Loss of TET proteins in regulatory T cells unleashes effector function

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Abstract

TET enzymes oxidize 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) and other oxidized methylcytosines, mediating DNA demethylation and serving as new epigenetic marks. Here we examine the role of TET proteins in Foxp3⁺ regulatory T (Treg) cells, a distinct lineage of CD4⁺ T cells that prevent autoimmunity and maintain immune homeostasis. We report that *Tet2/3^{fl/fl} Foxp3^{Cre}* mice that lack *Tet2* and *Tet3* specifically in Treg cells develop splenomegaly, leukocyte infiltration into tissues and inflammatory disease. Treg cells from these mice showed altered expression of Treg signature genes, with upregulation of genes involved in cell cycle regulation, DNA damage repair and cancer. In littermate mice with more severe inflammation, both CD4⁺ Foxp3⁺ and CD4⁺ Foxp3⁻ cells showed strong skewing towards Tfh and/or Th17 phenotypes. Notably, the presence of wild type Treg cells in mixed bone marrow chimeras and *Tet2/3^{fl/fl} Foxp3^{WT/Cre}* heterozygous female mice did not suppress the aberrant phenotype and function of *Tet2/3^{fl/fl} Foxp3^{Cre}* Treg cells. Fate-mapping experiments indicated that Treg cells from *Tet2/3^{fl/fl} Foxp3^{Cre}* mice were more prone to lose Foxp3 expression; moreover, transfer of total CD4⁺ T cells isolated from *Tet2/3^{fl/fl} Foxp3^{Cre}* mice could elicit inflammatory disease in fully immunocompetent mice. Together, these data indicate that *Tet2* and *Tet3* are guardians of Treg cell stability and immune homeostasis in mice.

Introduction

Regulatory T (Treg) cells are a distinct lineage of CD4⁺ T lymphocytes that prevent autoimmunity against self-antigens and maintain immune homeostasis ^{1, 2}. The lineage specification, development and suppressive function of Treg cells are controlled by Foxp3, an X-chromosome-encoded transcription factor that is the most specific marker distinguishing Treg cells from other T helper cell populations ³. Foxp3-expressing Treg cells have been reported to be stable in both steady state and inflammatory conditions *in vivo* ⁴, but a fraction of Treg cells show unstable Foxp3 expression ^{5, 6}. Fate mapping experiments have shown that many of these Treg cells are “ex-Treg” cells that derive from Foxp3-expressing Treg cells that subsequently lose Foxp3 expression ⁵, but some may be conventional T cells that transiently express Foxp3, thus representing a minor population of uncommitted Treg cells ⁶. Moreover, Treg-specific genetic ablation of many gene products – including cell surface proteins such as neuropilin1, transcription factors such as Nr4a, signaling molecules such as Pten, and epigenetic regulators such as Ezh2 – results in instability of Treg Foxp3 expression and/or loss of suppressive function ^{7, 8, 9, 10, 11}.

Epigenetic changes, including histone modifications, DNA methylation and chromatin accessibility, play a critical role in establishing and maintaining the Treg cell lineage ³. The best studied of these epigenetic changes is DNA demethylation at various Treg-specific demethylated regions (TSDRs), including two “conserved non-coding sequences” (CNS) *CNS1* and *CNS2* within the Foxp3 locus ^{12, 13}. The stability of Foxp3 expression is closely linked to the demethylated status of *CNS1* and *CNS2* ^{12, 13}, while Treg-specific gene expression and optimal Treg function are linked to the DNA methylation status of other relevant TSDRs. Thus, it is important to understand how DNA methylation is regulated at these elements, and how it in turn controls the stability and function of Treg cells.

TET family proteins are Fe(II) and 2-oxoglutarate-dependent dioxygenases that catalyze oxidation of the methyl group of 5-methylcytosine (5mC) in DNA to 5-hydroxymethylcytosine (5hmC) and the further oxidized products 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) ^{14, 15, 16, 17, 18}. These modified bases are intermediates in DNA demethylation as well as potential epigenetic marks that are capable of recruiting specific readers to exert specific functions ^{19, 20, 21}. Recent studies on the role of TET proteins in the immune and hematopoietic systems have revealed a profound influence of TET proteins and DNA modification on immune/hematopoietic cell development and function. Acute elimination of TET function by deletion of both *Tet2* and *Tet3* in hematopoietic stem cells induced the rapid development of an aggressive and fully-penetrant myeloid leukaemia in adult mice ²². Concomitant deletion of *Tet2* and *Tet3* by *Mb1-Cre* in early B cells resulted in developmental blockade at the pro-B to pre-B cell transition due to a defect in immunoglobulin light chain rearrangement ^{23, 24}. Deletion of *Tet2* and *Tet3* in T cells mediated by *CD4-Cre* led to an antigen-driven expansion of invariant NKT (iNKT) cells, which developed rapidly into CD1d-restricted iNKT cell lymphoma ²⁵. Treg cells in this *Tet2/3*-deficient mouse strain displayed unstable Foxp3 expression, concomitantly with DNA hypermethylation at *CNS1*, *CNS2*, and other TSDRs ¹². Combined deletion of *Tet1* and *Tet2* also resulted in *CNS2* hypermethylation and impaired Treg cell differentiation and function ²⁶.

Our previous study on the role of TET proteins in Treg cells ¹² was complicated by the iNKT cell expansion occurring in the same mouse strain, in which *Tet* gene deletion was mediated by *CD4-Cre* ^{12, 25}. In our current study, we used a mouse strain in which *Tet2* and *Tet3* deficiency were targeted specifically to Foxp3-expressing Treg cells using *Foxp3^{YFP-Cre}* (*Foxp3^{Cre}*). Here we show that *Tet2/3^{fl/fl}Foxp3^{Cre}* mice develop an inflammatory disease with splenomegaly and leukocyte infiltration into lung, and that CD4⁺Foxp3⁺ Treg cells, CD4⁺Foxp3⁻ and CD8⁺ T cells in these mice display an activated phenotype. Transcriptome analysis showed dysregulation of Treg signature genes as well as genes related to cell cycle, DNA damage repair and cancer in *Tet2/3 DKO* Treg cells compared to WT Treg cells. Perplexingly, however, a very similar inflammatory disease developed in *Tet2/3^{fl/fl}Foxp3^{WT/Cre}* heterozygous female mice, as well as in mixed bone marrow chimeras in which lethally irradiated mice were reconstituted with a 1:1 mixture of wildtype and

Tet2/3^{fl/fl}Foxp3^{Cre} bone marrow cells, indicating that the presence of wildtype (WT) Treg cells was not sufficient to suppress the inflammatory phenotype observed in *Tet2/3* DKO mice. Fate-mapping experiments showed that Treg cells from *Tet2/3^{fl/fl}Foxp3^{Cre}* DKO mice were more prone to lose Foxp3 expression and become “ex-Treg” cells. Furthermore, transfer of total CD4⁺ T cells from *Tet2/3^{fl/fl}Foxp3^{Cre}* DKO mice, which contained these ex-Treg cells, elicited inflammatory disease in immunocompetent mice. Thus TET deficiency in Treg cells resulted in a dominant inflammatory disease, in which the inflammatory phenotype was driven, at least in part, by ex-Treg cells that acquired effector function, and also conferred inflammatory function on endogenous host CD4⁺ T cells. Overall, our data indicate that Tet2 and Tet3 are essential for maintenance of Treg cell stability and immune homeostasis in mice.

