Science Advances
AAAS

advances.sciencemag.org/cgi/content/full/6/1/eaaw6443/DC1

Supplementary Materials for

Homeostasis and transitional activation of regulatory T cells require c-Myc

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Published 1 January 2020, *Sci. Adv.* **6**, eaaw6443 (2020)
DOI: 10.1126/sciadv.aaw6443

This PDF file includes:

Figure S1

A

Name	NES	FDR q-val
HALLMARK_E2F_TARGETS	4.4765	< 0.001
HALLMARK_G2M_CHECKPOINT	4.2489	< 0.001
HALLMARK_MYC_TARGETS_V1	4.1502	< 0.001
HALLMARK_OXIDATIVE_PHOSPHORYLATION	3.6944	< 0.001
HALLMARK_MTORC1_SIGNALING	3.5640	< 0.001
HALLMARK_MITOTIC_SPINDLE	3.4740	< 0.001
HALLMARK_MYC_TARGETS_V2	3.3775	< 0.001
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	3.1012	< 0.001
HALLMARK_DNA_REPAIR	2.9472	< 0.001
HALLMARK_FATTY_ACID_METABOLISM	2.3820	< 0.001

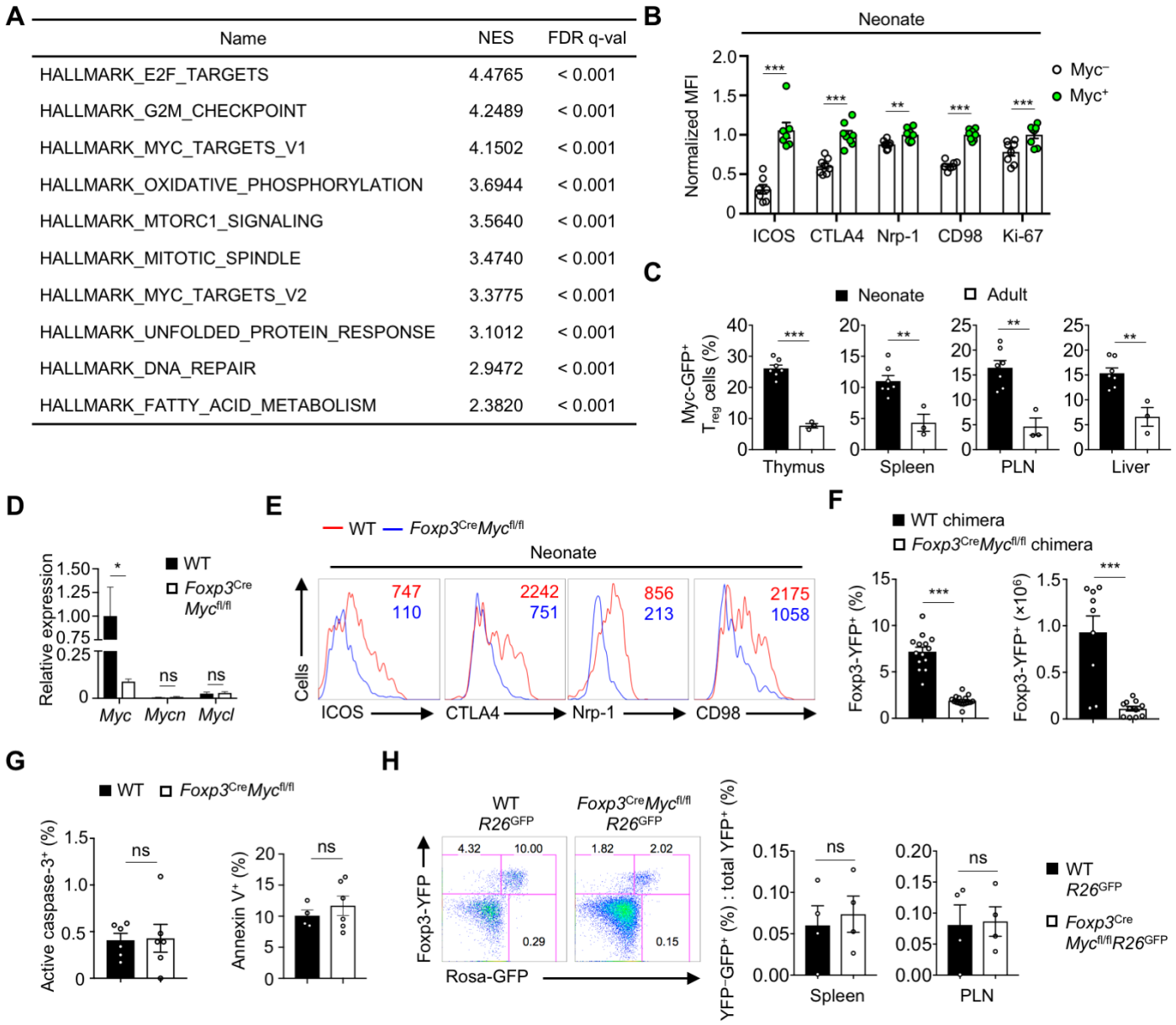

Fig. S1. Myc function is important for neonatal T_{reg} function and accumulation. (**A**) Enrichment of Hallmark gene sets in neonatal vs. adult T_{reg} cells (*22*). NES, normalized enrichment scores; FDR, false discovery rate. (**B**) Flow cytometry analysis of the indicated effector molecule expression by Myc^+ vs. Myc^- T_{reg} cells from neonatal mice. (**C**) Quantification of