Expanded View Figures

Figure EV1. Loss of Tet1 and Tet3 does not impair stable programs in naturally occurring Tregs.

A Representative flow plots and quantification of FoxP3 expression in cell proliferation dye-labeled Treg cells FACS sorted from *Tet1/2/3*$^{fl/fl}$ and *Rorc(t)*^{CreTg} *Tet1/2/3*$^{fl/fl}$ mice. Cells were sorted based on CD25 expression. Cells were activated *in vitro* for 72 h with anti-CD3/CD28 in the presence of 200 U/ml hIL-2 ($n = 3$ or 2 Figure Legends). Male mice were used in both experiments. Experiment was replicated two times.

B Representative flow plots and quantification of CD25 expression in cell proliferation dye-labeled Treg cells FACS sorted from *Tet1/2/3*$^{fl/fl}$; and *Rorc(t)*^{CreTg} *Tet1/2/3*$^{fl/fl}$ mice. Cells were activated as in (A). Experiment was replicated two times.

C ICOS MFI quantification on *in vitro* activated Tregs at 48 h ($n = 3$ biological replicates). Data are mean ± SEM, unpaired two-tailed *t*-test. Experiment was replicated three times.

D Representative flow plots depicting CFSE dilution upon proliferation of titrated CD4$^+$ T effectors in the presence of 30 K control or *Rorc(t)*^{CreTg} *Tet1/3*$^{fl/fl}$ Tregs, 3-day postactivation. Experiment was replicated three times.

E Weight curve of control *Tet1/3*$^{fl/fl}$ ($n = 14$ biological replicates) and *Rorc(t)*^{CreTg} *Tet1/3*$^{fl/fl}$ ($n = 9$ biological replicates) female littermates treated with 1.5% DSS in drinking water for 7 days. Data are a summary of two independent experiments and are mean ± SEM, two-way ANOVA with Bonferroni's multiple comparison test.

F Length of the small intestine at day 15 post-DSS treatment ($n = 5$ or 7 biological replicates). Data are mean ± SEM, unpaired Mann–Whitney *t*-test.

G Length of the colon as in (F).

H Weight curve of female Rag$^{-/-}$ mice after T cell transfer colitis induced via transfer of naïve WT CD45.1 conventional CD4$^+$ T cells alone ($n = 4$ recipient mice) or in combination with Tregs isolated from *Tet1/3*$^{fl/fl}$ ($n = 5$ recipient mice) and *Rorc(t)*^{CreTg} *Tet1/3*$^{fl/fl}$ littermates ($n = 4$ recipient mice). A ratio of 8:1 Teff: Treg cells was transferred. *$P < 0.05$, two-way ANOVA with Geisser–Greenhouse correction.

I Cytokine analysis by flow cytometry of CD45.1 CD4$^+$ effector cells isolated from the mLN of Rag$^{-/-}$ mice 7-weeks post-T cell transfer-induced colitis. Cells were stimulated with PMA/Ionomycin for 5 h in the presence of Brefeldin + Monensin A and pre-gated on Live CD45.1$^+$TCRβ$^+$CD4$^+$ cells for analysis. Data are mean ± SEM ($n = 7$ or 5 biological replicates), two-way ANOVA with Tukey's multiple comparison test. Experiment was replicated two times.

J Quantification of CTLA4 and CD25 MFI among Control CD45.2 *Tet1/3*$^{fl/fl}$ ($n = 5$ biological replicates) and CD45.2 *Rorc(t)*^{CreTg} *Tet1/3*$^{fl/fl}$ ($n = 4$ biological replicates) Tregs in the mLN 7-week post-T cell transfer-induced colitis. Data are mean ± SEM, two-way ANOVA with Sidak's multiple comparison test.

K Histopathological scoring of colon inflammation in Rag$^{-/-}$ mice 7-week post-T cell transfer-induced colitis ($n = 4$ or 5 biological replicates). Data are mean ± SEM, Kruskal–Wallis test with Dunn's correction.

