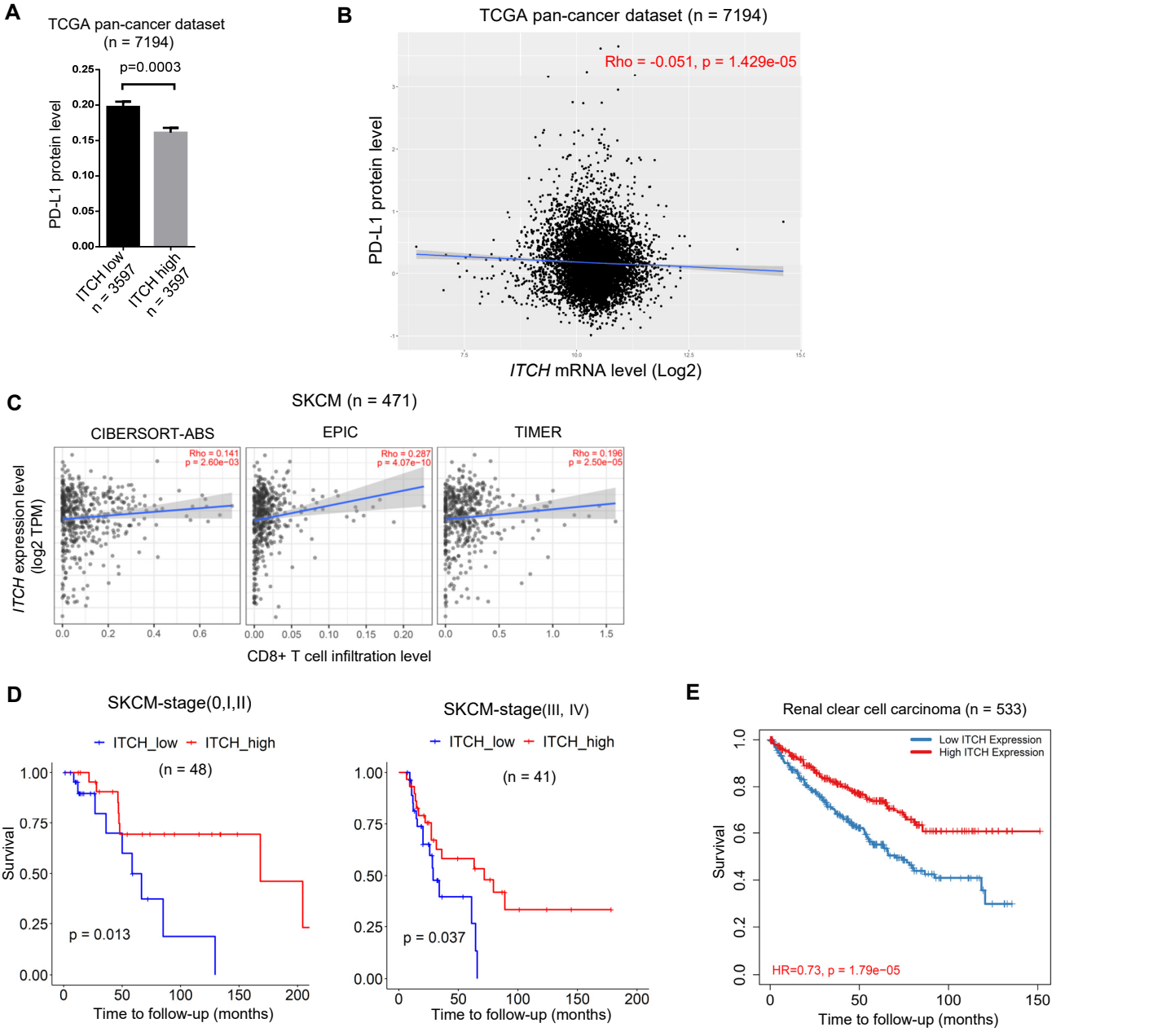


Supplementary Figure S1. Correlations between tumoral *ITCH* RNA expression and PD-L1 protein levels, CD8⁺ T-cell infiltration, or patient survival.