Myc-GFP expression in $CD4^+Foxp3\text{-}RFP^+$ T_{reg} cells from indicated tissues in neonatal (5–10 days) and adult (6–8 weeks) *Foxp3*^{RFP}Myc-GFP mice. (**D**) Deletion efficiency of Myc and expression of Myc family genes in T_{reg} cells from WT and *Foxp3*^{Cre}*Myc*$^{fl/fl}$ mice. (**E**) Flow cytometry analysis of indicated effector molecule expression on/in splenic T_{reg} cells from neonatal WT and *Foxp3*^{Cre}*Myc*$^{fl/fl}$ mice. (**F**) Flow cytometry analysis of Myc-deficient/WT ($CD45.2^+$) and congenic ($CD45.1^+$) $Foxp3^+$ T_{reg} cells in mixed bone marrow (BM) chimeric mice. (**G**) Flow cytometry analysis of active caspase-3 and Annexin V in/on splenic T_{reg} cells from WT and *Foxp3*^{Cre}*Myc*$^{fl/fl}$ mice. (**H**) Analysis for "ex-T_{reg}" cells using control or *Foxp3*^{Cre}*Myc*$^{fl/fl}$*R26*^{GFP} lineage tracing system. T_{reg} cells include YFP^+GFP^+ and YFP^+GFP^- populations, and ex-T_{reg} cells are YFP^+GFP^-. $^*P \le 0.05$; $^{**}P \le 0.01$; $^{***}P \le 0.001$; ns, not significant; unpaired Student's *t*-test. Data are representative of or pooled from 1 (A, C, and D), 4 (B), 9 (E and F), or 2 (G and H) independent experiments with 1-3 mice per group per experiment. Graphs show means ± SEM.

