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LETTER TO THE EDITOR

m^{6}A mRNA methylation sustains Treg suppressive functions

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Dear Editor,

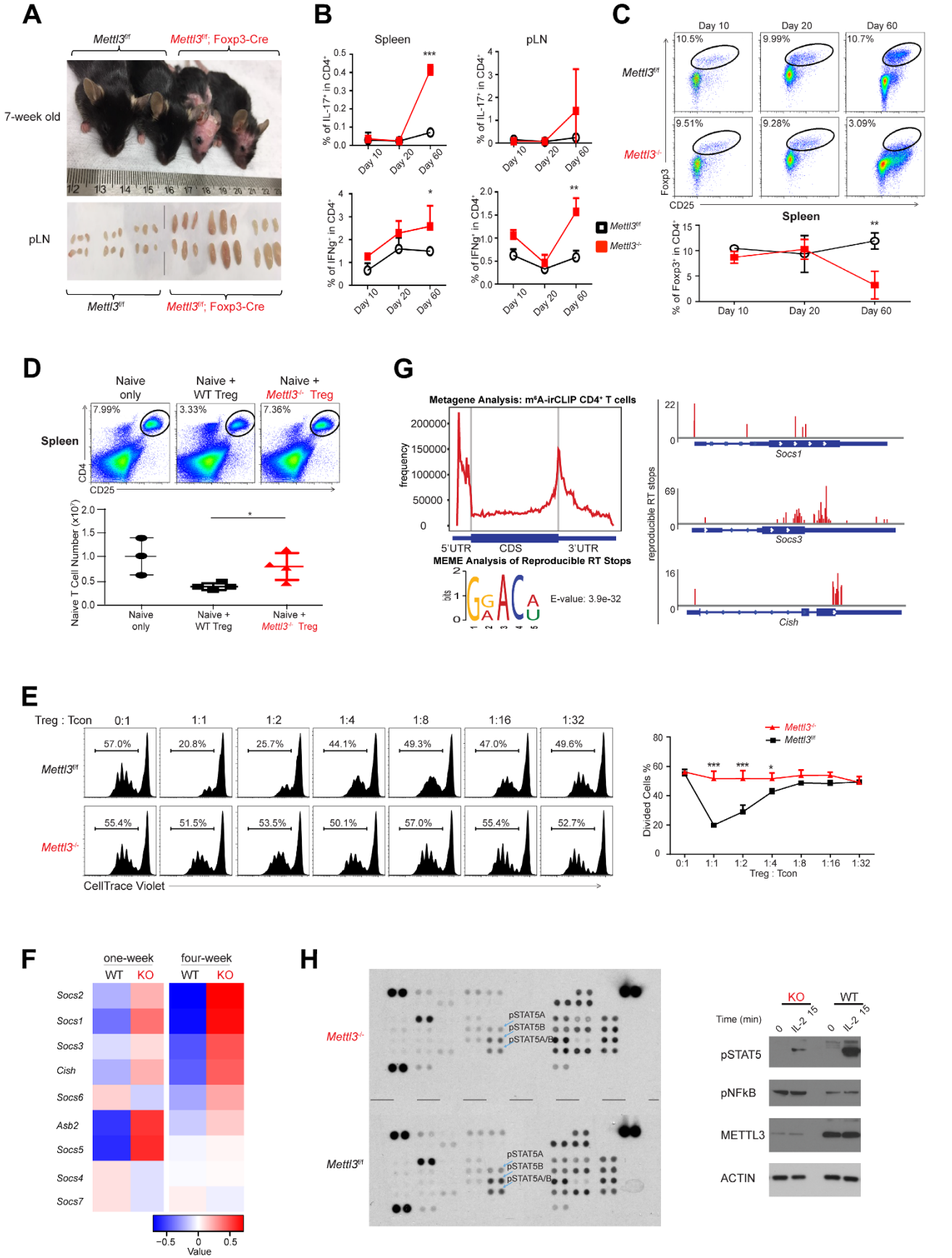
N6-methyladenosine (m^{6}A) is the most abundant mRNA chemical modification, and is modulated by m^{6}A 'writers', 'erasers' and 'readers' proteins [1-3]. *In vitro* experiments suggest that m^{6}A regulates several aspects of RNA metabolism, including RNA decay, splicing and translation [1]. Recent genetic analyses *in vivo* showed that m^{6}A functions in sex determination in *Drosophila* [4, 5], in maternal-to-zygotic transition and haematopoietic stem cell specification during zebrafish embryogenesis [6, 7], in mouse spermatogenesis [8-10], and in mouse brain development [11]. We recently discovered that lineage-specific deletion of the m^{6}A 'writer' enzyme METTL3 in CD4$^+$ T cells (*Mettl3*$^{f/f}$; CD4-Cre) led to disruption of naïve T cell homeostasis [12]. CD4$^+$ regulatory T cells (Tregs) comprise a critical subset of effector T cells, which are involved in resolution of inflammation and immunosuppression in tumor microenvironments [13]. However, the potential roles of m^{6}A mRNA modification in Treg functions *in vivo* are unknown.