Results

Uncontrolled T cell activation and tissue infiltration in mice lacking *Tet2* and *Tet3* in Treg cells

To investigate the role of TET proteins in Treg cells, we crossed mice carrying *LoxP*-flanked *Tet2* and *Tet3* alleles (*Tet2/3^{fl/fl}*) with *Foxp3^{YFP-Cre}* (*Foxp3^{Cre}*) mice in which a YFP-Cre fusion protein is knocked into the 3' UTR of the *Foxp3* gene²⁷, to generate mice with Treg-specific deletion of *Tet2* and *Tet3* (*Tet2/3^{fl/fl}Foxp3^{Cre}* mice). *Tet2* and *Tet3* mRNAs were specifically deleted in CD4⁺YFP⁺ Treg cells but not in CD4⁺YFP⁻ conventional T cells (Supplementary Fig. 1a). Mice lacking *Tet2* and *Tet3* in Treg cells did not survive past 8-22 weeks of age (Fig. 1a), although a fraction of male mice survived slightly longer than female mice (Supplementary Fig. 1b). *Tet2/3^{fl/fl}Foxp3^{Cre}* mice displayed splenomegaly and lymphadenopathy, primarily of mesenteric lymph nodes (mLN, Supplementary Fig. 1c), as evidenced by an increased cellularity (Fig. 1b). The slight increase in cellularity observed in peripheral lymph nodes (pLN) did not reach statistical significance (Fig. 1b). Histological analysis revealed disrupted splenic architecture in *Tet2/3^{fl/fl}Foxp3^{Cre}* mice with expansion of the white pulp areas, accompanied by leukocyte infiltration into the lung (Supplementary Fig. 1d). Examination of the peripheral blood showed an increase in neutrophils and a decrease in lymphocytes, which were within the normal range (Table S1). The concentration of red blood cells appeared normal (Table S1). *Tet2/3^{fl/fl}Foxp3^{Cre}* mice had significantly higher titers of anti-dsDNA antibodies in the serum compared to WT mice (Supplementary Fig. 2a), suggesting altered self-tolerance. In addition, the titer of serum IgG2b isotype was significantly higher in *Tet2/3^{fl/fl}Foxp3^{Cre}* mice than in WT mice, and there was a tendency towards increased titers of serum IgG1, IgG2a, IgG3 and IgM, which correlated with the severity of disease development in the mice. In contrast, the titer of serum IgA appeared slightly decreased in *Tet2/3^{fl/fl}Foxp3^{Cre}* mice compared to WT mice (Supplementary Fig. 2b).

Tet2/3^{fl/fl}Foxp3^{Cre} mice displayed a significant reduction in the percentages of CD4⁺ and CD8⁺ T cells in spleen and peripheral lymph nodes (Fig. 1c, *left panel*, Supplementary Fig. 3a), but the numbers of CD4⁺ and CD8⁺ T cells were roughly maintained except for an increase in CD4⁺ T cell numbers in the spleen (Fig. 1c, *right panel*). The percentage of CD62L^{hi}CD44^{lo} naïve cells was dramatically decreased in both CD4⁺ and CD8⁺ T cell populations, indicating that majority of the T cells were activated or became memory cells (Fig. 1d,e and Supplementary Fig. 3b). We also observed a reduction in the percentage of B cells and an increase in the percentage of CD11b⁺Gr1⁺ myeloid cells (Supplementary Fig. 4).

Dysregulated cell surface phenotype of *Tet2/3*-deficient Treg cells

We next examined the phenotypic features of *Tet2/3*-deficient Treg cells. The frequency of Foxp3⁺ Treg cells (as a percentage of total CD4⁺ T cells) was increased in spleen as well as in peripheral and mesenteric lymph nodes of *Tet2/3^{fl/fl}Foxp3^{Cre}* mice relative to that of WT mice (Fig. 2a,b). Despite the increase in frequency, *Tet2/3* DKO Treg cells had lower expression levels of CD25 (the IL-2 receptor α chain), a signature surface cytokine receptor of Treg and activated T cells; they also displayed a significant decrease in the

expression of neuropilin-1 (Nrp1), an important mediator of Treg homeostasis and function²⁸, in both spleen and lymph nodes (Fig. 2c). Flow cytometric analysis revealed varying degrees of increased expression and/or an increased proportion of cells expressing the transcription factor Helios (Ikzf2) and the surface proteins ICOS, CD103, GITR, CTLA-4 and PD-1 in *Tet2/3 DKO* compared to those in WT Treg cells, especially in the spleen; but similar expression of CD127, the IL-7 receptor α chain (Fig. 2d). Both Nrp1 and Helios have been reported to be markers of thymic-derived Treg cells and were used to distinguish thymic-derived Treg cells from peripherally-induced Treg cells^{29, 30, 31}; however, the reliability of these markers is still controversial^{32, 33}. The expression of Nrp1 was downregulated in Treg cells from mesenteric lymph nodes in *Tet2/3^{fl/fl}Foxp3^{Cre}* mice compared to that of WT mice, while the expression of Helios was increased (Supplementary Fig. 3c). The chemokine receptor CCR7, which is important for the migration and function of Treg cells³⁴, was also significantly downregulated in Treg cells from *Tet2/3^{fl/fl}Foxp3^{Cre}* compared to WT mice (Supplementary Fig. 3d). Moreover, compared to their WT counterparts, Treg cells from *Tet2/3^{fl/fl}Foxp3^{Cre}* mice showed a decreased proportion of CD62L^{hi}CD44^{lo} naïve cells (Fig. 2e) and a higher frequency of CD69⁺ recently activated cells (Fig. 2f), indicating that *Tet2* and *Tet3* deficiency in Treg cells results in an activated phenotype and dysregulated expression of multiple Treg activation and phenotypic molecules. The increased frequency and activation status of Treg cells that we observe in *Tet2/3^{fl/fl}Foxp3^{Cre}* mice is also commonly observed in mice with Treg perturbations, and may reflect a compensatory increase in Treg cell numbers observed under conditions of insufficient overall Treg cell function^{8, 35}.

Decreased long-term suppressive function of *Tet2/3*-deficient Treg cells

To investigate the *in vivo* function of Treg cells isolated from *Tet2/3^{fl/fl}Foxp3^{Cre} DKO* mice, we used a mouse model in which we attempted to correct the *scurfy* phenotype. *Scurfy* mice lack Treg cells because they carry a natural frame-shift mutation of in the *Foxp3* gene that abrogates Foxp3 protein expression³⁶. Transfer of peripheral CD4⁺ T cells from *scurfy* male mice into *Rag1*-deficient recipients leads to an expansion of autoreactive *scurfy* CD4⁺ T cells and severe autoimmune inflammation due to a lack of effective Treg cell suppression; both features can be suppressed by co-transfer of congenically-marked WT Treg cells³⁷. We transferred 5x10⁵ *scurfy* CD4⁺ T cells (CD45.1⁺) into *Rag1*-deficient recipient mice together with 1x10⁵ Treg cells from CD45.2⁺ mice, either WT *Foxp3^{Cre}* or *Tet2/3^{fl/fl}Foxp3^{Cre} DKO* mice (Supplementary Fig. 5a). In this short-term *in vivo* suppression assay, Treg cells isolated from *Tet2/3^{fl/fl}Foxp3^{Cre}* mice suppressed the expansion of *scurfy* CD4⁺ T cells to almost the same extent as Treg cells isolated from WT *Foxp3^{Cre}* mice, as evidenced by decreased expansion of CD45.1⁺ *scurfy* cells measured in spleen and lymph nodes 4-5 weeks after adoptive transfer (percentages, Supplementary Fig. 5b; total cell numbers, Supplementary Fig. 5c). As we previously observed for Treg cells from *Tet2/3^{fl/fl}CD4^{Cre}* mice¹², Treg cells isolated from *Tet2/3^{fl/fl}Foxp3^{Cre}* mice were more likely to lose Foxp3 expression compared to WT control Treg cells (Supplementary Fig. 5b,c).

To examine Treg function over a longer time course, we transferred bone marrow cells from *scurfy* mice into sublethally irradiated *Rag1^{-/-}* recipient mice, alone or together with bone marrow cells from WT or *Tet2/3^{fl/fl}Foxp3^{Cre} DKO* mice (Supplementary Fig. 5d,e). As expected³⁸, recipient mice receiving *scurfy* bone marrow cells alone started to lose weight at 4 weeks and succumbed 6-7 weeks after transfer. Co-transfer of WT bone marrow cells suppressed *scurfy* cell expansion and protected the recipient mice from death, whereas co-transfer of *Tet2/3 DKO* bone marrow cells failed to protect the recipient mice, which lost weight after 9 weeks and died at 11-13 weeks after transfer (Supplementary Fig. 5d,e). Together, these data indicate that *Tet2/3*-deficient Treg cells from *Tet2/3^{fl/fl}Foxp3^{Cre}* mice have normal *in vivo* suppressive function in the short-term that is not sustained in the long-term.

