Figure S1

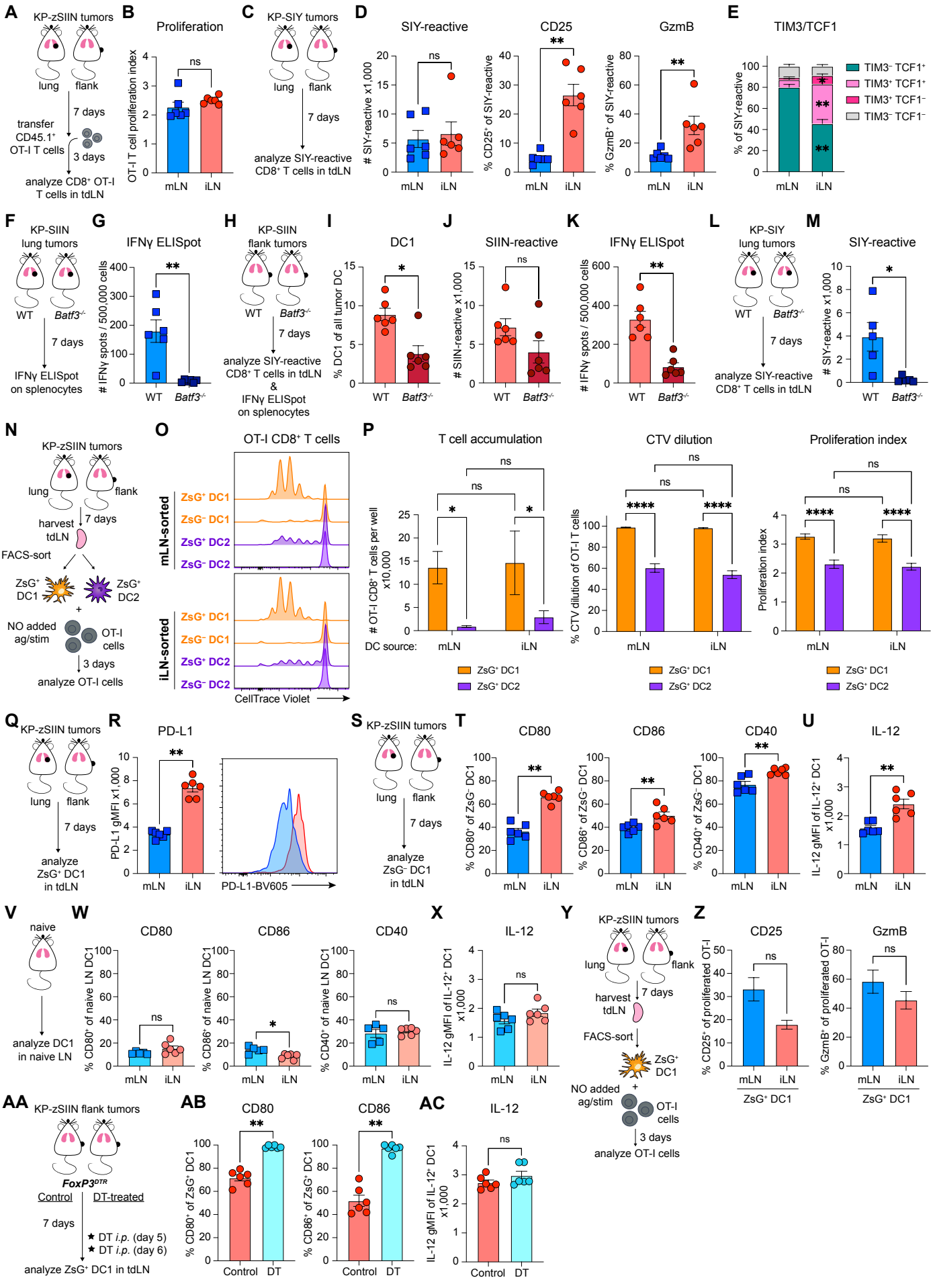

A
KP-zSIIN tumors
lung
flank
7 days
transfer CD45.1+ OT-I T cells
3 days
analyze CD8+ OT-I T cells in tdLN
B
Proliferation
OT-I T cell proliferation index
ns
mLN
iLN
C
KP-SIY tumors
lung
flank
7 days
analyze SIY-reactive CD8+ T cells in tdLN
D
SIY-reactive
SIY-reactive x1,000
ns
CD25
% CD25+ of SIY-reactive
**
GzmB
% GzmB+ of SIY-reactive
**
E
TIM3/TCF1
% of SIY-reactive
TIM3− TCF1+
TIM3+ TCF1+
TIM3+ TCF1−
TIM3− TCF1−
F
KP-SIIN lung tumors
WT
Batf3−/−
7 days
IFNγ ELISpot on splenocytes
G
IFNγ ELISpot
IFNγ spots / 500,000 cells
**
H
KP-SIIN flank tumors
analyze SIY-reactive CD8+ T cells in tdLN & IFNγ ELISpot on splenocytes
I
DC1
% DC1 of all tumor DC
*
J
SIIN-reactive
SIIN-reactive x1,000
ns
K
IFNγ ELISpot
IFNγ spots / 500,000 cells
**
L
KP-SIY lung tumors
analyze SIY-reactive CD8+ T cells in tdLN
M
SIY-reactive
SIY-reactive x1,000
*
N
KP-zSIIN tumors
harvest tdLN
FACS-sort
ZsG+ DC1
ZsG+ DC2
NO added ag/stim
OT-I cells
3 days
analyze OT-I cells
O
OT-I CD8+ T cells
mLN-sorted
iLN-sorted
ZsG+ DC1
ZsG− DC1
ZsG+ DC2
ZsG− DC2
CellTrace Violet
P
T cell accumulation
OT-I CD8+ T cells per well x10,000
CTV dilution
% CTV dilution of OT-I T cells
Proliferation index
DC source:
ZsG+ DC1
ZsG+ DC2
Q
KP-zSIIN tumors
analyze ZsG+ DC1 in tdLN
R
PD-L1
PD-L1 gMFI x1,000
PD-L1-BV605
S
KP-zSIIN tumors
analyze ZsG− DC1 in tdLN
T
CD80
% CD80+ of ZsG− DC1
CD86
% CD86+ of ZsG− DC1
CD40
% CD40+ of ZsG− DC1
U
IL-12
IL-12 gMFI of IL-12+ DC1 x1,000
V
naive
analyze DC1 in naive LN
W
CD80
% CD80+ of naive LN DC1
CD86
% CD86+ of naive LN DC1
CD40
% CD40+ of naive LN DC1
X
IL-12
IL-12 gMFI of IL-12+ DC1 x1,000
Y
KP-zSIIN tumors
harvest tdLN
FACS-sort
ZsG+ DC1
NO added ag/stim
OT-I cells
3 days
analyze OT-I cells
Z
CD25
% CD25+ of proliferated OT-I
GzmB
% GzmB+ of proliferated OT-I
ZsG+ DC1
AA
KP-zSIIN flank tumors
FoxP3DTR
Control
DT-treated
7 days
DT i.p. (day 5)
DT i.p. (day 6)
analyze ZsG+ DC1 in tdLN
AB
CD80
% CD80+ of ZsG+ DC1
CD86
% CD86+ of ZsG+ DC1
AC
IL-12
IL-12 gMFI of IL-12+ DC1 x1,000
Control
DT

Figure S1. Related to Main Figures 1-3: DC1 prime dysfunctional CD8$^+$ T cells against lung but not flank KP tumors.

(A) Experimental design for (B).

(B) Proliferation index of adoptively-transferred CTV-labelled OT-I T cells from tdLN, day 10 post-tumor implantation (n=3 mice/group; two independent experiments).