Mettl3$^{f/f}$; CD4-Cre mice were normal without obvious defects during the first three months after birth. However, at the age of three months and older, the *Mettl3*$^{f/f}$; CD4-Cre mice developed chronic inflammation in the intestine, evidenced by increased lymphocyte infiltration of the colon in Haematoxylin and eosin (H&E) staining analysis (Supplementary information, Figure S1). We hypothesized that the regulatory T cells might have lost their repressive functions in *Mettl3*$^{f/f}$; CD4-Cre knockout mice.

Therefore, we crossed *Mettl3*$^{f/f}$ mice with Foxp3Cre-YFP mice to specifically delete *Mettl3* and consequently the m^{6}A RNA modification in regulatory T cells. Because the Foxp3Cre-YFP transgene is on the X chromosome, we analyzed only male mice for all the following experiments in comparison to WT littermate controls. The *Mettl3*$^{f/f}$; Foxp3Cre mice developed severe autoimmune diseases (for both male and female mice) and thus were infertile. Strikingly, the *Mettl3*$^{f/f}$; Foxp3Cre mice had substantially larger peripheral lymph nodes and spleen, developed alopecia and severe systemic autoimmune disease after weaning, and started to die in 8~9 weeks, indicating a systematic loss of suppressive function of Tregs without m^{6}A RNA modification (Figure 1A).

To explore the cellular mechanisms of the severe autoimmunity of *Mettl3*$^{f/f}$; Foxp3Cre mice, we analyzed T cell effector cytokines in spleen and peripheral lymph nodes. The inflammatory Th1 and Th17 responses in *Mettl3*$^{f/f}$; Foxp3Cre were at basal levels 10 days after birth, and were still under control before weaning (day 20), but became significantly elevated compared to WT at day 60, which was consistent with the observed autoimmune phenotypes (Figure 1B). Uncontrolled inflammation in *Mettl3*$^{f/f}$; Foxp3Cre mice was not due to Treg differentiation defects, as the percentage and the number of Tregs in spleen and thymus at day 10 and day 20 were similar to WT littermate controls, but *Mettl3*$^{-/-}$ Tregs were exhausted at day 60 in the spleen due to excessive inflammation (Figure 1C and Supplementary information, Figures S2-S4). Because we previously showed that *in vitro* differentiation of *Mettl3*$^{f/f}$; CD4-Cre naïve T cells to Tregs was similar to littermate WT naïve T cells [12], these results suggest a potential role for m^{6}A in Treg function.

We surmise that *Mettl3*$^{-/-}$ Tregs lost their suppressive function over the effector T cells. To test this hypothesis, we first set up an *in vivo* co-suppression assay by co-transferring naïve T cells without Treg cells, or with WT Tregs, or with *Mettl3*$^{-/-}$ Tregs in different ratios into TCRβ$^{-/-}$ mice. The results showed that *Mettl3*$^{-/-}$ Tregs completely lost the ability to suppress naïve T cell *in vivo* proliferation comparing to WT Tregs (Figure 1D and Supplementary information, Figure S5). Second, we also performed the standard *in vitro* suppression assay by mixing Tregs isolated from *Mettl3*$^{f/f}$; Foxp3Cre mice or WT littermate control mice with WT naïve T cells labeled by CellTrace at different ratios. We measured the cell proliferation by FACS at day 4 after co-culture, and the results showed that naïve T cells co-cultured with m^{6}A KO Tregs proliferated much faster (Figure 1E). These data confirmed that *Mettl3*$^{f/f}$; Foxp3Cre Tregs lost their suppressive function over T cell proliferation.