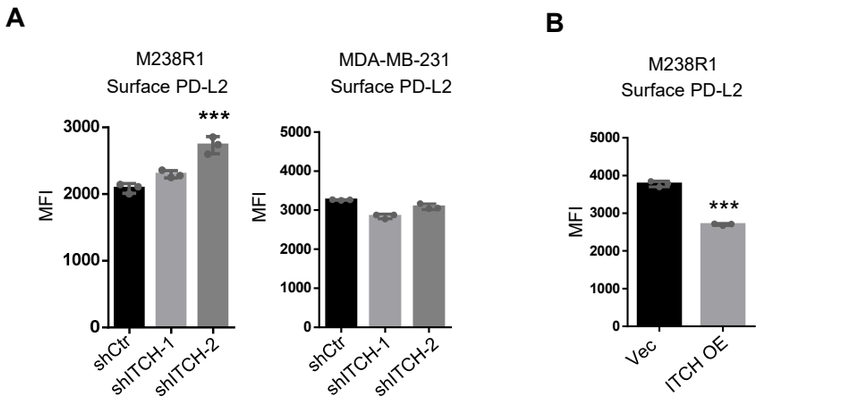
A, PD-L1 protein levels compared by splitting 7,194 patient-derived tumors with matched RNA-seq data and PD-L1 protein levels (measured by reverse-phase protein array from 32 TCGA cancer types) into top versus bottom 50% of *ITCH* RNA expression levels. P value as indicated, Student *t* test.

B, Spearman's correlation score (Rho) between intra-tumoral *ITCH* RNA levels and PD-L1 protein levels in 7,194 tumors of 32 TCGA cancer types. Rho = -0.051, negative correlation. P = 1.429e-05.

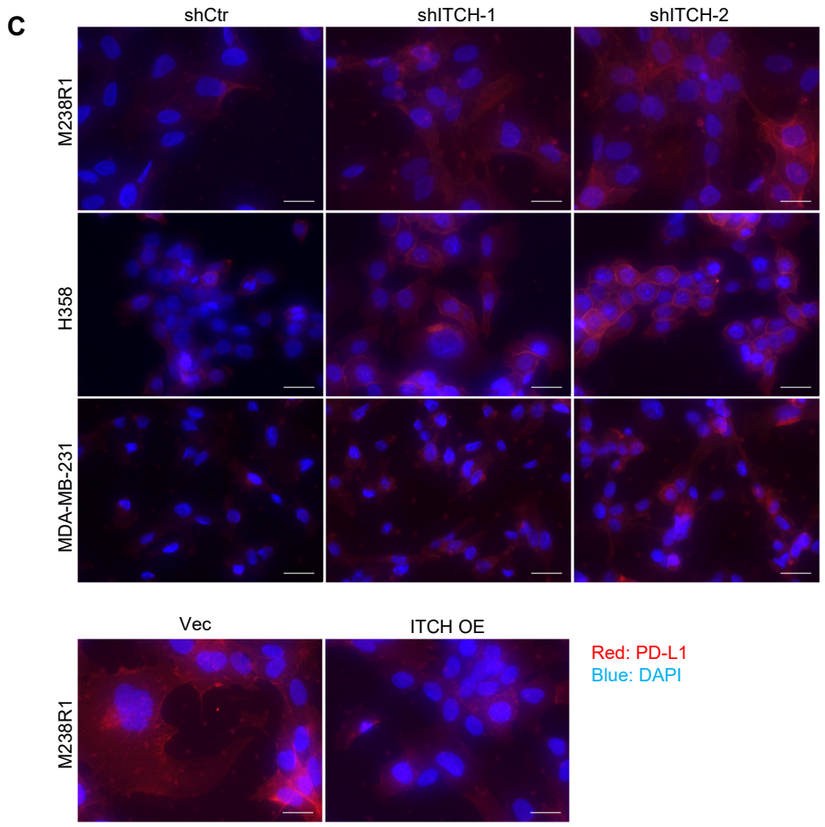
C, Spearman's correlation score (Rho) between intra-tumoral *ITCH* RNA levels and CD8+ T-cell infiltration levels in TCGA-SKCM dataset (n = 471) calculated by three different algorithms (CIBERSORT-ABS, EPIC and TIMER). Rho > 0, positive correlation. P values, as indicated.