(C) Experimental design for (D-E).

(D-E) Quantification of (D) numbers, CD25, GzmB, (E) TCF1 and TIM-3 expression of SIY-reactive CD8$^+$ T cells in tdLN, day 7 post-tumor implantation (n=3 mice/group; two independent experiments).

(F) Experimental design for (G).

(G) ELISpot quantification of IFNγ-producing splenocytes from WT and *Batf3*$^{-/-}$ mice bearing lung KP-zSIIN tumors, day 7 post-tumor inoculation (n=3 mice/group; two independent experiments).

(H) Experimental design for (I-K).

(I-J) Quantification of (I) DC1 frequency in tumors and (J) numbers of SIIN-reactive CD8$^+$ T cells in iLN from WT or *Batf3*$^{-/-}$ mice, day 7 post-tumor implantation (n=3 mice/group; two independent experiments).

(K) ELISpot quantification of IFNγ-producing splenocytes from WT and *Batf3*$^{-/-}$ mice bearing flank KP-zSIIN tumors, day 7 post-tumor inoculation (n=3 mice/group; two independent experiments).

(L) Experimental design for (M).

(M) Numbers of SIY-reactive CD8$^+$ T cells in mLN of tumor-bearing WT or *Batf3*$^{-/-}$ mice, day 7 post-tumor implantation (n=2-3 mice/group; two independent experiments).

(N) Experimental design for (O-P).

(O-P) (O) Representative histograms and (P) quantified proliferation of CTV-labelled OT-I T cells after 3-day co-culture with DC subsets sorted from tdLN (50 tdLN pooled for sorting, three independent experiments).

(Q) Experimental design for (R).

(R) Representative histogram and PD-L1 expression on ZsG$^+$ DC1 from tdLN, day 7 post-tumor implantation (LN from 3-4 mice pooled per datapoint; two independent experiments).

(S) Experimental design for (T-U).

(T-U) Expression of (T) CD80, CD86, CD40 and (U) IL-12 on ZsG$^-$ DC1 from tdLN, day 7 post-tumor implantation (LN from 3-4 mice pooled per datapoint; two independent experiments).

(V) Experimental design for (W-X).

(W-X) Expression of (W) CD80, CD86, CD40 and (X) IL-12 on DC1 from LN of naïve mice (n=2-3 mice/group; two independent experiments).

(Y) Experimental design for (Z).

(Z) CD25 and GzmB expression on proliferated OT-I T cells after co-culture with ZsG$^+$ DC1 sorted from tdLN at day 7 post-tumor implantation. (50 tdLN pooled for sorting, three independent experiments).

(AA) Experimental design for (AB-AC).

(AB-AC) Expression of (AB) CD80, CD86 and (AC) IL-12 on ZsG$^+$ DC1 from iLN of control or DT-treated tumor-bearing *FoxP3*^{DTR} mice, day 7 post-tumor implantation (iLN from 2 mice pooled per datapoint; two independent experiments).

*p<0.05, **p<0.01, ****p<0.0001, ns=not significant; MWU (B,D-E,G,I-K,M,R,T-U,W-X,Z,AB-AC), two-way ANOVA (P). Data shown as mean ± SEM.