Figure S2

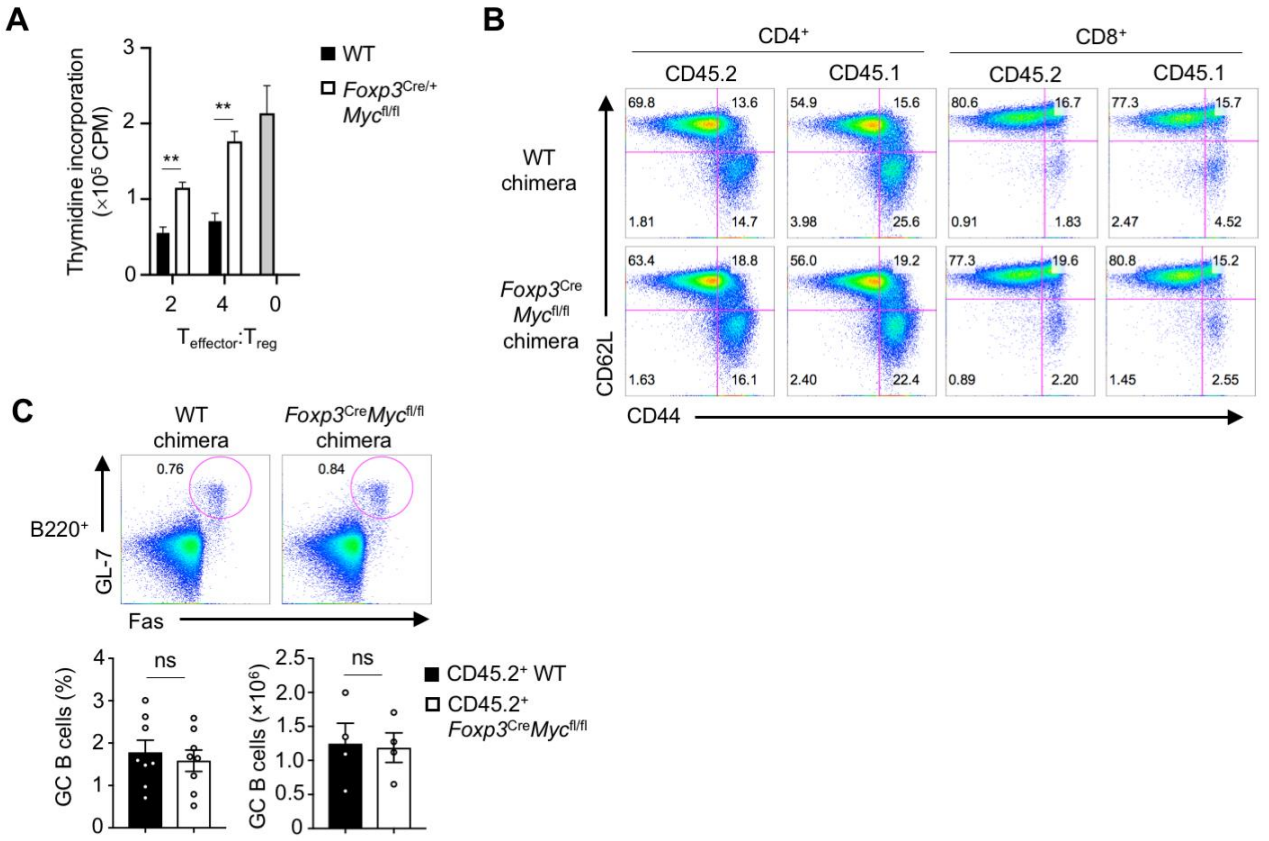

Fig. S2. Impaired in vitro T_{reg} suppression with Myc deficiency and rescue of immune homeostasis in mixed BM chimeric mice. (**A**) T_{reg} cell suppression assay comparing WT and Myc-deficient T_{reg} cells. (**B**) Flow cytometry analysis of naïve and effector $CD4^+$ and $CD8^+$ T cell populations in mixed BM chimeric mice. (**C**) Flow cytometry analysis and quantification of frequency and number of GC B cells in mixed BM chimeric mice. $^{**}P \leq 0.01$; ns, not significant; unpaired Student's *t*-test. Data are representative of or pooled from 4 (A) or 9 (B and C) independent experiments with 1-3 mice per group per experiment. Graphs show means ± SEM.