Moderate DNA hypermethylation at *Foxp3* CNS2 in *Tet2/3 DKO* Treg cells

The expression of *Foxp3* during Treg cell differentiation is regulated by three conserved noncoding sequence (CNS) elements located in the first intron of the *Foxp3* gene³⁹. Among these, *CNS1* and *CNS2* have been shown to control the stability of *Foxp3* expression in a manner linked to the DNA modification status of these elements^{12,13}. Given that TET proteins catalyze the process of loss of 5mC at *CNS1* and *CNS2* during Treg development *in vivo*¹², we examined the DNA modification status at *Foxp3* *CNS1* and *CNS2* in CD4⁺CD25⁺YFP⁺ peripheral Treg cells isolated from male WT or *Tet2/3^{fl/fl}Foxp3^{Cre}* mice using bisulfite-sequencing (BS-seq). The results clearly show impaired DNA demethylation (assessed as loss of 5mC) in *Foxp3* *CNS1* and *CNS2* in Treg cells isolated from *Tet2/3^{fl/fl}Foxp3^{Cre}* mice; however, this impairment was less pronounced (i.e. 5mC + 5hmC levels assessed by BS-seq were lower) than we had previously observed in Treg cells isolated from *Tet2/3^{fl/fl}CD4^{Cre}* mice (Fig. 3a,b). This result is likely to reflect the chronological order of CD4^{Cre} versus *Foxp3^{Cre}* expression: CD4^{Cre} mediates *Tet2* and *Tet3* deletion at an earlier developmental stage compared to *Foxp3^{Cre}*. Consistent with this difference, CpG sites #6 and #9-11 in the *CNS2* region, which have already initiated the process of loss of 5mC at the *Foxp3⁺CD25⁻* precursor stage¹², were efficiently demethylated in *Tet2/3^{fl/fl}Foxp3^{Cre}* Treg cells. We also examined several other regulatory regions that show Treg-specific hypomethylation: *Il2ra* intron 1a, *Tnfrsf18* exon 5, *Ikzf4* intron 1b and *Ctla4* exon 2⁴⁰. The DNA modification status at *Il2ra* intron 1a, *Tnfrsf18* exon 5 and *Ikzf4* intron 1b showed similar pattern compare to that of *Foxp3* *CNS1* and *CNS2* regions: DNA demethylation was impaired in Treg cells isolated from *Tet2/3^{fl/fl}Foxp3^{Cre}* mice, but to a lesser extent, compare to that in Treg cells isolated from *Tet2/3^{fl/fl}CD4^{Cre}* mice (Fig. 3c-3e). However, at *Ctla4* exon 2, the levels of impairment in DNA demethylation were similar regardless of whether Treg cells were isolated from *Tet2/3^{fl/fl}Foxp3^{Cre}* mice or *Tet2/3^{fl/fl}CD4^{Cre}* mice (Fig. 3f).

Dysregulated expression of genes involved in cell cycle, DNA damage repair and immune processes in *Tet2/3* DKO Treg cells

To further investigate the molecular mechanisms by which TET proteins control Treg cell identity and function, we compared the transcriptional profiles of CD4⁺YFP⁺ Treg cells sorted from 14-weeks old *Foxp3^{Cre}* WT and *Tet2/3^{fl/fl}Foxp3^{Cre}* DKO mice (2 biological replicates each, isolated from pooled spleen and peripheral lymph nodes). We isolated RNA from two *Tet2/3^{fl/fl}Foxp3^{Cre}* DKO littermates with the assumption that these mice would have comparable phenotypes. However, when sacrificed, one of these mice (labeled with an asterisk: DKO*) clearly showed more severe splenomegaly than its littermate (DKO). This difference was obvious in principal component analysis (PCA) of the RNA-seq data: the biological replicates of WT cells, whether Treg or CD4⁺ *Foxp3⁻* cells, clustered closely together, while those from Treg and CD4⁺ *Foxp3⁻* cells obtained from the two 14-weeks old *Tet2/3^{fl/fl}Foxp3^{Cre}* DKO littermates were not as closely aligned (Supplementary Fig. 6a). As expected, both Treg and CD4⁺ T cells from the *Tet2/3^{fl/fl}Foxp3^{Cre}* DKO mouse with less severe splenomegaly more closely resembled the corresponding WT cells, whereas cells from the mouse with more severe splenomegaly (marked with an asterisk) were less similar to WT (Supplementary Fig. 6a). Nevertheless, scatter plots of the data showed reasonably good correlation between the two replicates from *Tet2/3* DKO Treg and CD4⁺ T cells (0.92 and 0.90 respectively), although the correlation between the WT replicates was stronger (0.94 and 0.93 respectively) (Supplementary Fig. 6b,c). Based on these data, we averaged the two DKO replicates for subsequent analyses.

Based on a cut-off of FDR (false discovery rate) ≤ 0.05 and a fold change ≥ 1.5 , 1565 genes were differentially expressed, with 1156 genes being upregulated and 409 genes being downregulated in *Tet2/3* DKO Treg cells compared to WT Treg cells (Fig. 4a). Seventy-six Treg signature genes were differentially expressed (Table S2) and a selected subset (*Foxp3*, *Nrp1*, *Il1r1*, *Prg4*, *Dnahc7b* and *Klrg1*) are pointed out in the mean average (MA) plot (Fig. 4a). Ingenuity pathway analysis (IPA) of the differentially expressed genes showed that *Tet2/3* deficiency in Treg cells affected three main categories of canonical pathways (Fig. 4b and Table S3): genes involved in DNA damage, DNA repair (*Brca1*, *Atm*, *Rad50*) and cell cycle (*Cdkn1a*, *Cdkn2a*,

Hipk2) (green bars); genes implicated in immune cell function such as the antigen presentation pathway (*H2-Aa*, *H2-Ab* and *H2-Eb1*), Th1 and Th2 activation pathways, etc (blue bars); and genes related to molecular mechanisms of cancer (orange bars).

A recent study demonstrated that microbial-mediated inflammatory signals were critical for myeloid expansion of *Tet2*-deficient mice⁴¹. Therefore, we used RNA-seq to examine the transcriptional profiles of CD4⁺YFP⁺ Treg cells sorted from mesenteric lymph nodes of 14-week old WT and *Tet2/3^{fl/fl}Foxp3^{Cre}* DKO mice (3 biological replicates each). There was good correlation among the biological replicates (Table S4). Based on a cut-off of FDR (false discovery rate) ≤ 0.05 and a fold change ≥ 1.5 , 2073 genes were differentially expressed, with 1449 genes being upregulated and 624 genes being downregulated in *Tet2/3* DKO Treg cells compared to WT Treg cells (Supplementary Fig. 7a). A handful of them overlapped with the differentially expressed genes from *Tet2/3* DKO vs WT Treg cells isolated from pooled spleen and pLN (Supplementary Fig. 7b). IPA analysis of the differentially expressed genes indicated that similar canonical pathways were affected, including genes involved in cell cycle, DNA damage, immune cell function and cancer development (Supplementary Fig. 7c and Table S5).

The majority of cell cycle-related genes were upregulated in *Tet2/3* DKO Treg cells (see heatmap of Fig. 4c and Supplementary Fig. 7d), prompting us to assess the proliferation of *Tet2/3* DKO Treg cells *in vivo*. We injected WT and *Tet2/3^{fl/fl}Foxp3^{Cre}* DKO mice with BrdU, a thymidine analog that is incorporated into newly synthesized DNA during replication, and analyzed Treg cells 24 h later. Indeed, *Tet2/3* DKO Treg cells incorporated significantly more BrdU compared to WT Treg cells (Fig. 4d). The genes involved in DNA damage pathways also prompted us to examine the level of phosphorylated histone H2A.X (γ H2AX), an early marker of DNA damage⁴², by flow cytometry. In fact, the MFI (mean fluorescent intensity) of γ H2AX staining was significantly increased in both Treg cells and CD4⁺Foxp3⁻ cells in spleen and mLN isolated from *Tet2/3^{fl/fl}Foxp3^{Cre}* compared to WT mice (Supplementary Fig. 8). But we did not observe any increase in the level of active caspase-3, an early stage apoptosis marker, in Treg cells from *Tet2/3^{fl/fl}Foxp3^{Cre}* DKO mice compared to WT mice (Supplementary Fig. 9). Analysis of the TCR repertoire using RNA-seq reads mapping to the CDR3 region at *Tcrb* showed greater clonal expansion in both Treg cells and CD4⁺Foxp3⁻ cells isolated from *Tet2/3^{fl/fl}Foxp3^{Cre}* compared to WT mice (Supplementary Fig. 10 and Table S6 and S7), suggesting antigen-driven expansion.