Figure S2

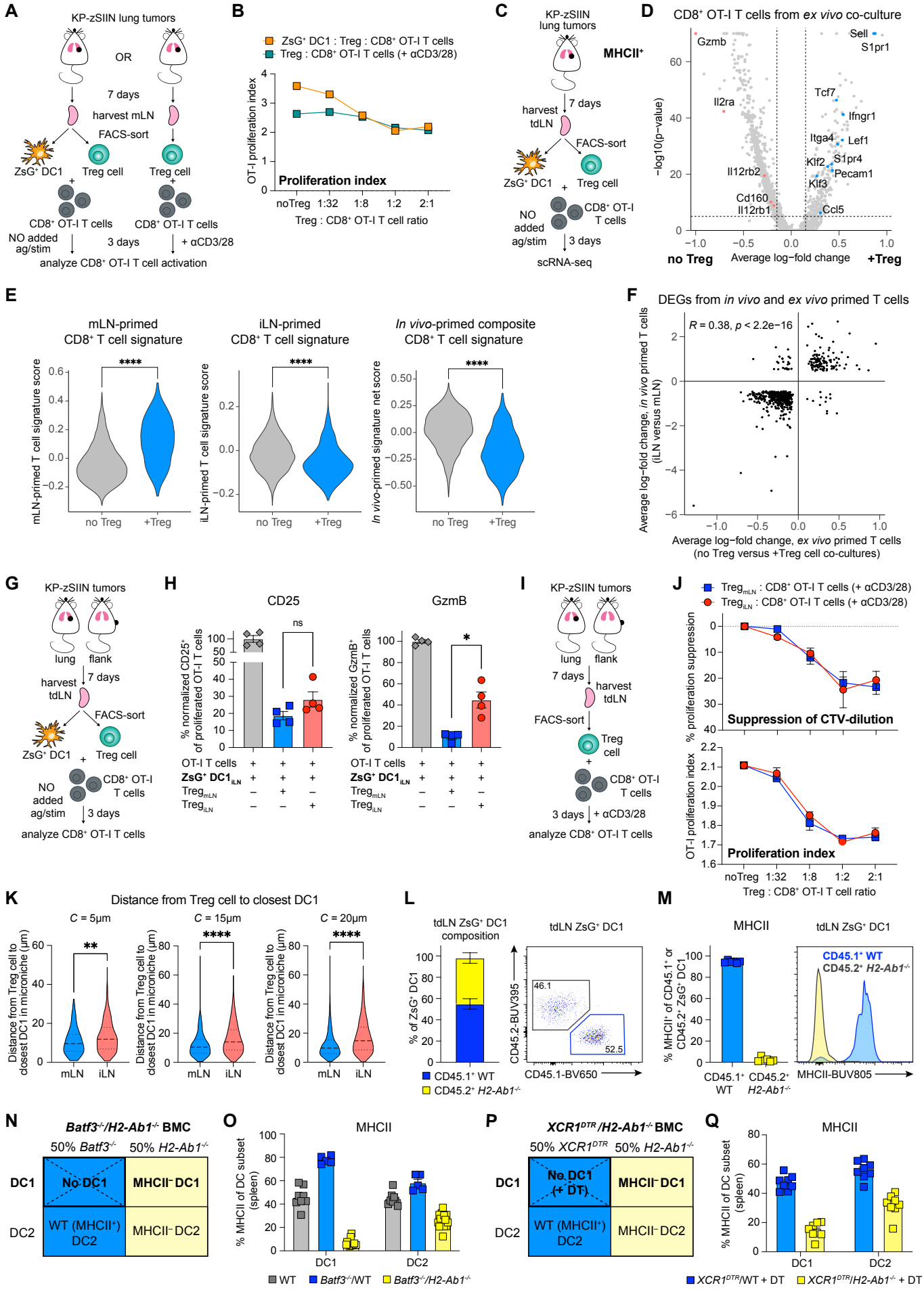

A
KP-zSIIN lung tumors
OR
7 days
harvest mLN
FACS-sort
ZsG⁺ DC1
Treg cell
Treg cell
CD8⁺ OT-I T cells
CD8⁺ OT-I T cells
NO added ag/stim
3 days
+ αCD3/28
analyze CD8⁺ OT-I T cell activation
B
ZsG⁺ DC1 : Treg : CD8⁺ OT-I T cells
Treg : CD8⁺ OT-I T cells (+ αCD3/28)
OT-I proliferation index
Proliferation index
noTreg 1:32 1:8 1:2 2:1
Treg : CD8⁺ OT-I T cell ratio
C
KP-zSIIN lung tumors
MHCII⁺
harvest tdLN
7 days
FACS-sort
ZsG⁺ DC1
Treg cell
CD8⁺ OT-I T cells
NO added ag/stim
3 days
scRNA-seq
D
CD8⁺ OT-I T cells from ex vivo co-culture
Gzmb
Sell
S1pr1
Tcf7
Il2ra
Ifngr1
Itga4
Lef1
Klf2
S1pr4
Il12rb2
Pecam1
Klf3
Cd160
Il12rb1
Ccl5
−log10(p−value)
no Treg
Average log−fold change
+Treg
E
mLN-primed CD8⁺ T cell signature
iLN-primed CD8⁺ T cell signature
In vivo-primed composite CD8⁺ T cell signature
mLN-primed T cell signature score
iLN-primed T cell signature score
In vivo-primed signature net score
****
no Treg
+Treg
F
DEGs from in vivo and ex vivo primed T cells
R = 0.38, p < 2.2e−16
Average log−fold change, in vivo primed T cells (iLN versus mLN)
Average log−fold change, ex vivo primed T cells (no Treg versus +Treg cell co-cultures)
G
KP-zSIIN tumors
lung
flank
7 days
harvest tdLN
FACS-sort
ZsG⁺ DC1
Treg cell
NO added ag/stim
CD8⁺ OT-I T cells
3 days
analyze CD8⁺ OT-I T cells
H
CD25
GzmB
ns
*
% normalized CD25⁺ of proliferated OT-I T cells
% normalized GzmB⁺ of proliferated OT-I T cells
OT-I T cells
ZsG⁺ DC1iLN
TregmLN
TregiLN
I
KP-zSIIN tumors
lung
flank
7 days
harvest tdLN
FACS-sort
Treg cell
CD8⁺ OT-I T cells
3 days
+ αCD3/28
analyze CD8⁺ OT-I T cells
J
TregmLN : CD8⁺ OT-I T cells (+ αCD3/28)
TregiLN : CD8⁺ OT-I T cells (+ αCD3/28)
% proliferation suppression
Suppression of CTV-dilution
OT-I proliferation index
Proliferation index
noTreg 1:32 1:8 1:2 2:1
Treg : CD8⁺ OT-I T cell ratio
K
Distance from Treg cell to closest DC1
C = 5μm
C = 15μm
C = 20μm
**
****
****
Distance from Treg cell to closest DC1 in microniche (μm)
mLN
iLN
L
tdLN ZsG⁺ DC1 composition
tdLN ZsG⁺ DC1
% of ZsG⁺ DC1
CD45.1⁺ WT
CD45.2⁺ H2-Ab1⁻/⁻
CD45.2-BUV395
CD45.1-BV650
46.1
52.5
M
MHCII
tdLN ZsG⁺ DC1
% MHCII⁺ of CD45.1⁺ or CD45.2⁺ ZsG⁺ DC1
CD45.1⁺ WT
CD45.2⁺ H2-Ab1⁻/⁻
CD45.1⁺ WT
CD45.2⁺ H2-Ab1⁻/⁻
MHCII-BUV805
N
Batf3⁻/⁻/H2-Ab1⁻/⁻ BMC
50% Batf3⁻/⁻
50% H2-Ab1⁻/⁻
DC1
DC2
No DC1
MHCII⁻ DC1
WT (MHCII⁺) DC2
MHCII⁻ DC2
O
MHCII
% MHCII of DC subset (spleen)
DC1
DC2
WT
Batf3⁻/⁻/WT
Batf3⁻/⁻/H2-Ab1⁻/⁻
P
XCR1DTR/H2-Ab1⁻/⁻ BMC
50% XCR1DTR
50% H2-Ab1⁻/⁻
DC1
DC2
No DC1 (+ DT)
MHCII⁻ DC1
WT (MHCII⁺) DC2
MHCII⁻ DC2
Q
MHCII
% MHCII of DC subset (spleen)
DC1
DC2
XCR1DTR/WT + DT
XCR1DTR/H2-Ab1⁻/⁻ + DT

Figure S2. Related to Main Figure 4 and 5: Treg cells from the tumor-draining mLN can induce CD8$^+$ T cell dysfunction by suppressing DC1.

(A) Experimental design for (B).

(B) Representative example of the proliferation index of CTV-stained OT-I T cells after 3-day co-culture with (*orange*) mLN-sorted ZsG$^+$ DC1 and Treg cells or (*teal*) mLN-sorted Treg cells with αCD3/αCD28-stimulation (50 tdLN pooled for sorting, three independent experiments).

(C) Experimental design for (D-F).

(D) Volcano plot of DEGs between OT-I T cells cultured *ex vivo* with mLN-sorted ZsG$^+$ DC1 in the absence or presence of mLN-sorted Treg cells. Select DEGs from no Treg (red) or Treg cell-containing (*red*) co-culture conditions are highlighted.

(E) Violin plots of *ex vivo*-primed OT-I T cell enrichment for signatures of *in vivo*-primed CD8$^+$ 2C T cells from the mLN and iLN of KP-SIY tumor-bearing mice, from Horton *et al.* (2021).

(F) Comparison between log-fold change values of DEGs of *ex vivo*-primed OT-I T cells (cultured for 3 days in the presence or absence of Treg cells) and *in vivo*-primed adoptively-transferred CD8$^+$ 2C T cells (isolated from KP-SIY tumor-draining iLN or mLN 3 days after adoptive transfer, day 10 post-tumor implantation). Spearman's correlation and the associated p-value are shown.

(G) Experimental design for (H).

(H) CD25 and GzmB expression on proliferated OT-I T cells after 3-day co-culture with iLN-sorted ZsG$^+$ DC1 and either mLN- or iLN-sorted Treg cells (50 tdLN pooled for sorting, three independent experiments).

(I) Experimental design for (J).

(J) Representative example of (*top*) suppression of proliferation frequency and (*bottom*) proliferation index of αCD3/αCD28-stimulated CTV-stained OT-I T cells after 3-day co-culture with (*blue*) mLN-sorted Treg cells or (*red*) iLN-sorted Treg cells (50 tdLN pooled for sorting, three independent experiments).

(K) Distance from Treg cell to closest DC1 within microniche in tdLN, using microniche radius equal to $r_{cluster}$ + *C*, where *C* is (*left*) 5μm, (*middle*) 15μm or (*right*) 20μm (n=4 mice/group including 399 mLN microniches and 74 iLN microniches; representative data from one of two independent experiments).

(L-M) Representative flow plot and quantification of (L) ZsG$^+$ DC1 composition and (M) MHCII expression on ZsG$^+$ DC1 in mLN of tumor-bearing WT/*H2-Ab1*$^{-/-}$ BMCs, day 7 post-tumor implantation (mLN from 3-4 mice pooled per datapoint; two independent experiments).

(N) Expected DC composition in *Batf3*$^{-/-}$/*H2-Ab1*$^{-/-}$ BMCs.

(O) Observed DC composition in spleens of tumor-bearing WT, *Batf3*$^{-/-}$/*WT* and *Batf3*$^{-/-}$/*H2-Ab1*$^{-/-}$ BMCs, day 10 post-tumor implantation (n=2-5 mice/group; 1-4 independent experiments).

(P) Expected DC composition in DT-treated *XCR1*^{DTR}/H2-*Ab1*$^{-/-}$ BMCs.

(Q) Observed DC composition in spleens of DT-treated tumor-bearing *XCR1*^{DTR}/*WT* and *XCR1*^{DTR}/*H2-Ab1*$^{-/-}$ BMCs, day 10 post-tumor implantation (n=3-4 mice/group; two independent experiments).