L Representative H&E staining of the colon of Rag$^{-/-}$ mice 7-week post-T cell transfer-induced colitis. Colon inflammation was evidenced by cellular infiltrates (arrows) in the lamina propria. Scale bar = 55 μm.

M MFI of intracellular Granzyme B and IL-10 in FACS-sorted CD25$^+$ natural Tregs activated for 48 h in the presence of 50 U IL-2. Brefeldin + Monensin A were added 6 h prior to staining. Data are mean ± SEM ($n = 3$ biological replicates), two-way ANOVA with uncorrected Fisher's test. Experiment was replicated three times.

Data information: ns = not significant, $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
Source data are available online for this figure.

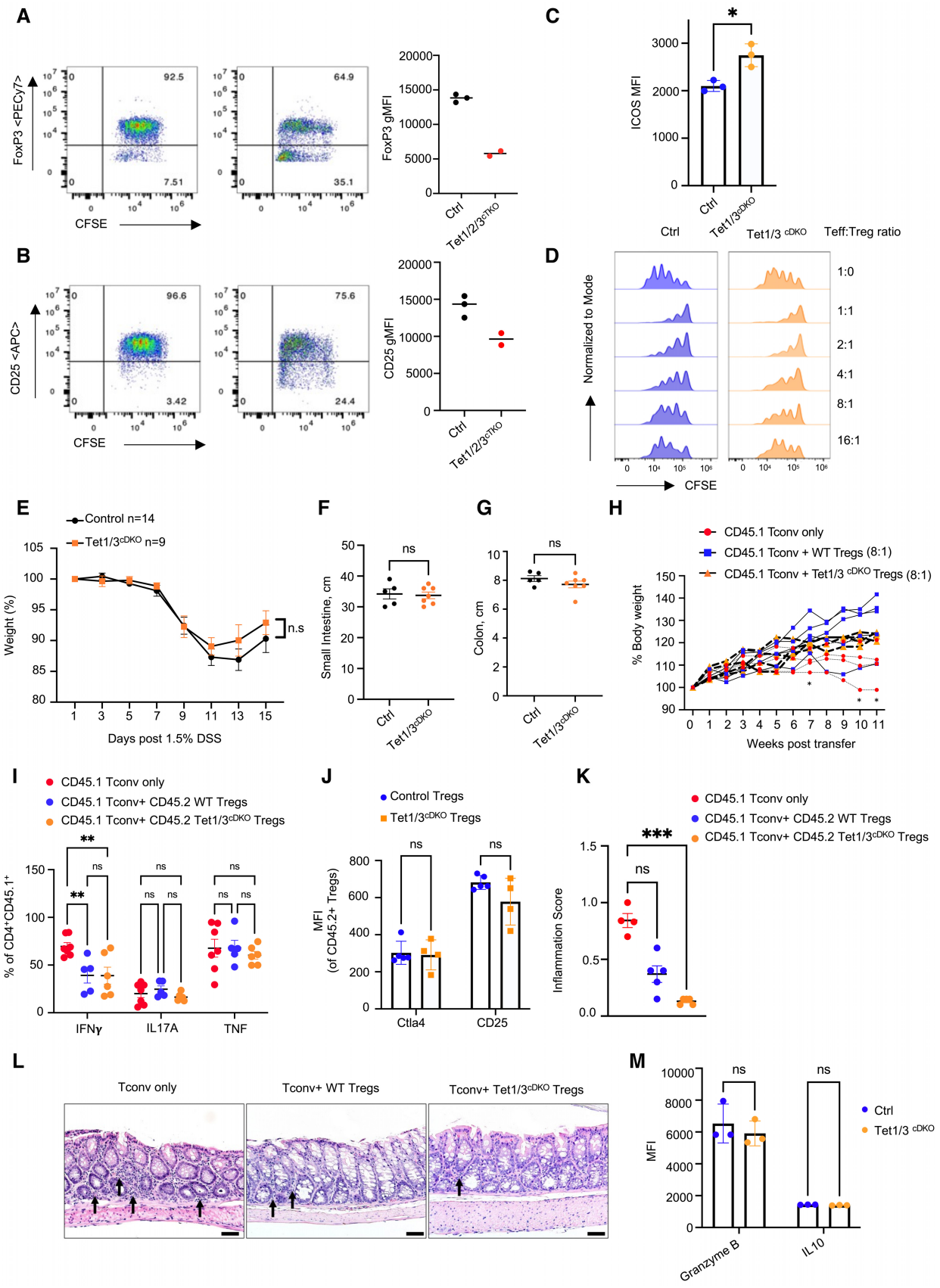
A
FoxP3 <PECy7>
CFSE
92.5
7.51
64.9
35.1
FoxP3 gMFI
Ctrl
Tet1/2/3cTKO
B
CD25 <APC>
96.6
3.42
75.6
24.4
CD25 gMFI
C
ICOS MFI
Ctrl
Tet1/3cDKO
D
Ctrl
Tet1/3 cDKO
Teff:Treg ratio
Normalized to Mode
1:0
1:1
2:1
4:1
8:1
16:1
E
Control n=14
Tet1/3cDKO n=9
Weight (%)
n.s
Days post 1.5% DSS
F
Small Intestine, cm
ns
G
Colon, cm
H
CD45.1 Tconv only
CD45.1 Tconv + WT Tregs (8:1)
CD45.1 Tconv + Tet1/3 cDKO Tregs (8:1)
% Body weight
Weeks post transfer
I
CD45.1 Tconv only
CD45.1 Tconv+ CD45.2 WT Tregs
CD45.1 Tconv+ CD45.2 Tet1/3cDKO Tregs
% of CD4+CD45.1+
IFNγ
IL17A
TNF
J
Control Tregs
Tet1/3cDKO Tregs
MFI (of CD45.2+ Tregs)
Ctla4
CD25
K
CD45.1 Tconv only
CD45.1 Tconv+ CD45.2 WT Tregs
CD45.1 Tconv+ CD45.2 Tet1/3cDKO Tregs
Inflammation Score
L
Tconv only
Tconv+ WT Tregs
Tconv+ Tet1/3cDKO Tregs
M
MFI
Ctrl
Tet1/3 cDKO
Granzyme B
IL10