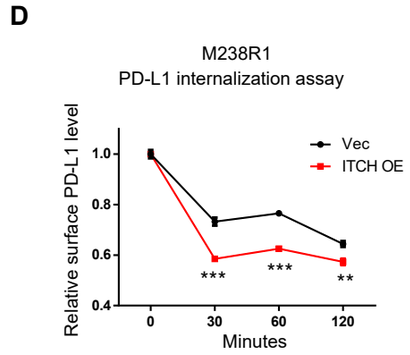
D, Kaplan-Meier survival curve of stage-matched patients from TCGA-SKCM dataset with high (top 10%) versus low (bottom 10%) intra-tumoral *ITCH* expression (early stage: stage 0, I, II combined, n = 231; left panel) or high (top 15%) versus low (bottom 15%) intra-tumoral *ITCH* expression (late stage: stage III, IV combined, n = 193; right panel). Time of diagnosis as starting time for follow-up. P values, log-rank test.

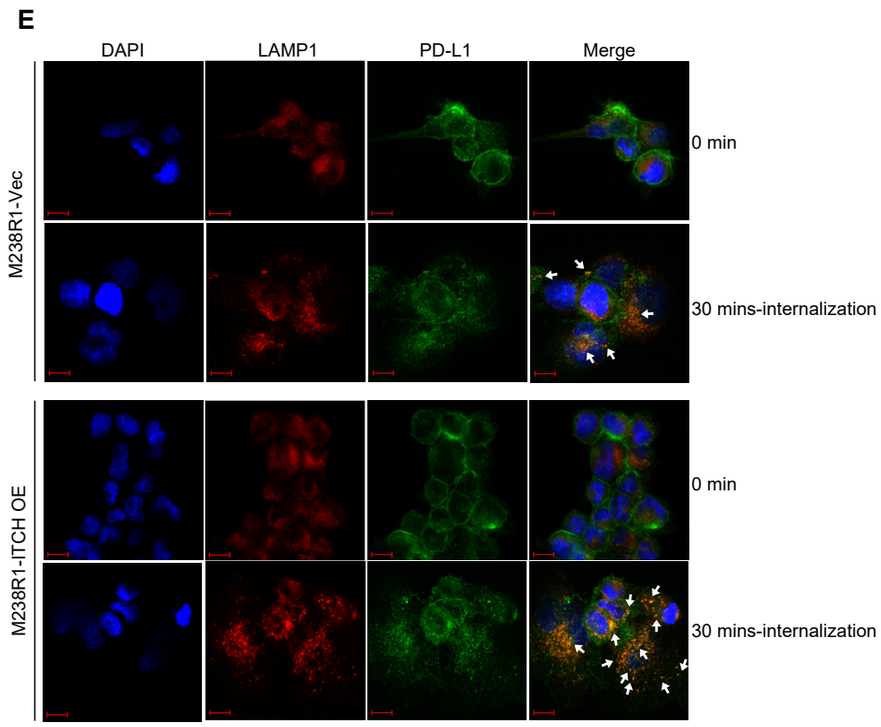
E, Kaplan-Meier survival curve of patients from TCGA renal clear cell carcinoma dataset with high (top 50%) versus low (bottom 50%) intra-tumoral *ITCH* expression. Time of diagnosis as starting time for follow-up. P values, log-rank test. HR, hazard ratio.

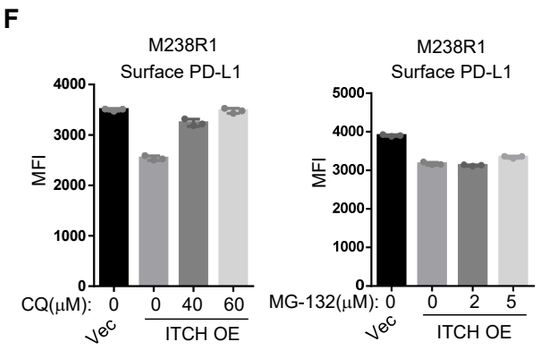

A
M238R1
Surface PD-L2
MDA-MB-231
Surface PD-L2
MFI
***
shCtr
shITCH-1
shITCH-2

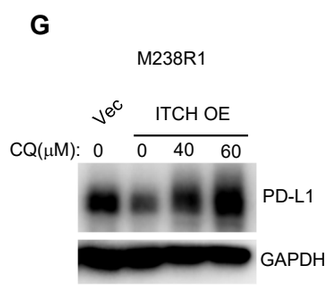
B
M238R1
Surface PD-L2
MFI
***
Vec
ITCH OE