*p<0.05, **p<0.01, ****p<0.0001, ns=not significant; MWU with Bonferroni correction (D), MWU (E,H,K). Data shown as mean ± SEM.

Figure S3

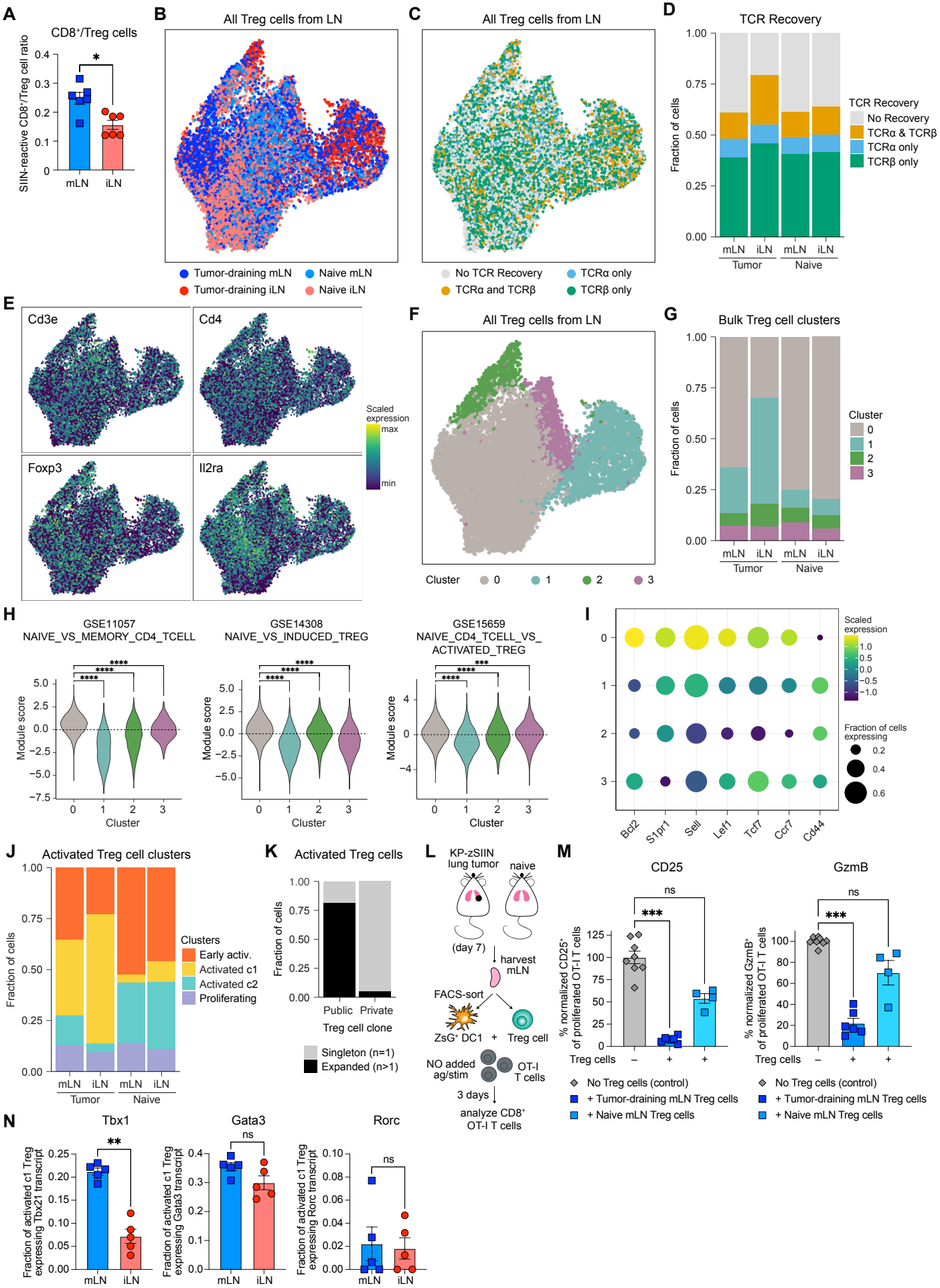

A
CD8⁺/Treg cells
SIIN-reactive CD8⁺/Treg cell ratio
mLN
iLN
B
All Treg cells from LN
Tumor-draining mLN
Tumor-draining iLN
Naive mLN
Naive iLN
C
All Treg cells from LN
No TCR Recovery
TCRα and TCRβ
TCRα only
TCRβ only
D
TCR Recovery
Fraction of cells
Tumor
Naive
No Recovery
TCRα & TCRβ
TCRα only
TCRβ only
E
Cd3e
Cd4
Foxp3
Il2ra
Scaled expression
max
min
F
All Treg cells from LN
Cluster 0 1 2 3
G
Bulk Treg cell clusters
Fraction of cells
Cluster
H
GSE11057
NAIVE_VS_MEMORY_CD4_TCELL
GSE14308
NAIVE_VS_INDUCED_TREG
GSE15659
NAIVE_CD4_TCELL_VS_
ACTIVATED_TREG
Module score
Cluster
I
Scaled expression
Fraction of cells expressing
Bcl2
S1pr1
Sell
Lef1
Tcf7
Ccr7
Cd44
J
Activated Treg cell clusters
Fraction of cells
Clusters
Early activ.
Activated c1
Activated c2
Proliferating
Tumor
Naive
K
Activated Treg cells
Fraction of cells
Public
Private
Treg cell clone
Singleton (n=1)
Expanded (n>1)
L
KP-zSIIN lung tumor
naive
(day 7)
harvest mLN
FACS-sort
ZsG⁺ DC1 + Treg cell
NO added ag/stim
OT-I T cells
3 days
analyze CD8⁺ OT-I T cells
M
CD25
GzmB
% normalized CD25⁺ of proliferated OT-I T cells
% normalized GzmB⁺ of proliferated OT-I T cells
Treg cells
No Treg cells (control)
+ Tumor-draining mLN Treg cells
+ Naive mLN Treg cells
N
Tbx1
Gata3
Rorc
Fraction of activated c1 Treg expressing Tbx21 transcript
Fraction of activated c1 Treg expressing Gata3 transcript
Fraction of activated c1 Treg expressing Rorc transcript

Figure S3. Related to Main Figure 6: T_H1-like Treg cells clonally expand in tdLN and are associated with suppression.

(A) SIIN-reactive $CD8^+$/Treg cell ratios in tdLN, day 7 post-tumor implantation (n=3 mice/group; two independent experiments).

(B) UMAP plot of all Treg cells from naïve and tdLN colored by tumor status and location (tdLN, n=5 mice/group; naïve LN, n=20 mice/group).

(C-D) Treg cell TCR recovery (C) mapped onto UMAP plot and (D) quantified as stacked bar chart for naïve and tdLN.

(E) UMAP feature plots of canonical Treg cell markers including *Cd3e*, *Cd4*, *FoxP3* and *Il2ra*.

(F-G) Treg cell cluster composition displayed on (F) UMAP plot and (G) stacked bar charts for naïve and tdLN.

(H) Violin plots of Treg cell cluster enrichment for naïve T cell gene sets.

(I) Dot plot of select naïve T cell marker genes displaying average expression and frequency of expression for each gene.

(J) Stacked bar plots depicting relative frequencies of activated Treg cell clusters across naïve and tdLN.

(K) Stacked bar plot of Treg cell expansion across public and private activated Treg cell clones. Expanded clones were defined as multiple cells recovered from the same mouse containing identical CDR3β junction nucleotide sequences.

(L) Experimental design for (M).