Compared to WT mice, expression of genes involved in Tfh differentiation, including *Gzmb*, *Pdcd1*, *Bcl6*, *Maf*, *Cxcr5* and *Il21*, was unaltered or only mildly increased in Treg cells isolated from the *Tet2/3^{fl/fl}Foxp3^{Cre}* mouse with moderate splenomegaly (DKO), but markedly increased in Treg cells isolated from the littermate mouse with more severe splenomegaly (DKO*) (Fig. 4e, left panel). Genes related to Tfh and Th17 cell differentiation (*Il17a*, *Il17f*, *Rorc*) in CD4⁺ Foxp3⁻ T cells displayed a similar trend (Fig. 4e, right panel), indicating that both CD4⁺Foxp3⁺ Treg cells and CD4⁺Foxp3⁻ T cells from *Tet2/3^{fl/fl}Foxp3^{Cre}* mice displayed skewing to Tfh and/or Th17 lineages in a manner that correlated directly with disease severity. The skewed Tfh and/or Th17 lineages were also observed in Treg cells and CD4⁺ Foxp3⁻ T cells isolated from mLN of *Tet2/3^{fl/fl}Foxp3^{Cre}* mice (Supplementary Fig. 7e).

In WT mice at steady state, fewer than 5% of CD4⁺TCR β ⁺ T cells from spleen and mesenteric lymph nodes stained positive for the Tfh signature molecules CXCR5 and PD-1, but this percentage was much higher in the spleen and mesenteric lymph nodes of *Tet2/3^{fl/fl}Foxp3^{Cre}* mice (Fig. 4f). In addition, spleen and mesenteric lymph nodes of *Tet2/3^{fl/fl}Foxp3^{Cre}* mice had more germinal center (GC) B cells, as judged by expression of the GC signature markers GL7 and CD95 (Fig. 4g).

Unexpected disease development in *Tet2/3^{fl/fl}Foxp3^{WT/Cre}* heterozygous females and mixed bone chimeras

Foxp3 is encoded on the X-chromosome, hence due to random X-inactivation, *Tet2/3^{fl/fl}* female mice that are heterozygous for *Foxp3^{Cre}* (*Foxp3^{WT/Cre}*) should harbor both *Tet2/3*-deleted Treg cells (*Foxp3^{Cre}*-positive) and WT Treg cells (*Foxp3^{Cre}*-negative). Thus, the presence of WT Treg cells should in theory prevent the mice from developing any autoimmune disease. Surprisingly, we found that female mice heterozygous for *Foxp3^{Cre}* also developed lymphoproliferative disease beginning as early as 15 weeks of age, and all mice succumbed by the age of 38 weeks (Fig. 5a). As expected, the total cellularity of the spleen from *Tet2/3^{fl/fl} Foxp3^{WT/Cre}* female mice was significantly increased compared to WT female mice (Fig. 5b).

To confirm this observation independently, we generated mixed bone marrow chimeras by adoptively co-transferring WT (CD45.1⁺) bone marrow cells together with bone marrow cells (CD45.2⁺) isolated from WT or *Tet2/3^{fl/fl} Foxp3^{Cre}* DKO mice at 1:1 ratio into lethally irradiated CD45.1⁺ WT mice (Fig. 5c). Chimeric mice given either WT or *Tet2/3^{fl/fl} Foxp3^{Cre}* DKO bone marrow cells were similar and appeared relatively normal at 14-16 weeks after bone marrow transfer (Supplementary Fig. 11). However at 18-20 weeks after adoptive transfer, the DKO bone marrow chimeras developed splenomegaly compared to the WT chimeras and showed a significant increase of total cellularity in the spleen (Fig. 5d).

Notably, CD45.2⁺ cells in mixed chimeras with *Tet2/3^{fl/fl} Foxp3^{Cre}* DKO bone marrow showed considerably higher capacity for expansion compared to CD45.2⁺ cells in WT mixed bone marrow chimeras (Fig. 5e,f, *leftmost panels*). In the DKO chimeric mice, the percentage of CD4⁺CD45.2⁺ T cells was significantly higher than expected, with over 80% of CD4⁺CD45.2⁺ T cells observed in one DKO chimeric mouse; however, the percentage of CD8⁺ T cells was strongly decreased (Fig. 5e, *second panels*; Fig. 5f, *second and third panels*). The frequency of *Foxp3*⁺ cells in the CD45.2⁺ CD4⁺ population varied widely in the DKO mixed chimeras, with some chimeras displaying a frequency as high as 60-70%, while others showed fewer than 10% of CD45.2⁺ CD4⁺ *Foxp3*⁺ cells (Fig. 5e, *middle panel*; Fig. 5f, *last panel*), suggesting that Treg cells in the DKO chimeras could manifest unstable *Foxp3* expression. Moreover, both CD4⁺*Foxp3*⁺ and CD4⁺*Foxp3*⁻ DKO cells in the chimeric mice showed an activated/memory phenotype, with a strong decrease in the proportion of “naïve” CD62L^{hi}CD44^{lo} cells (Fig. 5e, *last two panels*). Together these data indicate that the loss of T regulatory function in *Tet2/3* DKO Treg cells is dominant, and cannot be rescued or prevented in the presence of WT Treg cells.

Fate-mapping reveals unstable *Foxp3* expression in *Tet2/3* DKO Treg cells

Although *Foxp3*⁺ Treg cells are generally stable and are marked by sustained expression of *Foxp3*, they may lose *Foxp3* expression under certain conditions and develop into cells resembling effector T cells, designated “ex-Treg” cells⁵. To examine whether *Tet2/3*-deficient Treg cells indeed lose *Foxp3* expression and become ex-Treg effector cells, we introduced the *Rosa26-YFP^{LSL}* allele⁴³ into *Tet2/3^{fl/fl} Foxp3^{Cre}* mice. In the LSL (*LoxP*-STOP-*LoxP*) cassette, a strong transcriptional stop site is flanked by two *LoxP* sites and inserted into the *Rosa26* locus ahead of the cDNA sequence encoding YFP, ensuring that YFP expression is turned on only in Cre-expressing cells. Since YFP expression from the *Rosa26* locus is much brighter than YFP expression from the YFP-Cre fusion protein introduced into the 3' UTR of the *Foxp3* gene, cells expressing YFP from the *Rosa26* locus can be easily distinguished from cells expressing the YFP-Cre fusion protein encoded in the *Foxp3* gene⁴⁴ (Fig. 6a, where intermediate YFP expression derives from the YFP-Cre fusion protein whereas high YFP expression derives from the *Rosa26* fate mapping allele). Analysis of CD4⁺ T cells from spleen and mesenteric lymph nodes of *Tet2/3^{fl/fl} Foxp3^{Cre} Rosa26-YFP^{LSL}* mice showed clearly that a much higher proportion of *Rosa-YFP^{high}* cells – cells that had turned on Cre from the *Foxp3* gene, and thus had been Treg cells earlier in their developmental history – had lost *Foxp3* expression and turned into ex-Treg cells, compared to *Rosa-YFP^{high}* cells from heterozygous *Tet2^{+/fl} Tet3^{fl/fl} Foxp3^{Cre} Rosa26-YFP* mice (Fig. 6a, b). Further confirming the existence of ex-Treg cells in *Tet2/3^{fl/fl} Foxp3^{Cre}* mice, we showed by quantitative real-time PCR as well as RNA-seq that *Tet2* and *Tet3* mRNAs were both greatly reduced in expression in CD4⁺YFP⁻

cells isolated from *Tet2/3^{fl/fl}Foxp3^{Cre}* compared to WT mice (qPCR, Supplementary Fig. 12a; number of normalized reads mapping to the deleted exons; Supplementary Fig. 12b-c). Taken together with the data presented in the previous sections, this finding suggests strongly that a substantial fraction of the activated CD4⁺YFP⁺ T cells in *Tet2/3^{fl/fl}Foxp3^{Cre}* mice (Fig. 5e,f) are ex-Treg cells that have lost suppressive function and acquired deleterious effector function.