Figure S3

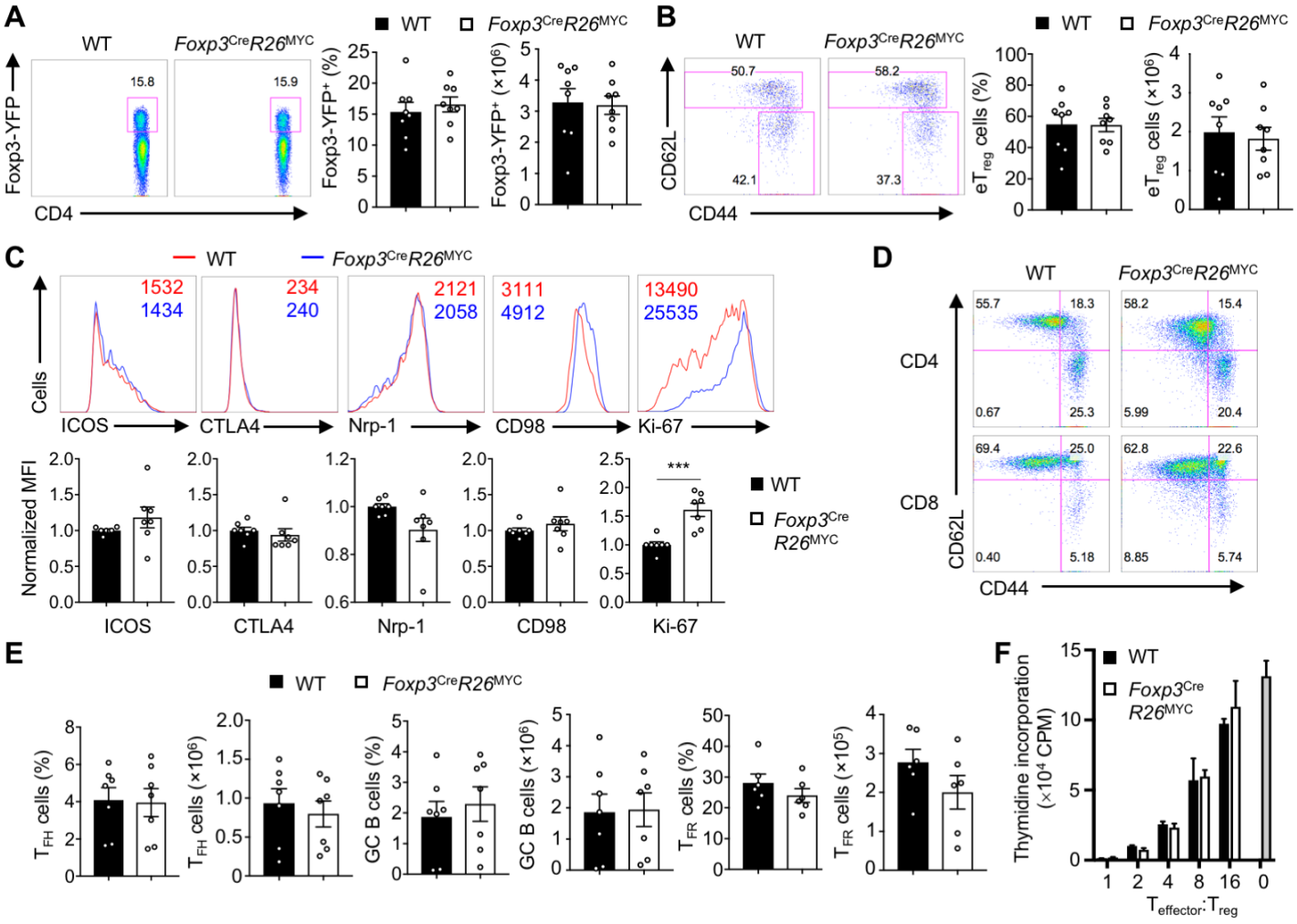

Fig. S3. Constitutive Myc expression in T_{regs} does not affect immune homeostasis. (**A** to **C**) Flow cytometry analysis and quantification of total T_{reg} cells (A), eT_{reg} cells (B), and indicated marker expression on/in T_{reg} cells (C) in WT and *Foxp3*^{Cre}*R26*^{MYC} mice. (**D**) Flow cytometry analysis of splenic naïve and effector $CD4^+$ and $CD8^+$ T cell populations in WT and *Foxp3*^{Cre}*R26*^{MYC} mice. (**E**) Quantification of splenic T_{FH}, GC B, and T_{FR} cells in WT and *Foxp3*^{Cre}*R26*^{MYC} mice. (**F**) T_{reg} cell suppression assay comparing purified WT and Myc-overexpressing T_{reg} cells. ***$P \leq 0.001$; unpaired Student's *t*-test. Data are representative of or pooled from 4 independent experiments with 1-5 mice per group per experiment. Graphs show means ± SEM.