(M) CD25 and GzmB expression on proliferated OT-I T cells after 3-day co-culture with mLN-sorted ZsG^+ DC1 and Treg cells isolated from tumor-draining or naïve mLN (40-70 mLN pooled for sorting, two independent experiments).

(N) *Tbx21*, *Gata3* and *Rorc* transcript expression on activated c1 Treg cells from tdLN, day 7 post-tumor implantation (n=5 mice/group).

**p<0.01, ***p<0.001, ****p<0.0001, ns=not significant; MWU (H,N), KW (M).

Figure S4

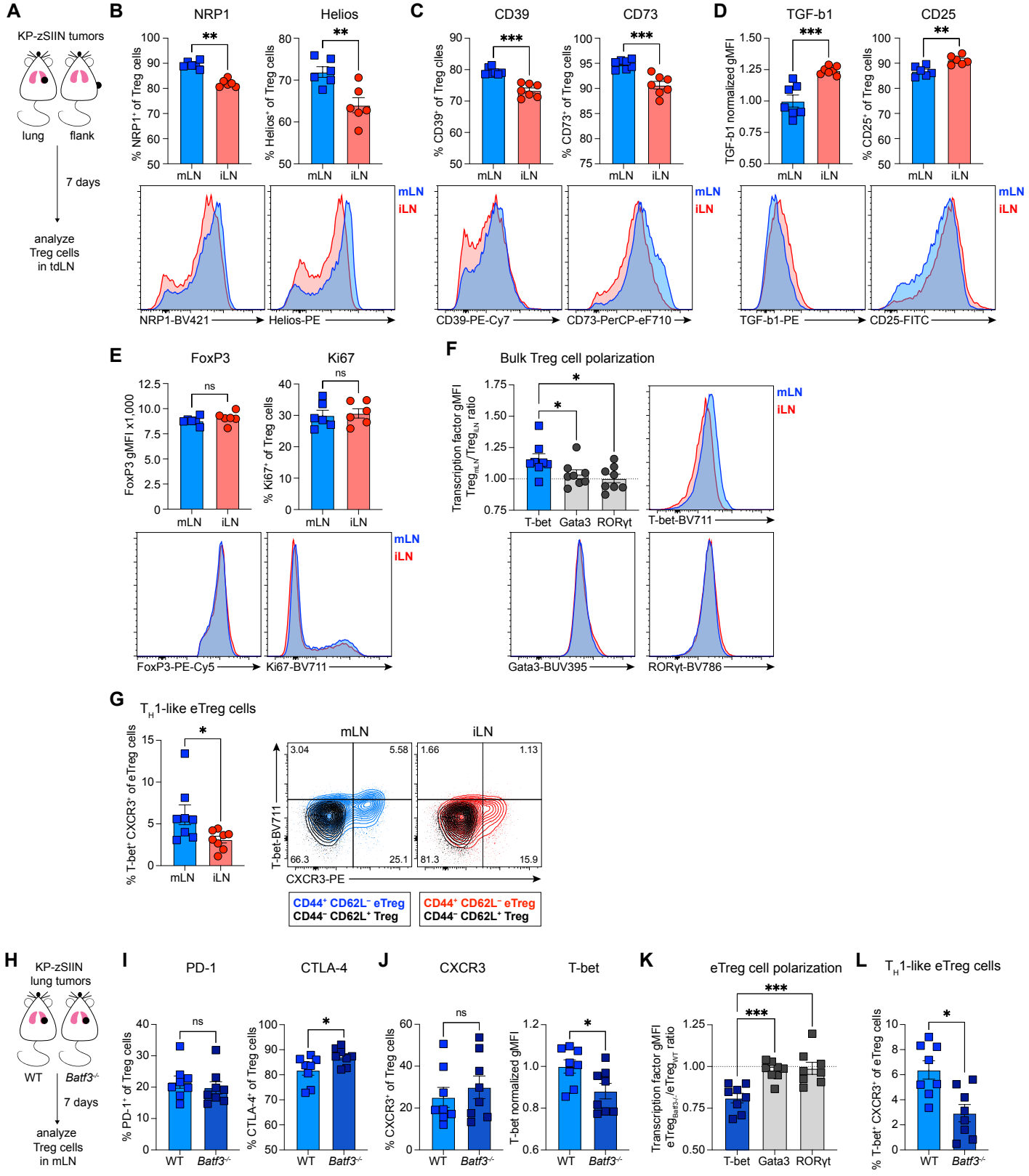

A
KP-zSIIN tumors
lung
flank
7 days
analyze
Treg cells
in tdLN
B
NRP1
Helios
% NRP1+ of Treg cells
% Helios+ of Treg cells
mLN
iLN
NRP1-BV421
Helios-PE
C
CD39
CD73
% CD39+ of Treg clles
% CD73+ of Treg cells
CD39-PE-Cy7
CD73-PerCP-eF710
D
TGF-b1
CD25
TGF-b1 normalized gMFI
% CD25+ of Treg cells
TGF-b1-PE
CD25-FITC
E
FoxP3
Ki67
FoxP3 gMFI x1,000
% Ki67+ of Treg cells
FoxP3-PE-Cy5
Ki67-BV711
F
Bulk Treg cell polarization
Transcription factor gMFI Treg_mLN/Treg_iLN ratio
T-bet
Gata3
RORγt
T-bet-BV711
Gata3-BUV395
RORγt-BV786
G
T_H1-like eTreg cells
% T-bet+ CXCR3+ of eTreg cells
T-bet-BV711
CXCR3-PE
3.04
5.58
66.3
25.1
1.66
1.13
81.3
15.9
CD44+ CD62L- eTreg
CD44- CD62L+ Treg
CD44+ CD62L- eTreg
CD44- CD62L+ Treg
H
KP-zSIIN
lung tumors
WT
Batf3-/-
7 days
analyze
Treg cells
in mLN
I
PD-1
CTLA-4
% PD-1+ of Treg cells
% CTLA-4+ of Treg cells
J
CXCR3
T-bet
% CXCR3+ of Treg cells
T-bet normalized gMFI
K
eTreg cell polarization
Transcription factor gMFI eTreg_Batf3-/-/eTreg_WT ratio
L
T_H1-like eTreg cells
% T-bet+ CXCR3+ of eTreg cells

Figure S4. Related to Main Figure 6: Characterization of Treg cells from tumor-draining mLN and iLN.

(A) Experimental design for (B-G).

(B-E) Representative histograms and expression of (B) NRP1, Helios, (C) CD39, CD73, (D) TGF-b1, CD25, (E) FoxP3 and Ki67 on Treg cells from tdLN, day 7 post-tumor implantation (n=3-4 mice/group; two independent experiments).

(F) Representative histograms and ratios of transcription factor gMFI values for bulk Treg cells from tdLN, day 7 post-tumor implantation (n=4 mice/group; two independent experiments).

(G) Representative flow plots and frequency of the T-bet^{+} CXCR3^{+} double-positive fraction of eTreg cells from tdLN, day 7 post-tumor implantation (n=4 mice/group; two independent experiments).

(H) Experimental design for (I-L).

(I-J) Expression of (I) PD-1, CTLA-4, (J) CXCR3 and T-bet on Treg cells from mLN of tumor-bearing WT and *Batf3*$^{-/-}$ mice, day 7 post-tumor implantation (n=4 mice/group; two independent experiments).

(K) Ratios of transcription factor gMFI values measured on eTreg cells from mLN of tumor-bearing WT and *Batf3*$^{-/-}$ mice, day 7 post-tumor implantation (n=4 mice/group; two independent experiments).

(L) Frequency of the T-bet^{+} CXCR3^{+} double-positive fraction of eTreg cells from mLN of tumor-bearing WT and *Batf3*$^{-/-}$ mice, day 7 post-tumor implantation (n=4 mice/group; two independent experiments).