Transfer of total CD4⁺ T cells from *Tet2/3 DKO* mice elicits disease in immunocompetent mice

To test the above hypothesis of deleterious effector function acquired by ex-Treg cells from *Tet2/3^{fl/fl}Foxp3^{Cre}* mice, we asked whether total CD4⁺ T cells from *Tet2/3^{fl/fl}Foxp3^{Cre}* mice, which contain ex-Treg cells as well as bystander activated CD4⁺ T cells, could elicit disease when transferred into healthy immunocompetent recipient mice. We transferred purified CD4⁺ T cells (5x10⁶; purity > 98.5%) from spleen and peripheral lymph nodes into fully immunocompetent (non-irradiated) congenic CD45.1⁺ recipient mice (Fig. 7a). All the recipients developed enlarged spleen and lymph nodes and developed disease 7-10 weeks after transfer (Fig. 7b). *Tet2/3 DKO* CD4⁺ T cells expanded 3-50 fold, from the starting transferred population of 5 million cells to as many as 250 million cells (Fig. 7b). The percentage of Foxp3⁺ cells after expansion of the transferred CD4⁺ population varied from mouse to mouse, with three mice showing high-level Foxp3 expression (mouse example 1 in Fig. 7c), and the others showing low to no expression of Foxp3 (mouse example 2 in Fig. 7c). We observed a compensatory expression of Foxp3⁺ cells in CD45.1⁺ host cells in each individual (Fig. 7b and 7c). Moreover, both CD4⁺Foxp3⁺ host Treg cells and CD4⁺Foxp3⁻ host cells displayed activated phenotype with low levels of CD62L expression and high levels of CD44 expression (Fig. 7c, Host Cells). Together these data suggest that *Tet2/3 DKO* CD4⁺ T cells contained the cell population responsible for transferring the diseases to immunocompetent mice. These might be ex-Treg effector cells, host CD4⁺ T cells that became activated and converted to effector cells, or both.

Discussion

Although ectopic expression of Foxp3 in conventional CD4⁺ T cells confers suppressive activity as well as expression of many Treg signature genes^{45, 46, 47}, Foxp3 expression by itself does not recapitulate all aspects of the *bona fide* Treg transcriptional and epigenetic landscape, nor does it result in stable, long-lasting immune suppressive function^{19, 48, 49}. Here, we investigated the role of two epigenetic regulators, the methylcytosine oxidases Tet2 and Tet3, in Treg function. We show that mice with Treg-specific deletion of *Tet2* and *Tet3* (*Tet2/3^{fl/fl}Foxp3^{Cre} DKO* mice) display defective Treg function and develop an inflammatory disease characterised by splenomegaly and lymphocyte infiltration into tissues. The inflammatory phenotype had three interrelated causes. First, TET loss-of-function in developing *Tet2/3^{fl/fl}Foxp3^{Cre} DKO* Treg cells led to increased methylation (5mC+5hmC) at the *Foxp3 CNS1* and *CNS2* enhancers compared to wildtype Treg cells. Second, increased methylation at these intronic enhancers resulted in decreased stability of Foxp3 expression and impaired Treg cell function. Third, both ex-Treg cells and conventional CD4⁺ and CD8⁺ T cells in *Tet2/3^{fl/fl}Foxp3^{Cre} DKO* mice developed an aberrant dominant effector phenotype, which could not be suppressed by the presence of wildtype Treg cells in *Tet2/3^{fl/fl}Foxp3^{WT/Cre}* heterozygous females or in mixed bone marrow chimeras. Our data emphasize the essential role of TET-mediated epigenetic modifications not only in maintaining the demethylated status of the *Foxp3 CNS1* and *CNS2* enhancers, thereby stabilizing Foxp3 expression, specifying Treg cell lineage and conferring stable Treg identity, but also in preventing indirectly the acquisition of aberrant effector function by bystander CD4⁺ and CD8⁺ T cells with otherwise normal TET function.

In our animal facility, mice lacking only *Tet2* or *Tet3* in Treg cells showed mild phenotypes if any¹², indicating that *Tet2* and *Tet3* have redundant functions in Treg cells as we previously observed for myeloid cells, B cells and iNKT cells^{22, 23, 25}. Consistent with this hypothesis of redundant function, Treg cells deficient

in both *Tet1* and *Tet2* also showed increased *CNS2* methylation, impaired Treg cell differentiation and impaired suppressor function *in vivo* and in mixed bone marrow chimeras²⁶. In contrast, a previous report suggested that Treg cells from mice lacking *Tet2* alone had a surprisingly high increase in the level of 5mC+5hmC at *CNS2*⁵⁰. Similarly variable phenotypes have been reported for myeloid expansion in *Tet2*-deficient mice, with some *Tet2*^{-/-} mouse strains showing no striking phenotypes compared to control littermate mice and others developing premalignant diseases or frank leukemias^{51, 52, 53, 54, 55, 56}. This unusually large variation in the myeloid and Treg cell phenotypes of *Tet2*-deficient mice may be related to the animal facility in which the mice are housed: myeloid expansion in *Tet2*-deficient mice has been attributed, at least in part, to the influence of cytokines such as IL-6 produced in response to gut microbiota, since it is eliminated or greatly attenuated if the mice are housed in a germ-free facility⁴¹. Even in 12-16 week-old *Tet2/3^{fl/fl}Foxp3^{Cre}* mice, the large increase in the cellularity of mesenteric lymph nodes compared to peripheral lymph nodes (Fig. 1b, Supplementary Fig. 1c) and the increased clonality of both *Foxp3*⁺ Tregs and *CD4*⁺*Foxp3*⁻ T cells (ex-Treg cells and/or activated *CD4*⁺ bystander T cells) (Supplementary Fig. 10) suggests a role for recognition of microbial or other antigens. We conclude that depending on the cell type, the effects of TET loss-of-function are most strikingly manifested when TET-deficient cells are subjected to stimulation, for instance through antigen recognition²⁵, cytokine stimulation¹¹, or the influence of the microbiota as discussed above⁴¹.

Tet2/3^{fl/fl}Foxp3^{Cre} mice showed later disease onset and lower levels of *CNS1/CNS2* methylation compared to *Tet2/3^{fl/fl}CD4^{Cre}* mice¹². This likely reflects the two distinguishing features of these two mouse strains. First, *CD4^{Cre}* expression precedes *Foxp3^{Cre}* expression during Treg development. Because 5hmC and 5fC are relatively stable epigenetic marks that are primarily lost by passive dilution during cell division^{57, 58, 59}, the later deletion of *Tet2* and *Tet3* in *Tet2/3^{fl/fl}Foxp3^{Cre}* compared to *Tet2/3^{fl/fl}CD4^{Cre}* mice will result in less extensive loss of TET proteins and oxidized methylcytosines, thus in turn leading to a less extensive increase of methylation at *CNS1* and *CNS2*. This translates into a slower loss of Treg function and concomitant slower increase of cytokine expression, and therefore a slower kinetics of disease development in *Tet2/3^{fl/fl}Foxp3^{Cre}* mice compared to *Tet2/3^{fl/fl}CD4^{Cre}* mice. Second, *Tet2/3^{fl/fl}CD4^{Cre}* mice show a dramatic expansion of iNKT cells that is not observed in *Tet2/3^{fl/fl}Foxp3^{Cre}* mice²⁵. This is known to lead to an aberrant increase in the production of IFN γ , IL-17 and other cytokines^{11, 25}, presumably further compromising immune homeostasis and T regulatory function and leading to more rapid disease onset in *Tet2/3^{fl/fl}CD4^{Cre}* mice. A similar process of gradually increased cytokine production is observed in adult *Tet2/3^{fl/fl}Foxp3^{Cre}* mice (Fig. 4e), but in this case the cytokines are produced by ex-Treg cells as well as activated T cells (see below).

Our fate-mapping experiments showed unambiguously that *Foxp3* expression in Treg cells from *Tet2/3^{fl/fl}Foxp3^{Cre}* DKO mice was considerably less stable than *Foxp3* expression in Treg cells from control mice (Fig. 6). Not only were Treg cells from *Tet2/3^{fl/fl}Foxp3^{Cre}* DKO mice more prone to lose *Foxp3* expression, they also showed dysregulated expression of many Treg signature genes including *Cd25*, *Nrp1* and *Il1r1*. Two points are particularly noteworthy. First, in *Tet2/3^{fl/fl}Foxp3^{WT/Cre}* heterozygous females or mixed bone marrow chimeras, the presence of wildtype Treg cells was not sufficient to suppress the aberrant inflammation observed in *Tet2/3^{fl/fl}Foxp3^{Cre}* DKO mice. Second, transfer of total *CD4*⁺ T cells from *Tet2/3^{fl/fl}Foxp3^{Cre}* DKO mice into immunocompetent recipient mice unexpectedly resulted in efficient transfer of the inflammatory disease. In both the mixed bone marrow chimeras and the *CD4*⁺ T cell transfer experiments, we noticed a large variation in *Foxp3* expression at the time of analysis, with some mice showing 60-70% of *Foxp3*-expressing cells but others showing fewer than 10% of *Foxp3*-expressing cells. The data indicate that *Foxp3*⁺ cells from *Tet2/3^{fl/fl}Foxp3^{Cre}* DKO mice expand somewhat more extensively than control cells in an early phase (see below) in which they maintain almost normal suppressive function; later, however, they lose *Foxp3* expression and suppressive function (Fig. 5) but acquire effector function (Fig. 4), leading to the accumulation of effector ex-Treg cells as well as activated bystander *CD4*⁺ and *CD8*⁺ T cells as the mice age. Overall, therefore, loss of TET function in Treg cells of *Tet2/3^{fl/fl}Foxp3^{Cre}* DKO mice results in a paradoxical dominant

disease, in which the defective TET-deficient Treg cells lose Foxp3 expression and suppressor function on the one hand, and convert to ex-Treg cells with dominant effector function on the other hand. Notably, these defective Treg cells and effector-like ex-Treg cells are able to confer an activated phenotype even to bystander CD4⁺ T cells (Fig. 7).