Figure EV1.

Figure EV2. Tet1 and 3 are required prior to lineage commitment of Treg cells from CD4$^+$ T lymphocytes.

A Quantification of CD24 MFI expression on DP, recently selected CD4$^+$ TCRβ^{hi} CD24^{hi} CD69$^+$ and mature CD4$^+$TCRβ^{hi} CD24^{lo} CD69$^+$ thymocytes (n = 12 or 10 biological replicates). Data are shown as mean ± SEM, ANOVA test with Sidak's multiple comparison test and is a summary of two experimental replicates.

B Representative flow plots and quantification of % dead cells among TCRβ^{hi} thymocytes measured by FLICA/Ghost dye or Annexin V/PI staining (n = 3 biological replicates/genotype). Data are shown as mean ± SEM, two-way ANOVA test with Sidak's multiple comparison test. Experiment was replicated two times.

C Quantification of CD25$^-$FoxP3^{lo}, CD25$^+$FoxP3$^-$, and CD25$^+$FoxP3$^+$ cells among Live CD4$^+$ T cells thymocytes of control *Tet1/3$^{fl/fl}$* (n = 3 biological replicates) and *Rorc(t)^{CreTg} Tet1/3$^{fl/fl}$* (n = 6 biological replicates) 6-day-old neonates. Data are shown as mean ± SEM, 2-way ANOVA with Sidak's multiple comparison test. Experiment was replicated two times.

D Quantification of ICOS MFI expression on FoxP3$^+$ T cells from the mesenteric lymph nodes of control *Tet1$^{fl/fl}$* (n = 5 biological replicates) and *Rorc(t)^{CreTg} Tet1$^{fl/fl}$* mice (n = 5 biological replicates). Data are mean ± SEM, unpaired two-tailed t-test. Experiment was replicated two times.