A
Mettl3f/f
Mettl3f/f; Foxp3-Cre
7-week old
pLN
B
Spleen
pLN
% of IL-17+ in CD4+
% of IFNg+ in CD4+
Day 10
Day 20
Day 60
Mettl3f/f
Mettl3-/-
C
Day 10
Day 20
Day 60
10.5%
9.99%
10.7%
9.51%
9.28%
3.09%
Foxp3
CD25
Spleen
% of Foxp3+ in CD4+
D
Naive only
Naive + WT Treg
Naive + Mettl3-/- Treg
7.99%
3.33%
7.36%
Spleen
CD4
CD25
Naive T Cell Number (x10^7)
G
Metagene Analysis: m6A-irCLIP CD4+ T cells
frequency
5'UTR
CDS
3'UTR
MEME Analysis of Reproducible RT Stops
bits
E-value: 3.9e-32
reproducible RT stops
Socs1
Socs3
Cish
E
Treg : Tcon
0:1
1:1
1:2
1:4
1:8
1:16
1:32
57.0%
20.8%
25.7%
44.1%
49.3%
47.0%
49.6%
55.4%
51.5%
53.5%
50.1%
57.0%
55.4%
52.7%
CellTrace Violet
Divided Cells %
F
one-week
four-week
WT
KO
Socs2
Socs1
Socs3
Cish
Socs6
Asb2
Socs5
Socs4
Socs7
Value
H
pSTAT5A
pSTAT5B
pSTAT5A/B
Time (min)
IL-2
pSTAT5
pNFkB
METTL3
ACTIN

We next sought to understand the molecular mechanism of the loss of suppressive function of Tregs from *Mettl3* conditional knockout mice. We used FACS to sort Tregs from spleen by the Foxp3Cre-YFP marker from *Mettl3*$^{f/f}$; Foxp3Cre-YFP and littermate Foxp3Cre-YFP mice at one week and four weeks of age, and subjected the Tregs to RNA sequencing analysis. Consistent with our previous observation in naïve T cells from *Mettl3*$^{f/f}$; CD4-Cre mice [12], members of the inhibitory *Socs* gene family, including *Cish*, *Socs1*, *Socs2*, *Socs3*, *Asb2*, were among the top up-regulated genes in *Mettl3*$^{f/f}$; Foxp3Cre CD4$^+$ Tregs comparing to WT CD4$^+$ Tregs. Elevation of *Socs* gene family mRNA levels in *Mettl3*$^{-/-}$ Tregs was evident at the very early age of one week, and further increased by four weeks of age (Figure 1F). RT-qPCR analysis confirmed the increase of *Socs* family mRNA levels without changes in levels of transcripts of other genes in the IL-2-STAT5 pathway (Supplementary information, Figure S6). Furthermore, we applied m^{6}A irCLIP-Seq, a newly developed and sensitive method to map m^{6}A modification at near nucleotide resolution, in CD4$^+$ T cells. We confirmed that m^{6}A modification was enriched at 3′ UTR and 5′ UTR regions, with a consensus sequence of GG/AACA/U (Figure 1G). In addition, *Socs* gene mRNAs, including *Socs3* and *Cish*, were indeed modified by m^{6}A (Figure 1G). We have previously shown that these *Socs* gene mRNAs are m^{6}A targets in CD4$^+$ T cells, and deletion of *Mettl3* led to a global decrease of mRNA m^{6}A levels and loss of m^{6}A modification in specific *Socs* gene transcripts. Decreased m^{6}A modification led to enhanced *Socs* mRNA stability, increased levels of SOCS proteins, and blockage in cytokine signal transduction [12].