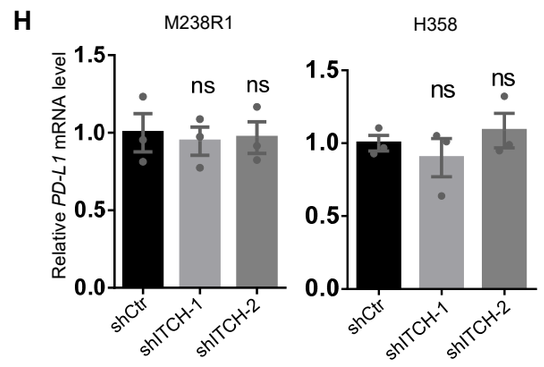

C
shCtr
shITCH-1
shITCH-2
M238R1
H358
MDA-MB-231
Vec
ITCH OE
M238R1
Red: PD-L1
Blue: DAPI

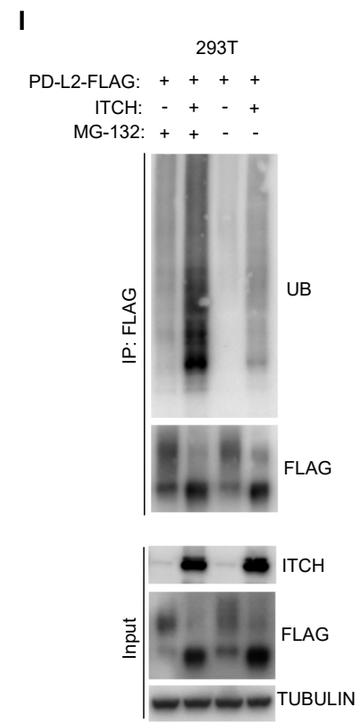

D
M238R1
PD-L1 internalization assay
Relative surface PD-L1 level
Vec
ITCH OE
***
***
**
Minutes

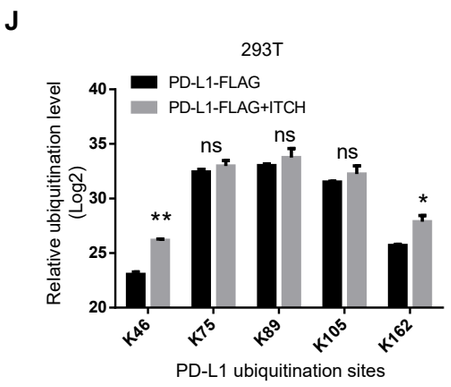

E
DAPI
LAMP1
PD-L1
Merge
M238R1-Vec
0 min
30 mins-internalization
M238R1-ITCH OE
0 min
30 mins-internalization

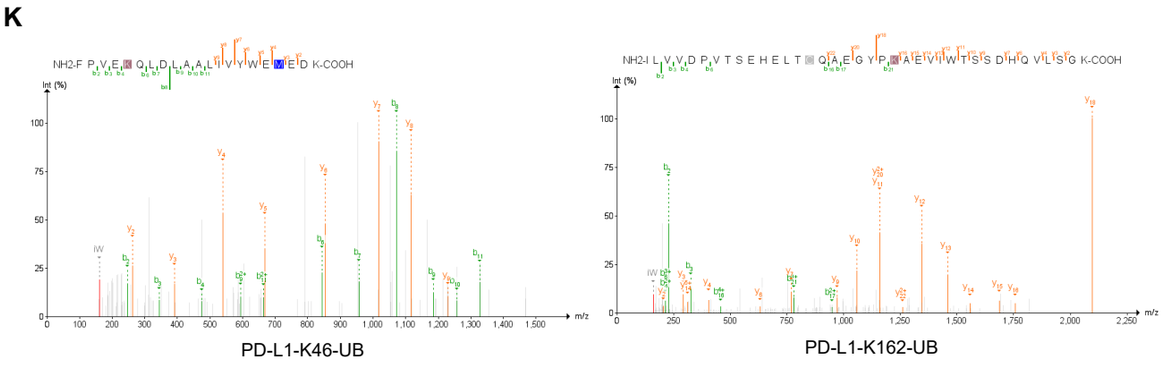

F
M238R1
Surface PD-L1
MFI
CQ(μM): 0 0 40 60
Vec
ITCH OE
M238R1
Surface PD-L1
MG-132(μM): 0 0 2 5
Vec
ITCH OE