A notable feature of Treg cells from *Tet2/3^{fl/fl}Foxp3^{Cre}* DKO mice was aberrantly high proliferation and expansion compared to wildtype Treg cells (Fig. 2b, 4d). This phenomenon appears to be a consistent feature of TET-deficient cells: *Tet2/3* DKO Treg cells showed a significantly higher proliferation rate compared to WT Treg cells (Fig. 4d); both Treg cells and CD4⁺Foxp3⁻ cells from *Tet2/3^{fl/fl}Foxp3^{Cre}* mice showed greater clonal expansion compared to WT mice (Supplementary Fig. 10); and both iNKT cells from *Tet2/3^{fl/fl}CD4^{Cre}* mice²⁵ and myeloid cells from polyI:polyC-treated *Tet2/3^{fl/fl}Mx1^{Cre}* and tamoxifen-treated *Tet2/3^{fl/fl}ERT2-Cre* mice²² displayed a pronounced tendency to expansion and proliferation compared to their WT counterparts. Even Treg cells from younger (14 week) *Tet2/3^{fl/fl}Foxp3^{Cre}* DKO mice, isolated from apparently healthy mice before the development of severe inflammatory disease, showed altered expression of genes involved in cell cycle, DNA damage repair, immune processes and cancer compared to WT Treg cells (Fig. 4a-c). While the increased expansion of cells with TET loss-of-function appears to be facilitated by stimulation as discussed above, the underlying mechanisms remain to be elucidated.

In addition to simply losing suppressive function, ex-Treg cells appear to be prone to acquire effector function⁴⁹. Again, the underlying mechanisms are not yet completely clear, but it is known that mice with Treg perturbations frequently compensate for insufficiency of T regulatory function by becoming hyperproliferative and hyperactivated^{8, 35}. For instance, ex-Treg cells arising as a result of deficiency in the mTOR-signaling regulator TSC1 produce increased levels of IL-17 and acquire Th17 effector features under inflammatory conditions⁶⁰; and neuropilin1-deficient Treg cells produce high levels of IFN γ , which feeds back to suppress Treg cell function¹¹. In our case, we have shown that *Tet2/3* deficiency leads to increased expression of genes related to Tfh and/or Th17 cell differentiation, both in CD4⁺Foxp3⁻ T cells which include both ex-Treg cells and CD4⁺ bystander cells, and in CD4⁺Foxp3⁺ Treg cells, in a manner that correlated directly with disease severity.

Taken together, our data emphasize the critical role of TET proteins in the maintenance of stable Foxp3 expression and the integrity of Treg gene expression and function. The absence of TET proteins in Treg cells sets off a cascade of events that begins with increased DNA methylation of *CNS1* and *CNS2* and unstable Foxp3 expression, both facilitated by cell division occurring as a result of antigen and cytokine stimulation; and continues into compromised suppressor function and acquisition of effector function by ex-Treg cells as well as bystander T cells. The aberrant acquisition of these dysregulated phenotypes eventually results in a fatal inflammatory/ lymphoproliferative disease.

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Author contributions: A.R., X.Y. and C.W.L. conceived the project. A.R. supervised project planning and execution. X.Y. and X.L. performed experiments. D.S.C performed bioinformatic analyses of RNA-seq data. C.W.L. provided input for experimental design and critically evaluated the manuscript. A.R. and X.Y. wrote the manuscript.

Materials and Methods

Mice

Foxp3^{YFP-Cre} (*Foxp3*^{Cre}) mice, congenic mice (strain B6.SJL-Ptprc^aPepc^b/BoyJ) and scurfy mice were obtained from Jackson Laboratory. *Tet2*^{fl/fl}*Tet3*^{fl/fl}*Foxp3*^{Cre} mice were generated in our laboratory by crossing *Tet2*^{fl/fl}*Tet3*^{fl/fl} with *Foxp3*^{Cre} mice. For the fate-mapping experiments, *Tet2*^{fl/fl}*Tet3*^{fl/fl}*Foxp3*^{Cre} or *Tet2*^{fl/fl}*Tet3*^{fl/fl}*Foxp3*^{Cre} female mice were crossed with *Tet2*^{fl/fl}*Tet3*^{fl/fl}*Rosa26-YFP*⁺ male mice to generate *Tet2*^{fl/fl}*Tet3*^{fl/fl}*Foxp3*^{Cre}*Rosa26-YFP*⁺ male mice for further analysis. Age of the mice used for each experiment was stated in the figure legends. All breeding and experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the La Jolla Institute for Allergy and Immunology.

Cell Preparation and Flow cytometry

Single-cell suspensions were prepared from spleen, peripheral lymph nodes and mesenteric lymph nodes for staining or cell sorting. All the antibodies used for flow cytometry analyses and FACS sorting were purchased from eBioscience and Biolegend except for the CD95 (Fas) antibody which was purchased from BD Biosciences. For analysis of T cell compartments and Treg cell features, single-cell suspensions were stained with anti-mouse antibodies CD4 (GK1.5), CD8 (53-6.7), CD62L (MEL-14), CD25 (PC61), CD44 (IM7), CD45.1 (A20), CD45.2 (104), Nrp1 (3E12), ICOS (C398.4A), CD103 (2E7), GITR (YGITR765), PD1 (29F.1A12), CD127 (A7R34), CD69 (H1.2F3), CCR7 (4B12). Anti-mouse CCR7 staining was performed at 37°C for 30min. For analysis of Tfh and germinal center B cells, single-cell suspensions were stained with anti-mouse antibodies TCRβ (H57-597), CD19 (6D5), CXCR5 (L138D7), PD1 (29F.1A12), GL7 (GL7) and CD95 (Fas, Jo2). For intracellular staining, cells were surface-stained and then stained with anti-Foxp3 (FJK-16S, eBioscience), anti-active caspase-3 (C92-605, BD Pharmingen), anti-Helios (22F6), anti-CTLA4 (UC104.89) and anti-H2A.X phosphorylated (Ser139, 2F3) antibodies (Biolegend) using the Foxp3 Fixation/Permeabilization kit (eBioscience) and analysed by flow cytometry.

BrdU incorporation assay

Mice were injected with 1mg of BrdU (100μl of 100mg/ml stock solution) intraperitoneally. 24 h later, mice were sacrificed and the cells from spleen and peripheral lymph nodes were stained for surface markers and then for BrdU according to the manufacturer's protocol (BD Pharmingen, APC BrdU flow kit 552598).

Anti-dsDNA antibody ELISA

Anti-dsDNA autoantibodies in the serum from *Foxp3*^{Cre} WT and *Tet2*^{fl/fl}*Tet3*^{fl/fl}*Foxp3*^{Cre} mice (11-16 weeks old) were measured with kit from Alpha Diagnostic International according to the manufacturer's protocol (ELISA Kit 5110).

LEGENDplex Mouse Immunoglobulin Isotyping Panel

The serum immunoglobulin levels were measured with LEGENDplex Mouse Immunoglobulin Isotyping Panel according to the manufacturer's protocol (Biolegend, 740493).

Analysis of Peripheral bloods from *Tet2/3^{fl/fl}Foxp3^{Cre}* DKO mice

Peripheral bloods from WT and *Tet2/3^{fl/fl}Foxp3^{Cre}* DKO mice (12-14 weeks old) were collected into tubes pretreated with EDTA (BD Biosciences, 365974). Hematological parameters including the concentrations of white blood cells (WBCs), red blood cells (RBCs) and platelets were analyzed by Hemavet 950FS (Drew Scientific).