*p<0.05, **p<0.01, ***p<0.001, ns=not significant; MWU (B-G,I-L).

Figure S5

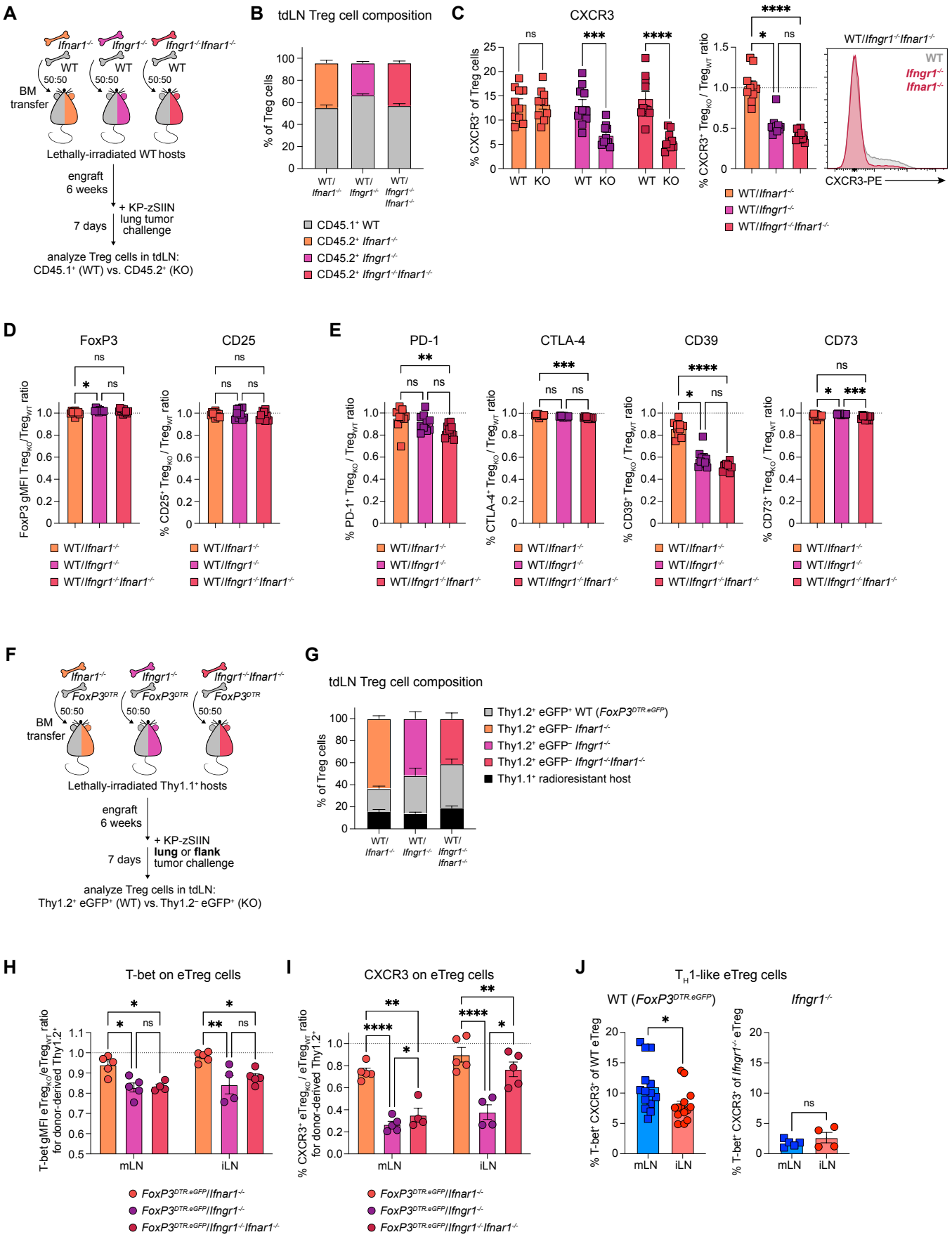

A
Ifnar1-/-
Ifngr1-/-
Ifngr1-/-Ifnar1-/-
WT
50:50
BM transfer
Lethally-irradiated WT hosts
engraft 6 weeks
+ KP-zSIIN lung tumor challenge
7 days
analyze Treg cells in tdLN: CD45.1+ (WT) vs. CD45.2+ (KO)
B
tdLN Treg cell composition
% of Treg cells
CD45.1+ WT
CD45.2+ Ifnar1-/-
CD45.2+ Ifngr1-/-
CD45.2+ Ifngr1-/-Ifnar1-/-
C
CXCR3
% CXCR3+ of Treg cells
% CXCR3+ Treg KO / Treg WT ratio
WT/Ifngr1-/-Ifnar1-/-
CXCR3-PE
D
FoxP3
CD25
FoxP3 gMFI Treg KO/Treg WT ratio
% CD25+ Treg KO / Treg WT ratio
E
PD-1
CTLA-4
CD39
CD73
% PD-1+ Treg KO / Treg WT ratio
% CTLA-4+ Treg KO / Treg WT ratio
% CD39+ Treg KO / Treg WT ratio
% CD73+ Treg KO / Treg WT ratio
F
FoxP3DTR
Lethally-irradiated Thy1.1+ hosts
+ KP-zSIIN lung or flank tumor challenge
analyze Treg cells in tdLN: Thy1.2+ eGFP+ (WT) vs. Thy1.2- eGFP+ (KO)
G
tdLN Treg cell composition
% of Treg cells
Thy1.2+ eGFP+ WT (FoxP3DTR.eGFP)
Thy1.2+ eGFP- Ifnar1-/-
Thy1.2+ eGFP- Ifngr1-/-
Thy1.2+ eGFP- Ifngr1-/-Ifnar1-/-
Thy1.1+ radioresistant host
H
T-bet on eTreg cells
T-bet gMFI eTreg KO/eTreg WT ratio for donor-derived Thy1.2+
mLN
iLN
FoxP3DTR.eGFP/Ifnar1-/-
FoxP3DTR.eGFP/Ifngr1-/-
FoxP3DTR.eGFP/Ifngr1-/-Ifnar1-/-
I
CXCR3 on eTreg cells
% CXCR3+ eTreg KO / eTreg WT ratio for donor-derived Thy1.2+
J
TH1-like eTreg cells
WT (FoxP3DTR.eGFP)
Ifngr1-/-
% T-bet+ CXCR3+ of WT eTreg
% T-bet+ CXCR3+ of Ifngr1-/- eTreg

Figure S5. Related to Main Figure 7: Induction of T_H1-like Treg cells is dependent on IFNγ-sensing.

(A) Experimental design for (B-E).

(B) Treg cell composition in mLN of tumor-bearing WT/*Ifnar1*$^{-/-}$, WT/*Ifngr1*$^{-/-}$ and WT/*Ifngr1*$^{-/-}$ *Ifnar1*$^{-/-}$ BMCs, day 7 post-tumor implantation (n=5 mice/group; two independent experiments).

(C) Representative histogram and CXCR3 expression on WT and IFN receptor-deficient (KO) Treg cells from mLN of tumor-bearing WT/*Ifnar1*$^{-/-}$, WT/*Ifngr1*$^{-/-}$ and WT/*Ifngr1*$^{-/-}$*Ifnar1*$^{-/-}$ BMCs, day 7 post-tumor implantation (n=5 mice/group; two independent experiments).

(D-E) Ratios of IFN receptor-deficient (KO) and WT Treg cell expression of (D) FoxP3, CD25, (E) PD-1, CTLA-4, CD39 and CD73 in mLN of tumor-bearing WT/*Ifnar1*$^{-/-}$, WT/*Ifngr1*$^{-/-}$ and WT/*Ifngr1*$^{-/-}$*Ifnar1*$^{-/-}$ BMCs, day 7 post-tumor implantation (n=5 mice/group; two independent experiments).