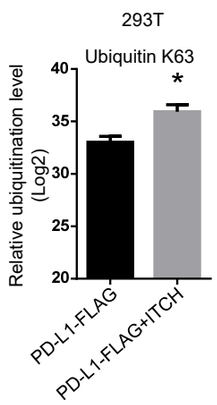

G
M238R1
Vec
ITCH OE
CQ(μM): 0 0 40 60
PD-L1
GAPDH

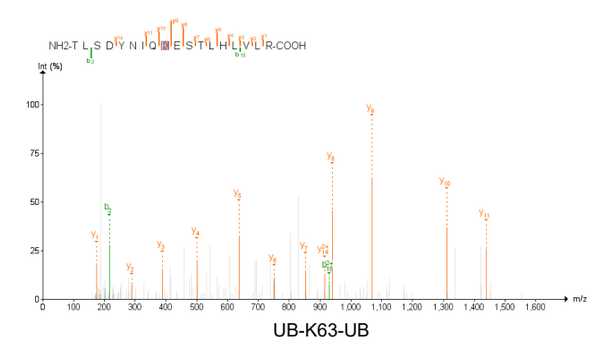

H
M238R1
H358
Relative PD-L1 mRNA level
ns
ns
ns
ns
shCtr
shITCH-1
shITCH-2

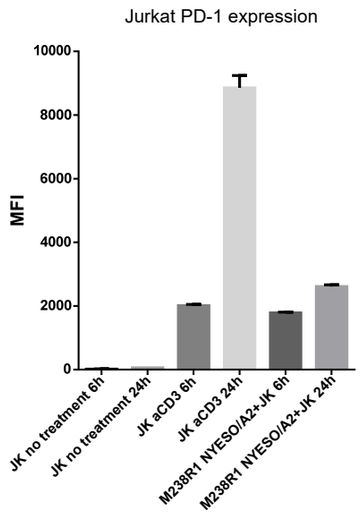

I
293T
PD-L2-FLAG: + + + +
ITCH: - + - +
MG-132: + + - -
IP: FLAG
UB
FLAG
Input
ITCH
FLAG
TUBULIN


J
293T
PD-L1-FLAG
PD-L1-FLAG+ITCH
Relative ubiquitination level (Log2)
**
ns
ns
ns
*
K46
K75
K89
K105
K162
PD-L1 ubiquitination sites