Generation of mixed bone marrow chimeras

Bone marrow cells were obtained from tibia and fibula from *Foxp3^{Cre}* WT mice, *Tet2/3^{fl/fl}Foxp3^{Cre}* mice and CD45.1 congenic mice. Erythrocytes and mature T cells were then depleted from the bone marrow cells using MACS LS columns. The purified cells (2.5×10^6) from *Foxp3^{Cre}* WT and *Tet2/3^{fl/fl}Foxp3^{Cre}* mice were then mixed together with cells (2.5×10^6) isolated from tibia and fibula from CD45.1 congenic mice at 1:1 ratio and transferred intravenously (i.v.) into lethally-irradiated CD45.1 congenic mice. Reconstituted mice were then sacrificed for analysis 14 to 20 weeks after adoptive transfer of bone marrow cells.

Scurfy CD4⁺ T cell adoptive transfer

Scurfy CD4⁺ T cells were isolated from spleen and peripheral lymph nodes from male *Scurfy* mice and purified using Dynabeads (Life Tech, purity > 98%). 5×10^5 CD45.1⁺CD4⁺ *scurfy* T cells were injected into *Rag1*-deficient mice alone or mixed with 1×10^5 Treg cells isolated from *Foxp3^{Cre}* WT or *Tet2/3^{fl/fl}Foxp3^{Cre}* mice. 4 to 5 weeks after adoptive transfer, the percentage of CD45.1⁺CD4⁺ cells and CD45.2⁺CD4⁺Foxp3⁺ cells were analyzed by flow cytometry. Alternatively, we used bone marrow cells from scurfy mice, *Foxp3^{Cre}* WT or *Tet2/3^{fl/fl}Foxp3^{Cre}* mice for the adoptive transfer.

Tet2/3 DKO CD4⁺ T cell adoptive transfer

Total CD4⁺ T cells were isolated from spleen and peripheral lymph nodes from CD45.2⁺ *Tet2/3^{fl/fl}Foxp3^{Cre}* mice and double purified using Dynabeads (Life Tech, purity > 98.5%). 5×10^6 CD4⁺ T cells were adoptively transferred into immunocompetent congenic mice and analyzed 7 to 9 weeks after adoptive transfer. As a control, total CD4⁺ T cells isolated from CD45.2⁺ *Foxp3^{Cre}* WT mice were also transferred into immunocompetent congenic mice, which did not show any cell expansion.

Bisulfite (BS) sequencing

Bisulfite sequencing was performed as described previously¹². Briefly, DNA samples were treated with sodium bisulfite (MethylCode Bisulfite Conversion Kit, Invitrogen). The PCR amplicons were generated using PyroMark PCR kit (Qiagen), and quantified using Quant-iT PicoGreen dsDNA reagent (Invitrogen). PCR amplicons were then used for library preparation using NEBNext DNA Library Modules for Illumina platform (NEB). The final libraries were quantified using KAPA library quantification kit for Illumina (KAPA Biosystems), and sequenced on Miseq (300bp, paired end, Illumina). The data are based on thousands of sequence reads per amplicon. Bisulfite sequencing results were analyzed as described previously¹².

RNA-seq library preparation

Total RNA were isolated from CD4⁺YFP⁺ (Foxp3⁺) Treg cells and CD4⁺YFP⁻ (Foxp3⁻) T cells (from pooled spleen and pLNs in Figure 4 or from mLN in Supplementary Figure 6) from *Foxp3*^{Cre} WT mice and *Tet2/3*^{fl/fl}*Foxp3*^{Cre} DKO mice (14-weeks old) using RNeasy plus mini kit (Qiagen). RNA-sequencing libraries were prepared using Truseq stranded mRNA kit (Illumina) according to the manufacture's protocol and sequenced at the La Jolla Institute sequencing core using Illumina HiSeq2500 single end 50bp platform.

RNA-seq and TCR-seq analysis

RNA-seq data were mapped against the UCSC mouse genome mm9 using TopHat2⁶¹ (v2.1.1) with the following parameters “-p 16 -N 2 --max-multihits 1 --read-gap-length 1 --transcriptome-index” and the RefSeq gene annotation was obtained from the UCSC genome Bioinformatics database. The number of reads mapping to each gene was counted using featureCounts⁶² (subread-1.4.3-p1) with the following parameters “-g gene_name -s 2”. Differentially expressed genes (DEGs) between WT and Tet2/3 DKO cell types were determined using the Bioconductor package DESeq2⁶³ with adjusted P value < 0.05 and a fold change threshold of > 1.5 or <0.67. And genes with total counts < 1 in the sum of all conditions were removed from the analysis. Canonical pathway analysis was performed using Ingenuity Pathway Analysis software (license for La Jolla Institute).

TCR sequences were retrieved from RNA-Seq data sets, and the frequency of TCRβ chain clonotypes (CDR3 regions) was determined using MiXCR⁶⁴ (mixcr-1.7-2.1) package for RNA-seq analysis with the default parameters “align -I TCR -s mmu -p rnaseq -OallowPartialAlignments=true”. Two rounds of contig assembly were performed by employing the “assemblePartial” function; extension of incomplete TCR CDR3s with uniquely determined V and J genes using germline sequences was done using the “extendAlignments” function; assembly and export of the clonotypes was performed using the “assemble” and the “exportClones” (-preset min -fraction -targets -vHits -dHits -jHits -vAlignments -dAlignments -jAlignments) functions, respectively.

Quantitative real-time PCR

Total RNA was isolated using RNeasy plus mini kit (Qiagen); cDNA was synthesized using SuperScript III reverse transcriptase (Thermo Fisher). Quantitative real-time PCR was performed using FastStart Universal SYBR Green Master mix (Roche) on a StepOnePlus real-time PCR machine (Thermo Fisher). Gene expression was normalized to *Hprt*. Primers used to detect the expression levels of *Tet2*, *Tet3* and *Foxp3* are as following:

Tet2 forward primer: AACCTGGCTACTGTCATTGCTCCA

Tet2 reverse primer: ATGTTCTGCTGGTCTCTGTGGGAA

Tet3 forward primer: GTCTCCCCAGTCCTACCTCCG

Tet3 reverse primer: GTCAGTGCCCCACGCTTCA

Foxp3 forward primer: GGCCCTTCTCCAGGACAGA

Foxp3 reverse primer: GCTGATCATGGCTGGGTTGT

Hprt forward primer: CTGGTGAAAAGGACCTCTCG

Hprt reverse primer: TGAAGTACTCATTATAGTCAAGGGCA

Histology

Lungs, livers and spleens were isolated from 12-14 week-old *Tet2/3*^{fl/fl}*Foxp3*^{Cre} DKO mice and littermate controls. Samples were fixed using 10% formalin overnight, preserved in 70% ethanol and embedded in paraffin. The paraffin blocks were then cut and sections were stained with haematoxylin and eosin (H&E).

Statistics

P values from unpaired two-tailed Student's t test were used for all the statistical comparisons between different groups and data were displayed as mean $\pm$ SD (Prism). P values are denoted in corresponding figures as: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Data Availability

The authors declare that all data supporting the findings of this study are available within the article and its Supplementary information files or from the corresponding author upon reasonable request. RNA-seq data have been deposited in the Gene Expression Omnibus database under accession number GSE113694. A reporting summary for this Article is available as a Supplementary Information file.

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Figure Legends

Figure 1. Phenotypic analysis of mice with Treg-specific deletion of *Tet2* and *Tet3*.

a. Survival curves for control WT and *Tet2/3^{fl/fl}Foxp3^{Cre}* DKO mice (n=28). **b.** Total cell numbers in spleen, peripheral lymph nodes (pLN) and mesenteric lymph nodes (mLN) of WT and *Tet2/3^{fl/fl}Foxp3^{Cre}* DKO mice (13-16 weeks old, n=9). **c.** Percentages (*left panel*) and cell numbers (*right panel*) of CD4⁺ and CD8⁺ T cells in the spleen and pLN from WT and *Tet2/3^{fl/fl}Foxp3^{Cre}* DKO mice (13-16 weeks old, n=9). **d.** Representative flow cytometry analysis of CD62L and CD44 expression in CD4⁺Foxp3⁺ T cells from spleen (*upper panels*) and pLN (*lower panels*). **e.** Representative flow cytometry analysis of CD62L and CD44 expression in CD8⁺ T cells from spleen (*upper panels*) and pLN (*lower panels*). Statistical analysis was performed using two-tailed unpaired student's t test (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001). Data are mean ± S.D. from at least three independent experiments.