(F) Experimental design for (G-J).

(G) Treg cell composition in tdLN of tumor-bearing *FoxP3*^{DTR}/*Ifnar1*$^{-/-}$, *FoxP3*^{DTR}/*Ifngr1*$^{-/-}$ and *FoxP3*^{DTR}/*Ifngr1*$^{-/-}$*Ifnar1*$^{-/-}$ BMCs, day 7 post-tumor implantation (n=4-5 mice/group; two independent experiments).

(H-I) Ratios of Thy1.2^{+} donor-derived IFN receptor-deficient (KO) and WT eTreg cell expression of (H) T-bet and (I) CXCR3 in tdLN of tumor-bearing *FoxP3*^{DTR}/*Ifnar1*$^{-/-}$, *FoxP3*^{DTR}/*Ifngr1*$^{-/-}$ and *FoxP3*^{DTR}/*Ifngr1*$^{-/-}$*Ifnar1*$^{-/-}$ BMCs, day 7 post-tumor implantation (n=2-3 mice/group; two independent experiments).

(J) T-bet^{+} CXCR3^{+} double-positive percentage of eTreg cells for (*left*) Thy1.2^{+} eGFP^{+} donor-derived WT fraction from tumor-bearing *FoxP3*^{DTR}/*Ifnar1*$^{-/-}$, *FoxP3*^{DTR}/*Ifngr1*$^{-/-}$ and *FoxP3*^{DTR}/*Ifngr1*$^{-/-}$*Ifnar1*$^{-/-}$ BMCs and (*right*) Thy1.2^{+} eGFP^{-} fraction from tumor-bearing *FoxP3*^{DTR}/*Ifngr1*$^{-/-}$ BMCs, from tdLN at day 7 post-tumor implantation (n=2-7 mice/group; two independent experiments).

*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns=not significant; two-way ANOVA (C, *left*; G-H), KW (C, *middle*; D), MWU (I). Data shown as mean ± SEM.

Figure S6

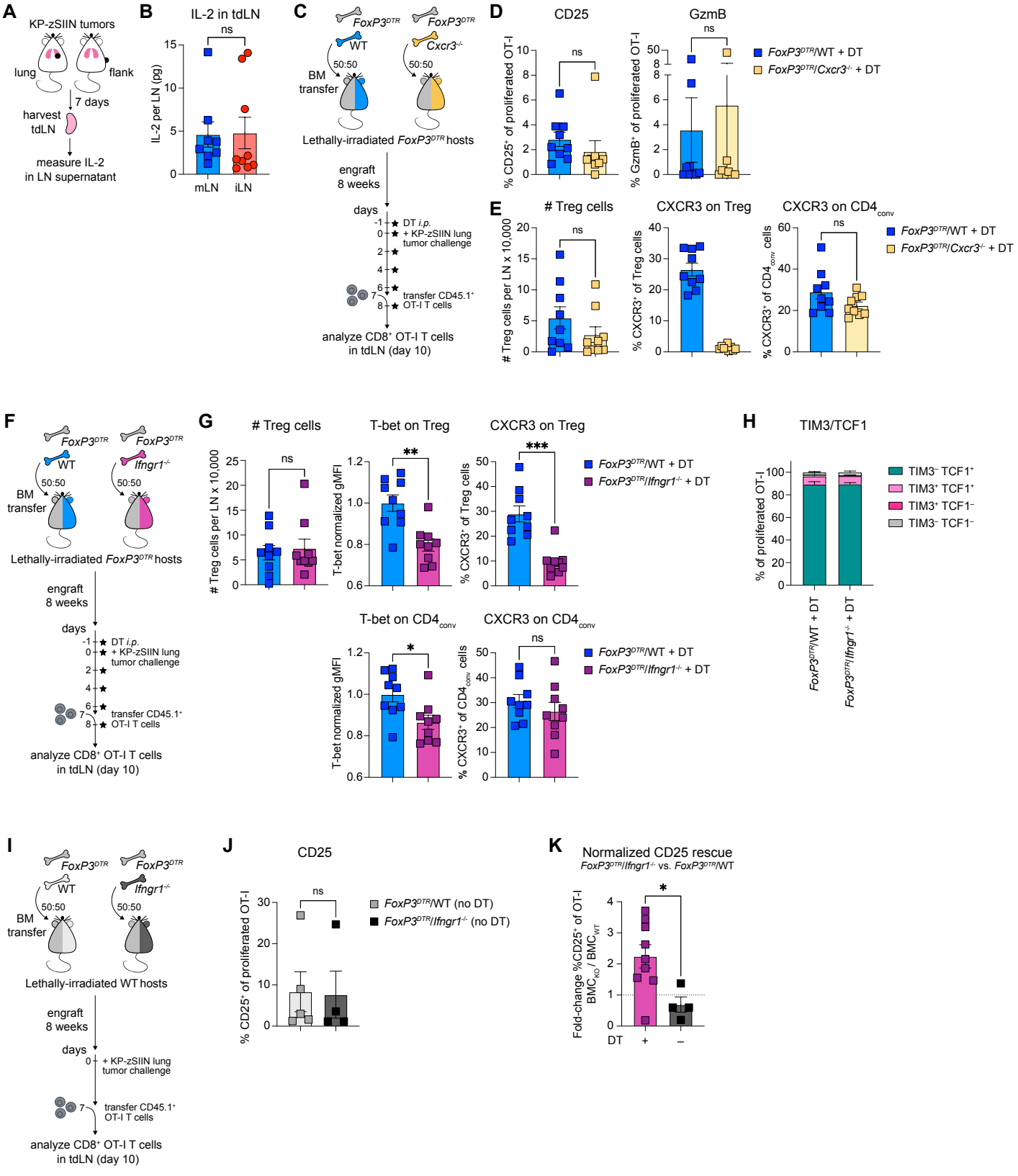

A
KP-zSIIN tumors
lung
flank
7 days
harvest tdLN
measure IL-2 in LN supernatant
B
IL-2 in tdLN
ns
IL-2 per LN (pg)
mLN
iLN
C
FoxP3DTR
WT
Cxcr3-/-
50:50
BM transfer
Lethally-irradiated FoxP3DTR hosts
engraft 8 weeks
days
DT i.p.
+ KP-zSIIN lung tumor challenge
transfer CD45.1+ OT-I T cells
analyze CD8+ OT-I T cells in tdLN (day 10)
D
CD25
GzmB
% CD25+ of proliferated OT-I
% GzmB+ of proliferated OT-I
FoxP3DTR/WT + DT
FoxP3DTR/Cxcr3-/- + DT
E
Treg cells
CXCR3 on Treg
CXCR3 on CD4conv
Treg cells per LN x 10,000
% CXCR3+ of Treg cells
% CXCR3+ of CD4conv cells
F
Ifngr1-/-
Lethally-irradiated FoxP3DTR hosts
G
T-bet on Treg
T-bet on CD4conv
T-bet normalized gMFI
FoxP3DTR/Ifngr1-/- + DT
H
TIM3/TCF1
% of proliferated OT-I
TIM3− TCF1+
TIM3+ TCF1+
TIM3+ TCF1−
TIM3− TCF1−
FoxP3DTR/WT + DT
FoxP3DTR/Ifngr1-/- + DT
I
Lethally-irradiated WT hosts
J
CD25
FoxP3DTR/WT (no DT)
FoxP3DTR/Ifngr1-/- (no DT)
K
Normalized CD25 rescue
FoxP3DTR/Ifngr1-/- vs. FoxP3DTR/WT
Fold-change %CD25+ of OT-I BMKO / BMWT
DT

Figure S6. Related to Main Figure 7: IFNγ-sensing in Treg cells drives dysfunctional CD8$^+$ T cell responses against lung cancer.