E Quantification of ICOS MFI expression on FoxP3$^+$ T cells from the mesenteric lymph nodes of control *Tet3$^{fl/fl}$* (n = 5 biological replicates) and *Rorc(t)^{CreTg} Tet3$^{fl/fl}$* mice (n = 3 biological replicates). Data are mean ± SEM, unpaired two-tailed t-test. Experiment was replicated two times.

Data information: ns = not significant, $P > 0.05$, ***$P < 0.001$, ****$P < 0.0001$.
Source data are available online for this figure.

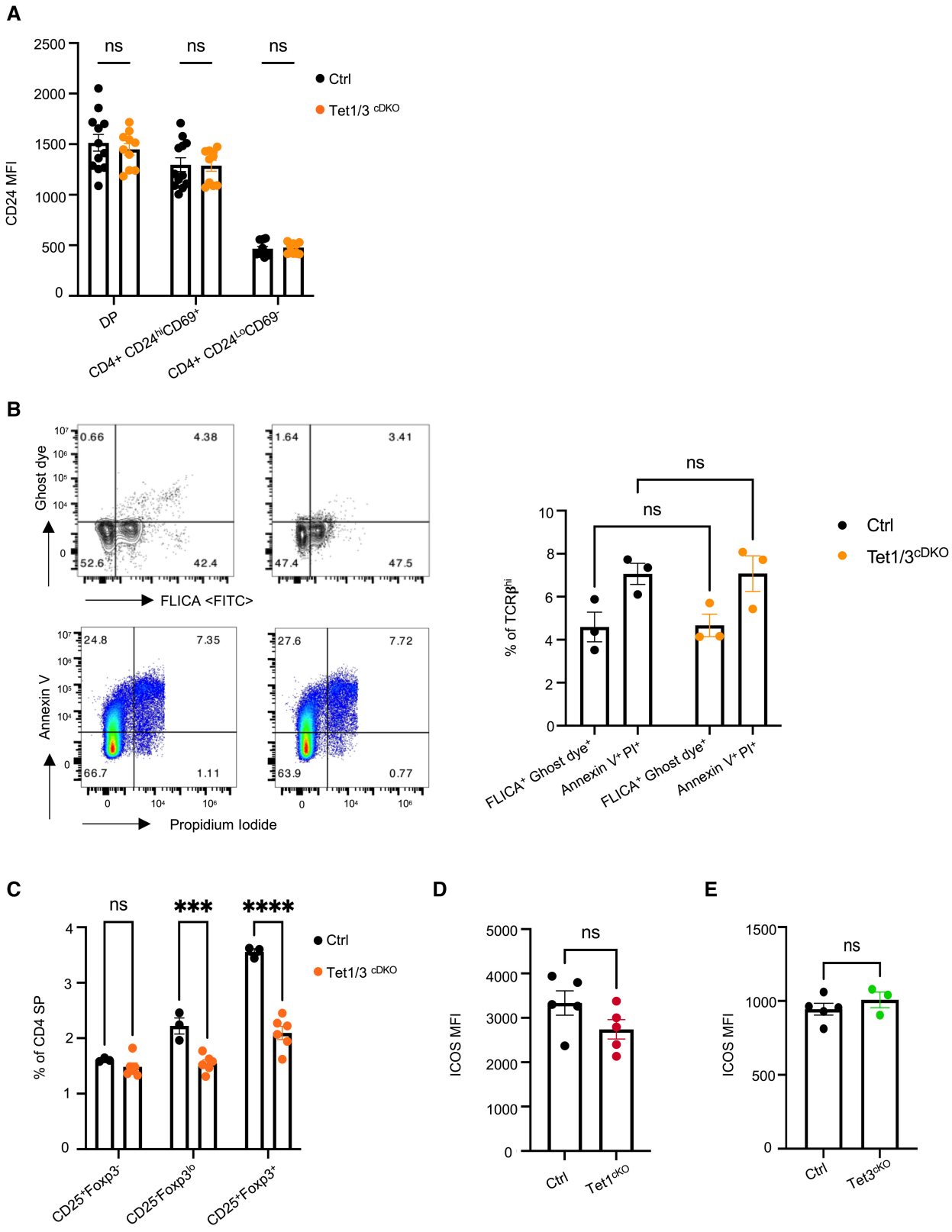
A
ns
ns
ns
Ctrl
Tet1/3 cDKO
CD24 MFI
2500
2000
1500
1000
500
0
DP
CD4+ CD24hiCD69+
CD4+ CD24LoCD69-
B
Ghost dye
FLICA <FITC>
0.66
4.38
52.6
42.4
1.64
3.41
47.4
47.5
Annexin V
Propidium Iodide
24.8
7.35
66.7
1.11
27.6
7.72
63.9
0.77
% of TCRβhi
ns
ns
FLICA+ Ghost dye+
Annexin V+ PI+
FLICA+ Ghost dye+
Annexin V+ PI+
Ctrl
Tet1/3cDKO
C
ns
***
****
% of CD4 SP
Ctrl
Tet1/3 cDKO
CD25+Foxp3-
CD25+Foxp3lo
CD25+Foxp3+
D
ns
ICOS MFI
Ctrl
Tet1cKO
E
ns
ICOS MFI
Ctrl
Tet3cKO