Figure S4

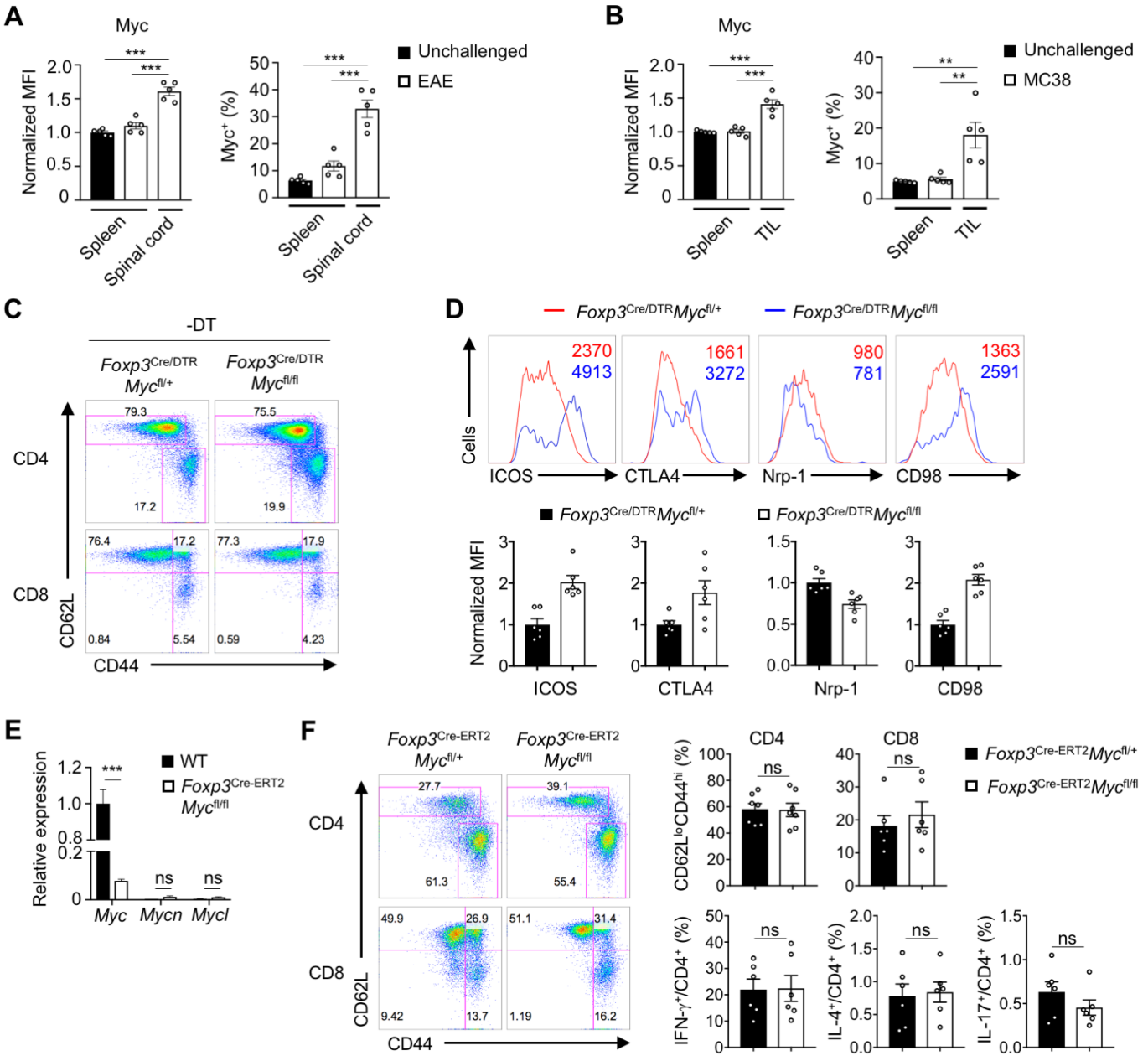

Fig. S4. CD4$^+$ and CD8$^+$ T cell responses in *Foxp3*$^{Cre/DTR}$ mosaic mice and mice with tamoxifen-induced Myc deletion. (**A**) Flow cytometry analysis of Myc expression (left graph) in T_{reg} cells or the frequency of Myc$^+$ T_{reg} cells in the spleen of unchallenged mice (black bars) or the spleen (middle white bars) or spinal cords (right white bars) of mice with EAE. (**B**) Flow cytometry analysis of Myc expression in T_{reg} cells or frequency of Myc$^+$ T_{reg} cells from the spleen of unchallenged mice (black bar), MC38-incoculated mice (middle white bars), or TIL of MC38-challenged mice (right white bars). Data are from five mice per group. (**C**) Flow cytometry analysis of naïve and effector non-T_{reg} CD4$^+$ and CD8$^+$ T cells in Foxp3-DTR mosaic mice at steady state. (**D**) Flow cytometry analysis and quantification of indicated marker expression on/in splenic CD4$^+$Foxp3-YFP$^+$ T_{reg} cells in DT-treated mosaic mice. (**E**) Deletion efficiency of *Myc* and expression of Myc family genes in CD4$^+$GFP$^+$YFP$^+$ T_{reg} cells sorted from tamoxifen-treated WT and *Foxp3*$^{Cre-ERT2}$*Myc*$^{fl/fl}$ mice. (**F**) Flow cytometry analysis and quantification