(A) Experimental design for (B).

(B) IL-2 quantification in tdLN, day 7 post-tumor implantation (n=2-3 mice/group; three independent experiments).

(C) Experimental design for (D-E).

(D-E) Expression of (D) CD25 and GzmB on adoptively-transferred proliferated OT-I T cells and (E) numbers of Treg cells and CXCR3 expression on Treg cells and $CD4_{conv}$ cells from mLN of DT-treated tumor-bearing *FoxP3^{DTR}*/WT or *FoxP3^{DTR}*/*Cxcr3$^{-/-}$* BMCs, day 10 post-tumor implantation (n=3 mice/group; three independent experiments).

(F) Experimental design for (G-H).

(G) Numbers of Treg cells and expression of T-bet and CXCR3 on Treg cells and $CD4_{conv}$ cells from mLN of DT-treated tumor-bearing *FoxP3^{DTR}*/WT or *FoxP3^{DTR}*/*Ifngr1$^{-/-}$* BMCs, day 10 post-tumor implantation (n=3 mice/group; three independent experiments).

(H) TCF1 and TIM-3 expression on adoptively-transferred proliferated OT-I T cells from mLN of DT-treated tumor-bearing *FoxP3^{DTR}*/WT or *FoxP3^{DTR}*/*Ifngr1$^{-/-}$* BMCs, day 10 post-tumor implantation (n=3 mice/group; three independent experiments).

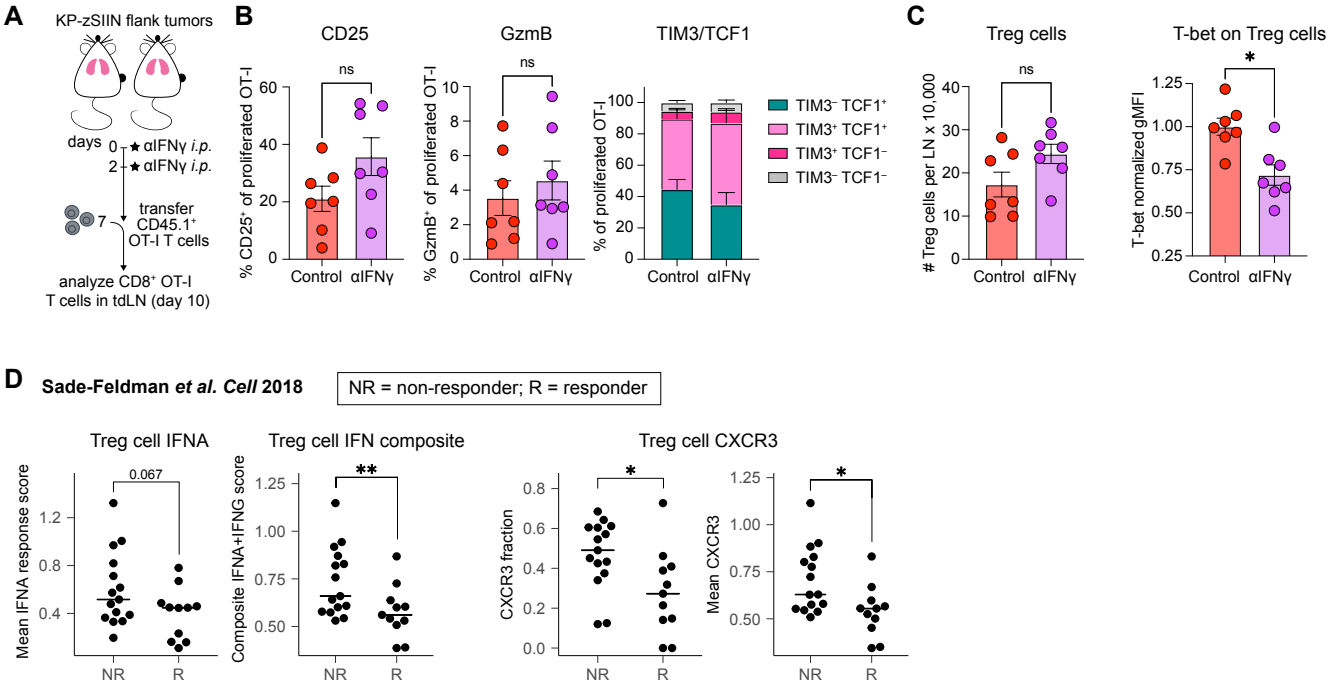
(I) Experimental design for (J).

(J) CD25 expression on adoptively-transferred proliferated OT-I T cells from mLN of untreated tumor-bearing *FoxP3^{DTR}*/WT or *FoxP3^{DTR}*/*Ifngr1$^{-/-}$* BMCs, day 10 post-tumor implantation (n=2-3 mice/group; two independent experiments).

(K) Normalized CD25 expression on adoptively-transferred proliferated OT-I T cells (values from untreated *FoxP3^{DTR}*/WT BMCs were divided by values from untreated *FoxP3^{DTR}*/*Ifngr1$^{-/-}$* BMCs; normalization was performed separately for each independent replicate for the DT-treated condition in Fig. 7I and for the untreated control experiment in Figure S6J), (n=2-3 mice/group; two independent experiments).

*p<0.05, **p<0.01, ***p<0.001, ns=not significant; MWU (B,D-E,G,J-K). Data shown as mean ± SEM.

Figure S7


A
KP-zSIIN flank tumors
days
0 ★ αIFNγ i.p.
2 ★ αIFNγ i.p.
7 transfer CD45.1+ OT-I T cells
analyze CD8+ OT-I T cells in tdLN (day 10)
B
CD25
% CD25+ of proliferated OT-I
ns
Control αIFNγ
GzmB
% GzmB+ of proliferated OT-I
ns
Control αIFNγ
TIM3/TCF1
% of proliferated OT-I
TIM3− TCF1+
TIM3+ TCF1+
TIM3+ TCF1−
TIM3− TCF1−
Control αIFNγ
C
Treg cells
Treg cells per LN x 10,000
ns
Control αIFNγ
T-bet on Treg cells
T-bet normalized gMFI
*
Control αIFNγ

D
Sade-Feldman et al. Cell 2018
NR = non-responder; R = responder
Treg cell IFNA
Mean IFNA response score
0.067
NR R
Treg cell IFN composite
Composite IFNA+IFNG score
**
NR R
Treg cell CXCR3
CXCR3 fraction
*
NR R
Mean CXCR3
*
NR R

Figure S7. Related to Main Figure 7: IFNγ blockade has no effect on $CD8^+$ T cell responses in the iLN and T_H1-like Treg cells correlate with ICB resistance in patients with melanoma.
(A) Experimental design for (B-C).
(B-C) Expression of (B) CD25, GzmB, TIM3 and TCF1 on adoptively-transferred proliferated OT-I T cells and (C) numbers and T-bet expression for Treg cells from iLN of control and αIFNγ-treated tumor-bearing mice, day 10 post-tumor implantation (n=3-4 mice/group; two independent experiments).
(D) Reanalysis of human melanoma T cell scRNA-seq data from Sade-Feldman *et al.* (2018); IFNA and composite IFNA+IFNG response hallmark signature scores and CXCR3 expression on intratumoral Treg cells from melanoma patients, including ICB-responders (R) and ICB-non-responders (NR).
*p<0.05, **p<0.01, ns=not significant; MWU (B-D). Data shown as mean ± SEM, except in (D) where the median is shown.