Figure EV2.

Figure EV3. Tet1 and 3 regulate Treg differentiation by modulating TCR signaling and IL-2 production.

A Quantification of % FoxP3$^+$ cells among CD4 T cells, 72-h postactivation with anti-CD3/CD28 and 100 U/ml rhIL-2 and indicated doses of TGF-β ($n = 3$ biological replicates). Data are shown as mean ± SEM, two-way ANOVA with Bonferroni multiple comparison tests. Experiment was replicated three times.

B Representative flow plots and quantification of FoxP3 MFI among CD4$^+$ T cells described in Fig 4A.

C FoxP3 MFI on CD4$^+$ T cells activated as described in Fig 4A. Experiment was replicated three times.

D Representative flow plot and quantification of YFP$^+$FoxP3$^+$ cells 72-h postactivation of sorted YFP$^-$ naïve CD4$^+$ T cells activated with anti-CD3/CD28 and 20 ng/ml TGF-β in the absence exogenous rhIL-2 ($n = 5$ biological replicates). Data are a summary of two independent experiments and shown as mean ± SEM, unpaired two-tailed *t*-test.

E Quantification and representative flow plots of % Live cells across each division in CFSE-labeled CD4$^+$ T cells 72-h postactivation with anti-CD3/CD28 in the absence of exogenous IL-2 and in the presence or absence of 20 ng/ml TGF-β ($n = 2$ or 3 biological replicates). Two-way ANOVA with Bonferroni multiple comparison test. Experiment was replicated at least three times.

F Representative flow plot and quantification of FoxP3$^+$ cells (bottom) 72-h postactivation with anti-CD3/CD28 (i) in the absence of TGF and IL-2 (ii) 20 ng/ml TGF-β and no exogenous IL-2 (iii) 20 ng/ml TGF-β and 10 μg/ml of anti-mIL-2 blocking antibody and (iv) 20 ng/ml TGF-β, 10 μg/ml of anti-mIL-2 blocking antibody and 100 U rhIL-2 ($n = 2$ or 3 biological replicates). Data are shown as means ± SEM, ANOVA test with Bonferroni multiple comparison tests. Experiment was replicated three times.

Data information: ns = not significant, $^{**}P < 0.01$, $^{****}P < 0.0001$.
Source data are available online for this figure.