of naïve and effector CD4$^+$ and CD8$^+$ T cells, and quantification of cytokine production in T helper cell subsets in tamoxifen-treated *Foxp3* $^{Cre-ERT2}$*Myc*$^{fl/+}$ and *Foxp3* $^{Cre-ERT2}$*Myc*$^{fl/fl}$ mice. Data are representative of or pooled from 1 (A, B, and E), 4 (C and D) or 5 (F) independent experiments with 1-5 mice per group per experiment. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; ns, not significant; unpaired Student's *t*-test. Graphs show means ± SEM.

Figure S5

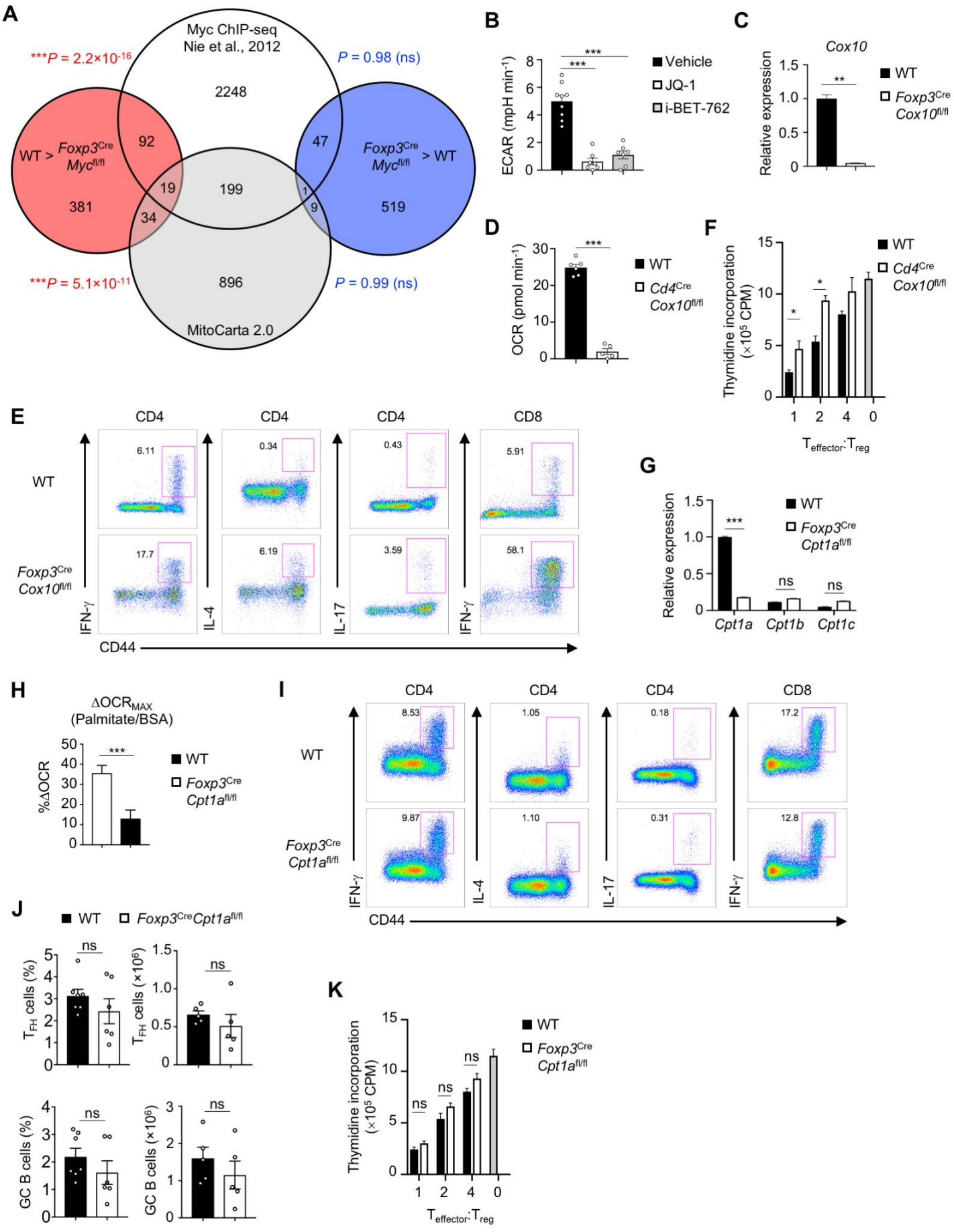
A
Myc ChIP-seq
Nie et al., 2012
2248
***P = 2.2×10-16
P = 0.98 (ns)
WT > Foxp3Cre Mycfl/fl
381
92
19
199
47
1
9
34
Foxp3Cre Mycfl/fl > WT
519
***P = 5.1×10-11
896
P = 0.99 (ns)
MitoCarta 2.0
B
ECAR (mpH min-1)
Vehicle
JQ-1
i-BET-762
C
Cox10
Relative expression
WT
Foxp3Cre Cox10fl/fl
D
OCR (pmol min-1)
WT
Cd4Cre Cox10fl/fl
F
Thymidine incorporation (×10⁵ CPM)
Teffector:Treg
E
CD4
CD8
WT
Foxp3Cre Cox10fl/fl
IFN-γ
IL-4
IL-17
CD44
6.11
0.34
0.43
5.91
17.7
6.19
3.59
58.1
G
Relative expression
Foxp3Cre Cpt1afl/fl
Cpt1a
Cpt1b
Cpt1c
H
ΔOCRMAX (Palmitate/BSA)
%ΔOCR
I
8.53
1.05
0.18
17.2
9.87
1.10
0.31
12.8
J
Foxp3Cre Cpt1afl/fl
TFH cells (%)
TFH cells (×10⁶)
GC B cells (%)
GC B cells (×10⁶)
K

