IL2 in maintaining the level of Tet2, up-regulated by TCR. Tet3 showed the opposite tendency: it was down-regulated (Fig. 3E).

3.4. Over-expression of Tet2 restored TSDR demethylation

Next, we investigated whether over-expression of Tet2 can restore TSDR demethylation. Partially purified *Il2*$^{-/-}$ thymocytes were transfected with the empty vector or the plasmid encoding mouse Tet2, and subjected to FACS-sorting. Each sorted GFP^{+} Treg cells and GFP^{-}CD25^{+} Treg precursors were cultured in the presence of anti-CD3/CD28 + rIL2 and TSDR was analyzed in 6 days. TSDRs in vector-transfected *Il2*$^{-/-}$ Treg and Treg precursors were fully methylated even after culture with rIL2 (Fig. 4A). However, Tet2-transfected cells showed different behaviors: TSDR of Tet2-transfected *Il2*$^{-/-}$ Treg cells was methylated, but yet that of Tet2-transfected *Il2*$^{-/-}$ Treg precursors was demethylated (Fig. 4A), suggesting that the up-regulation of Tet2 is required for TSDR demethylation but should occur early. Lastly, since our results suggest the essential role of Tet2 in TSDR demethylation in Treg cells, we checked TSDR demethylation in Tet2 deficient Treg cells. We crossed floxed Tet2 transgenic mice [20] with Lck-Cre transgenic mice to delete *Tet2* in T cells (LckCre-*Tet2*$^{fl/fl}$, called here as Tet2 knock-out mice). Tet2 was down-regulated in Tet2 deficient thymocytes, but not Tet1 and Tet3 (Tet2 qPCR: WT/knock-out, 1/0.03). WT and Tet2 deficient tTreg cells (CD4^{+}CD25^{+}) were FACS-sorted and used for TSDR analysis. Since GFP could not be used for gating, Foxp3 was checked in sorted cells and about 70% was Foxp3 positive. Interestingly, TSDR in Tet2 deficient tTreg cells was much less demethylated (Fig. 4B), suggesting the essential role of Tet2 in TSDR demethylation during the development of Treg cells.

In this study, we demonstrated that TSDR was not demethylated in *Il2*$^{-/-}$ Treg cells, which resulted from the down-regulation of Tet2 and over-expression of Tet2 fully induced TSDR demethylation. These results uncover the relationships between IL2 and Tet2, and their roles in Foxp3 expression. Currently, it is unclear what up-regulates Tet2 *in vivo*. However, since Tet2 was up-regulated by TCR signals *in vitro*, it looks tempting to speculate that 1) agonistic self-antigen in the thymus up-regulates Tet2 in Treg precursors or CD4SP cells, and 2) IL2 keeps the level of Tet2 high by delivering survival signals to the Tet2-expressing cells (which received strong TCR stimulations). This speculation predicts that Tet2 can be up-regulated in *Il2*$^{-/-}$ thymocytes, if they are resistant to apoptosis. Indeed, human Bcl-2 transgene up-regulated Tet2 in *Il2*$^{-/-}$ Treg and CD4SP cells, albeit in a small way (Supplemental Fig. 2). Further study using Tet2 reporter mice which can visualize Tet2 protein will help us to understand how Tet2 expression is regulated *in vivo* more deeply.

Disclosures

The authors have no conflicting financial interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.110.

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