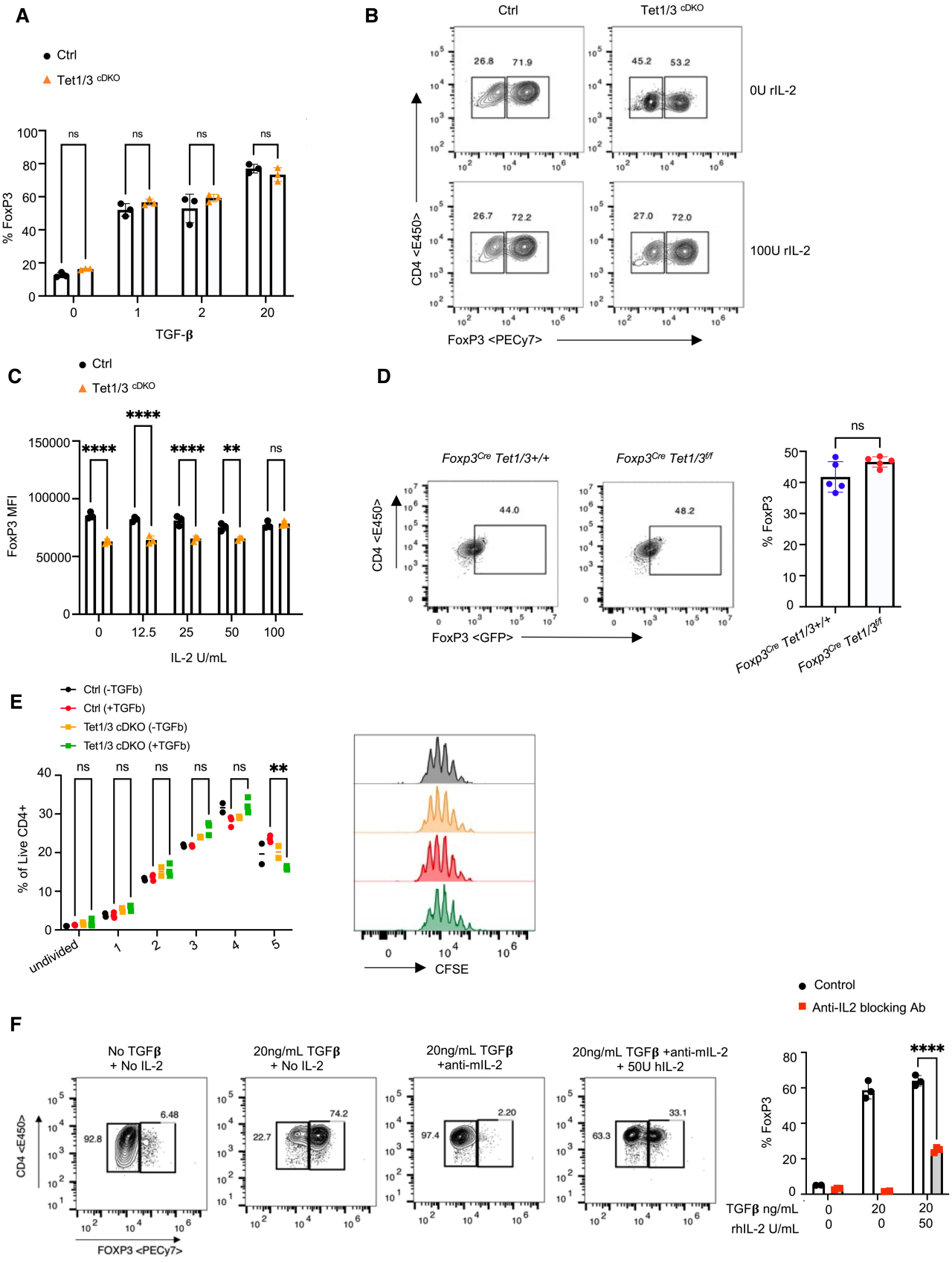
A
Ctrl
Tet1/3 cDKO
% FoxP3
TGF-β
ns
B
Ctrl
Tet1/3 cDKO
0U rIL-2
100U rIL-2
CD4 <E450>
FoxP3 <PECy7>
26.8
71.9
45.2
53.2
26.7
72.2
27.0
72.0
C
FoxP3 MFI
IL-2 U/mL
****
**
D
Foxp3Cre Tet1/3+/+
Foxp3Cre Tet1/3f/f
FoxP3 <GFP>
44.0
48.2
% FoxP3
E
Ctrl (-TGFb)
Ctrl (+TGFb)
Tet1/3 cDKO (-TGFb)
Tet1/3 cDKO (+TGFb)
% of Live CD4+
undivided
CFSE
F
No TGFβ + No IL-2
20ng/mL TGFβ + No IL-2
20ng/mL TGFβ +anti-mIL-2
20ng/mL TGFβ +anti-mIL-2 + 50U hIL-2
FOXP3 <PECy7>
6.48
92.8
74.2
22.7
2.20
97.4
33.1
63.3
Control
Anti-IL2 blocking Ab
TGFβ ng/mL
rhIL-2 U/mL