Fig. S5. Immune homeostasis in mice with Cox10- or Cpt1a-deficient T_{regs}. (**A**) Negative enrichment of direct Myc targets (defined in published Myc ChIP-seq data (*10*); top *P*-values), and mitochondria-related genes (defined in MitoCarta 2.0 database; bottom *P*-values) in Myc-deficient T_{reg} cells. (**B**) Seahorse analysis of ECAR in T_{reg} cells activated *in vitro* for 6 h in the presence of Myc inhibitors. (**C**) Deletion efficiency of *Cox10* in T_{reg} cells. (**D**) Seahorse analysis of OCR in Cox10-deficient T_{reg} cells. (**E**) Flow cytometry analysis of cytokine production in splenic non-T_{reg} $CD4^+$ and $CD8^+$ T cells in WT and $Foxp3^{Cre}Cox10^{fl/fl}$ mice. (**F**) T_{reg} cell suppression assay comparing WT and Cox10-deficient T_{reg} cells. (**G**) Deletion efficiency and additional Cpt1 isoform expression in T_{reg} cells from WT and $Foxp3^{Cre}Cpt1a^{fl/fl}$ mice. (**H**) Seahorse analysis of the difference in maximum OCR (following FCCP treatment) when assayed with BSA-conjugated palmitate. (**I** and **J**) Flow cytometry analysis of cytokine production in non-T_{reg} $CD4^+$ and $CD8^+$ T cells (I), and quantification of GC responses (J) in WT and $Foxp3^{Cre}Cpt1a^{fl/fl}$ mice. (**K**) T_{reg} cell suppression assay comparing WT and Cpt1a-deficient T_{reg} cells. Data are representative of or pooled from 3 (A, G), 1 (B, C, F, and K), 2 (D and E), or 4 (H to J) independent experiments with 1-2 mice per group per experiment. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; ns, not significant; unpaired Student's *t*-test. Graphs show means ± SEM.