Figure 2. Characterization of *Tet2/3*-deficient Treg cells.

a. Representative flow cytometry analyses of the percentage of Treg cells (% of Foxp3⁺ cells in gated CD4⁺ T cells) in spleen and pLN from WT and *Tet2/3^{fl/fl}Foxp3^{Cre}* DKO mice (13-16 weeks old). **b.** Quantification of the percentages (*left panel*) and cell numbers (*right panel*) of Treg cells in spleen, pLN and mLN from WT and *Tet2/3^{fl/fl}Foxp3^{Cre}* DKO mice (13-16 weeks old, n=9). **c-f.** Flow cytometry analysis of *Tet2/3*-deficient Treg cells from spleen and pLN for the expression of CD25 and Nrp1 (**c**); Helios, ICOS, CD103, GITR, CTLA4, PD1 and CD127 (**d**); CD62L versus CD44 (**e**); and CD69 (**f**). Shown are WT CD4⁺ T cells (shaded grey); WT Tregs (black line); DKO Tregs (red line). Statistical analysis was performed using two-tailed unpaired student's t test (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001). Data are mean ± S.D. from at least three independent experiments.

Figure 3. DNA methylation status of *Foxp3* CNS1 and CNS2 in WT and *Tet2/3^{fl/fl}Foxp3^{Cre}* DKO Treg cells.

a-b. DNA methylation status of *Foxp3* CNS1 and CNS2. Graphs depict the percentage of 5mC+5hmC determined by BS-seq in peripheral Tregs in: (**a**) 4 CpGs in *Foxp3* CNS1, and (**b**) 11 CpGs in *Foxp3* CNS2, (**c**) 4 CpGs in *Il2ra* intron 1a, (**d**) 7 CpGs in *Tnfrsf18* exon 5, (**e**) 6 CpGs in *Ikzf4* intron 1b and (**f**) 5 CpGs in *Ctla4* exon 2. Error bars show mean ± S.D. of thousands of sequencing reads from two independent experiments. Wild type (WT) Treg cells (8-10 weeks old) are shown in black, CD4^{Cre} mediated *Tet2/3* DKO Treg cells (3-4

weeks old) are shown in red ¹² and *Foxp3*^{Cre} mediated *Tet2/3* DKO Treg cells (8-10 weeks old) are shown in blue.

Figure 4. RNA-seq analysis for Treg cells isolated from WT or *Tet2/3*^{fl/fl}*Foxp3*^{Cre} DKO mice.

a. Mean average (MA) plot of genes differentially expressed in *Tet2/3* DKO Tregs (14-weeks old, isolated from pooled spleen and pLNs) relative to their expression in WT Treg cells. **b.** IPA analysis of canonical pathways for differentially expressed genes in *Tet2/3* DKO Treg cells. Green, categories related to DNA repair, DNA damage and cell cycle; blue, categories related to immune cell function; orange, category related to cancer. **c.** Heatmap for the expression of selected genes encoding cell cycle regulators with differential expression. The color gradient indicates Log2 Fold Change (DKO/WT). **d.** *Left panel*, Representative flow cytometry analysis of BrdU incorporation in CD4⁺Foxp3⁺ Treg cells in spleen and pLN from WT and *Tet2/3*^{fl/fl}*Foxp3*^{Cre} DKO mice (10 weeks old). The gates show the percentage of cells with BrdU incorporation. *Right panel*, Quantification of the frequency of CD4⁺Foxp3⁺ Treg cells that incorporated BrdU. **e.** Heatmaps showing expression (row z score of log2 TPM values) of Tfh-related genes in WT and DKO Tregs (*left panel*) and of Tfh and Th17 related genes for WT and DKO CD4⁺Foxp3⁺ cells (*right panel*). **f.** Flow cytometry analysis of CXCR5⁺PD-1⁺ Tfh cells (gated on CD4⁺TCRβ⁺ T cells) in the spleen and mLN of WT and *Tet2/3*^{fl/fl}*Foxp3*^{Cre} DKO mice (13-16 weeks old). **g.** Flow cytometry analysis of GL7⁺CD95⁺ germinal center B cells (gated on CD19⁺ B cells) in the spleen and mLN of WT and *Tet2/3*^{fl/fl}*Foxp3*^{Cre} DKO mice (13-16 weeks old). Statistical analysis was performed using two-tailed unpaired student's t test (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001). Data are mean ± S.D. from two to three independent experiments.

Figure 5. The presence of WT Treg cells fails to rescue the inflammatory phenotype of *Tet2/3*^{fl/fl}*Foxp3*^{Cre} DKO mice.

a. Survival curves for control WT (n=9) and *Tet2/3*^{fl/fl}*Foxp3*^{WT/Cre} heterozygous female (n=9) mice. **b.** Total cell numbers in spleen from WT and *Tet2/3*^{fl/fl}*Foxp3*^{WT/Cre} heterozygous female mice (15-30 weeks old, n=5). **c.** Schematic illustration for the generation of mixed bone marrow chimeras. The flow cytometry plots show the ratios of WT CD45.1⁺ congenic bone marrow cells and co-transferred CD45.2⁺ WT or *Tet2/3* DKO bone marrow cells immediately prior to transfer. **d.** Total cell numbers in spleen from WT and *Tet2/3* DKO mixed bone marrow chimeras with splenomegaly (n=7) 18-20 weeks after transfer. **e.** Representative flow cytometry analysis for WT and DKO mixed bone marrow chimeras 18-20 weeks after transfer. **f.** The graphs from left to right show the quantifications for the percentage of CD45.2⁺ cells, the percentage of CD4⁺ and CD8⁺ cells within the CD45.2⁺ cells and the percentage of Foxp3⁺ cells within the CD45.2⁺CD4⁺ cells (n=7). Statistical analysis was performed using two-tailed unpaired student's t test (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

Figure 6. Fate mapping experiments show that *Tet2/3* DKO Treg cells are less stable than Treg cells from heterozygous littermates

a. Representative lineage tracing of Treg cells in spleen and mLN from littermate mice of *Tet2*^{+/fl}*Tet3*^{fl/fl}*Foxp3*^{YFP-Cre}*Rosa26*^{YFP} (Het) and *Tet2/3*^{fl/fl}*Foxp3*^{YFP-Cre}*Rosa26*^{YFP} genotypes. *Left panels*, Contour plots showing that *Rosa26*^{YFP} is much brighter than, and so can be distinguished from, *Foxp3*^{YFP-Cre}. *Right panels*, The percentage of Foxp3-negative cells is much higher in sorted CD4⁺*Rosa26*^{YFP}^{High} cells from *Tet2/3*^{fl/fl}*Foxp3*^{YFP-Cre}*Rosa26*^{YFP} (*Tet2/3* DKO) mice compared to those from *Tet2*^{+/fl}*Tet3*^{fl/fl}*Foxp3*^{YFP-Cre}*Rosa26*^{YFP} mice, indicating that *Tet2/3* DKO Treg cells that had originally turned on Foxp3 and Cre from the *Foxp3*^{YFP-Cre} locus later lose Foxp3 and become ex-Treg cells. **b.** Quantification of the percentage of Foxp3^{neg}-*Rosa26*^{YFP}^{High} cells as shown in **a** (11-12 weeks old, n=3 from three independent experiments). Statistical analysis was performed using two-tailed unpaired student's t test (*P<0.05).

Figure 7. Transfer of total CD4⁺ T cells from *Tet2/3^{fl/fl}Foxp3^{Cre}* mice elicits disease in immunocompetent recipient mice.

a. Schematic illustration for the transfer of total CD4⁺ T cells (5×10^6 cells) from WT or *Tet2/3^{fl/fl}Foxp3^{Cre}* DKO mice (14 weeks old) into immunocompetent congenic recipient mice (n=11 recipient mice), the mice were analyzed 7-10 weeks after transfer. **b.** From left to right, the graphs show the numbers of total splenocytes and of transferred and expanded CD45.2⁺ cells, the fold increase in the number of transferred and expanded DKO CD45.2⁺ cells, the percentage of Foxp3⁺ cells in CD45.2⁺ cells after transfer, and the percentage of Foxp3⁺ cells in CD45.2⁺ and CD45.1⁺ cells in each individual with connection lines. **c.** Representative flow cytometry analyses of the percentage of CD45.2⁺ T cells, the percentage of Foxp3⁺ cells in CD45.2⁺ cells (example 1 showing an expansion of Foxp3⁺ cells, example 2 showing a loss of Foxp3⁺ cells), the percentage of Foxp3⁺ cells in CD45.1⁺ host cells, CD62L and CD44 expression in host Treg cells and CD4⁺Foxp3⁺ cells. Statistical analysis was performed using two-tailed unpaired student's t test (**P<0.001).

Figure 1

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