Figure EV3.

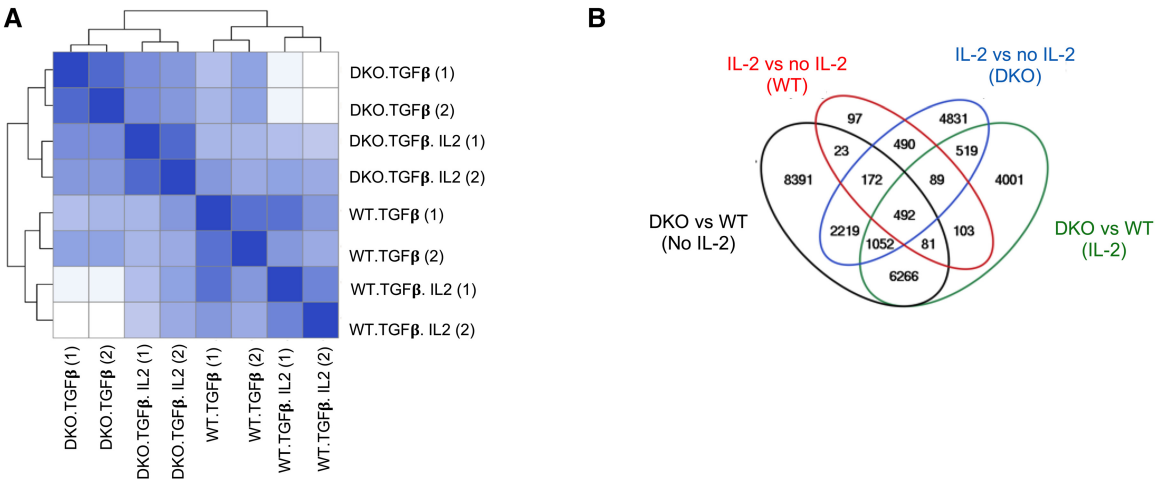

Figure EV4. Tet1 and 3-dependent IL-2 secretion shapes the early chromatin landscape of differentiating Tregs.

A Pearson correlations between accessibility profiles with a dendrogram showing the similarity between samples via hierarchical clustering. For each sample, the accessibility profile is the vector of log2-transformed normalized counts in peaks. DKO = *Rorc(t)*^{CreTg} *Tet1/3*$^{fl/fl}$.

B Venn diagram depicting common differentially accessible peaks among four comparisons: *Rorc(t)*^{CreTg} *Tet1/3*$^{fl/fl}$ versus WT CD4^{+} T cells with no IL-2 treatment (DKO vs. WT (no IL-2)), IL-2-treated versus untreated WT CD4^{+} T cells (IL-2 vs. no IL-2 (WT)), IL-2-treated versus untreated *Rorc(t)*^{CreTg} *Tet1/3*$^{fl/fl}$ CD4^{+} T cells (IL-2 vs. no IL-2 (DKO)), and *Rorc(t)*^{CreTg} *Tet1/3*$^{fl/fl}$ versus WT CD4^{+} T cells with IL-2 treatment (DKO vs. WT (no IL-2)).