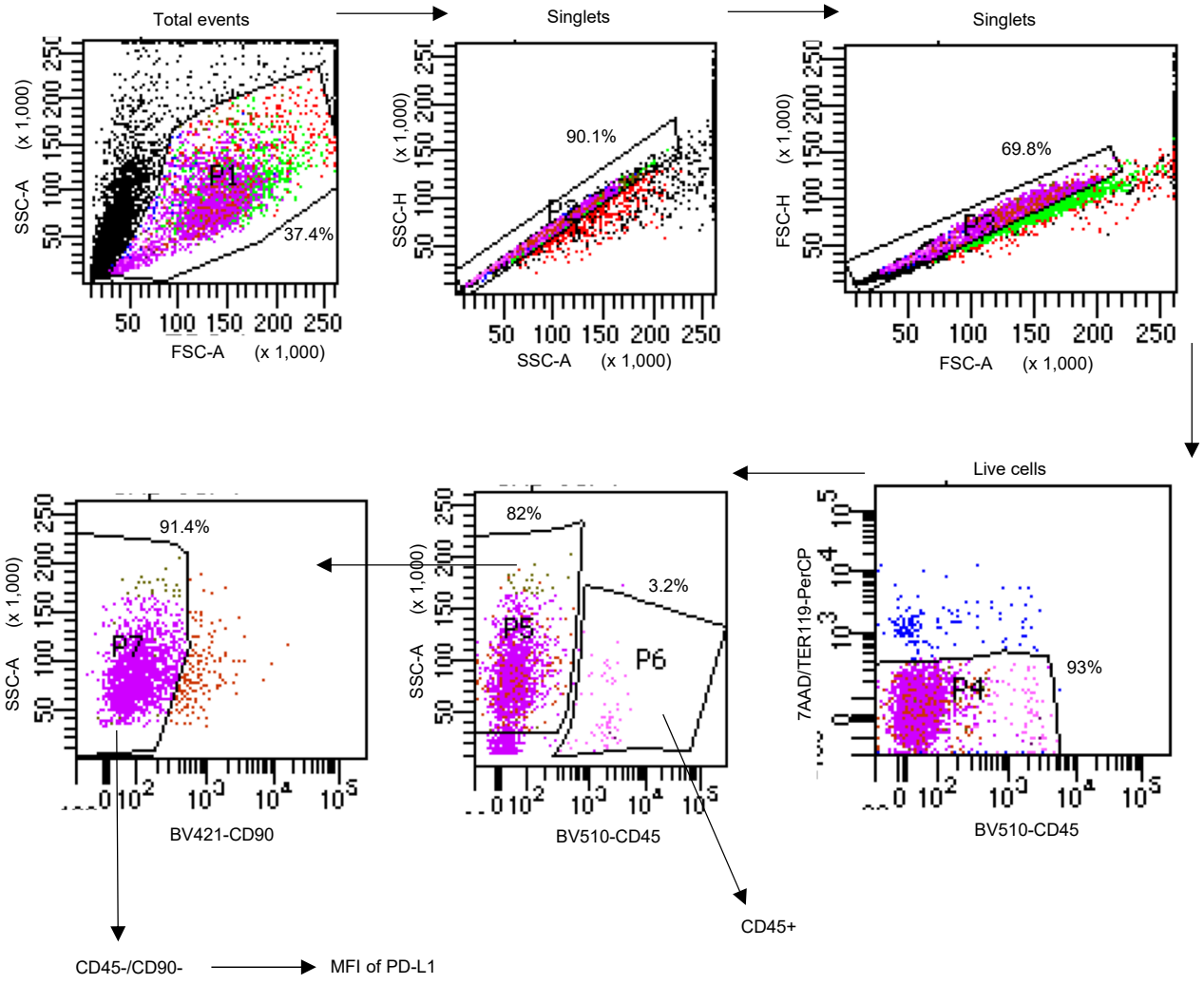

K
PD-L1-K46-UB
PD-L1-K162-UB


L
293T
Ubiquitin K63
Relative ubiquitination level (Log2)
*
PD-L1-FLAG
PD-L1-FLAG+ITCH