The IL2-STAT5 signaling axis is critical for Treg function [13]. The increased *Socs* function in Tregs in *Mettl3*$^{f/f}$; Foxp3Cre mice is likely to inhibit the IL2-STAT5 pathway, similar to the scenario that depletion of m^{6}A leads to increased *Socs* mRNAs and inhibition of IL7-STAT5 signaling in CD4$^+$ naïve T cells [12]. To test this hypothesis, we FACS-sorted YFP+ Tregs from *Mettl3*$^{f/f}$; Foxp3Cre-YFP mice and Foxp3Cre-YFP control mice, and stimulated these cells *in vitro* with IL-2. 15 minutes after stimulation, we applied the cell lysates to the phospho-kinase array to interrogate multiple signaling pathways. As shown in Figure 1H, phosphorylation of STAT5A and STAT5B decreased in *Mettl3* KO Tregs comparing to WT Tregs (Quantification shown in Supplementary information, Figure S7). Western blots confirmed that the phosphorylation levels of STAT5 in m^{6}A KO Tregs were severely diminished comparing to those in littermate control WT cells isolated from 6-week-old mice (Figure 1I). Taken together, we conclude that depletion of *Mettl3*/m^{6}A in Tregs leads to increased *Socs* mRNA levels, thus suppresses the IL-2-STAT5 signaling pathway which is essential to Treg functions and stability.

We reported in our previous work that m^{6}A controlled the rapid degradation of transcripts of early inducible genes in naïve T cells, and here again we observed similar elevated *Socs* mRNA levels in *Mettl3*$^{f/f}$; Foxp3Cre Tregs. But in the latter case of Tregs, elevated *Socs* function targeted the IL-2-STAT5 signaling pathway, which critically controls Treg cell functions. Our results show that the m^{6}A RNA modification specifically targets the same class of genes encoding components of essential signaling pathways in different T cell subtypes, thereby controlling the differentiation of naïve T cells [12] and also sustaining the suppressive functions of Tregs. Since Tregs in the tumor microenvironment suppress the tumor-killing functions of CD8 T cells, it is possible that selective depletion of m^{6}A in tumor-infiltrated Tregs may be beneficial in combination with other forms of cancer immunotherapy.

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Figure 1 Specific deletion of *Mettl3* in Tregs led to loss of Treg suppression function and severe autoimmune diseases. **(A)** 7-week-old *Mettl3*$^{f/f}$; Foxp3Cre mice developed severe autoimmune disease, lost hair, and had enlarged periphery lymph nodes (pLN). **(B)** Th1 and Th17 cytokine analysis by FACS of the spleen and periphery lymph nodes from *Mettl3*$^{f/f}$; Foxp3Cre and WT control mice. **(C)** FACS analysis of Treg population (Foxp3$^+$CD25$^+$) at different days after birth, and the statistics is shown below (n = 7). **(D)** *In vivo* suppression assay by co-transferring naïve T cells only, naïve T cells with WT or *Mettl3*$^{-/-}$ Tregs into TCRβ$^{-/-}$ mice (n = 4). **(E)** *In vitro* suppression assay by co-culturing the WT naïve T cells with either KO Tregs or WT Treg cells at different ratios. The mixing ratio is shown above the graph, and the percentage of divided cells and statistics are shown on the right graph (n = 3). **(F)** RNA-Seq analysis of WT and *Mettl3* KO Tregs isolated from spleen of one-week-old and four-week-old mice, and the *Socs* genes are among the most up-regulated genes. **(G)** m^{6}A irCLIP-Seq analysis of CD4$^+$ T cells shows m^{6}A enrichment at 3′ UTR and 5′ UTR regions and confirms *Socs* genes are m^{6}A targets (n = 2). **(H)** Phospho-kinase array with lysates of FACS-sorted WT or *Mettl3*$^{-/-}$ Tregs from 4-week-old mice; pSTAT5 signals decrease in the *Mettl3* KO Tregs and are highlighted. **(I)** Signaling assay of the IL-2-STAT5 pathway with FACS-sorted pure WT and *Mettl3* KO Tregs from 6-week-old mice (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)