M
UB-K63-UB

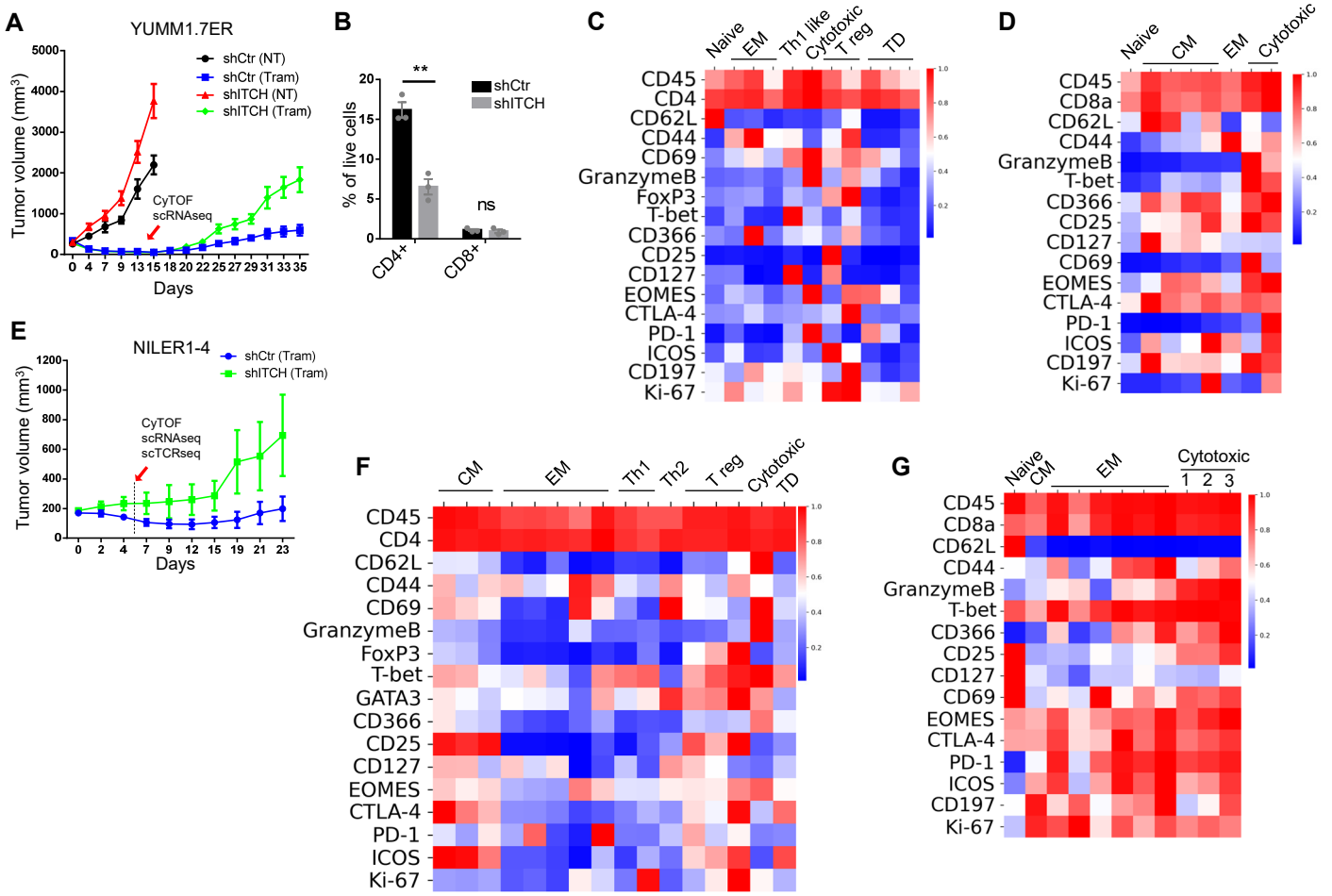

N
Jurkat PD-1 expression
MFI
JK no treatment 6h
JK no treatment 24h
JK aCD3 6h
JK aCD3 24h
M238R1 NYESO/A2+JK 6h
M238R1 NYESO/A2+JK 24h

Supplementary Figure S2. Physical and functional interactions between ITCH and PDL1/L2.
A, Cell-surface levels of PD-L2 in M238R1 (left) and MDA-MB-231 (right) cells stably expressing control shRNA (shCtr) or ITCH-targeting shRNAs (shITCH-1, shITCH-2), as measured by cell-surface staining and FACS analysis (PD-L2 is not detectable in H358 cells). MFI, mean fluorescence intensity. Mean ± SEMs (n = 3).
B, Cell-surface levels of PD-L2 in M238 R1 cells stably expressing empty vector (Vec) or over-expressing (OE) ITCH, as measured by cell-surface staining and FACS analysis. Mean ± SEMs (n = 3).
C, Cell-surface levels of PD-L1 in M238R1, H358, and MDA-MB-231 stably expressing control shRNA (shCtr) or ITCH-targeting shRNAs (shITCH-1, shITCH-2) and M238R1 stably expressing empty vector (Vec) or OE ITCH, as measured by immunofluorescence staining. Scale bar = 20 μm.
D, PD-L1 internalization in M238 R1 cells stably expressing empty vector (Vec) or OE ITCH, as measured by FACS analysis. Mean ± SEMs (n = 3).
E, Confocal microscopic imaging of M238 R1 cells stably expressing empty vector (Vec) or OE ITCH before (0 min) and 30 minutes after initiating surface PD-L1 internalization. Small white arrows, co-localization of PD-L1 and the lysosome marker, LAMP1. Scale bar = 10 μm.
F, M238 R1 cells stably expressing Vec or OE ITCH were treated with indicated concentrations of chloroquine (CQ) (left) or MG-132 (right) for 16 hours, followed by measurement of cell-surface levels of PD-L1 by cell-surface staining and FACS analysis. Mean ± SEMs (n = 3).
G, M238 R1 cells stably expressing Vec or ITCH were treated with indicated concentrations of chloroquine (CQ) for 16 hours, followed by measurement of total PD-L1 level by Western blots (WBs).
H, Real-time PCR of *PD-L1* mRNA levels in M238 R1 or H358 stably expressing control and ITCH-shRNAs. Mean ± SEMs (n = 3).
I, HEK 293T cells expressing PD-L2-FLAG, with or without ITCH co-transfection, were pre-treated with or without MG-132 (20 μM) for 4 hours followed by anti-FLAG IP and detection of UB by WBs.
J, HEK 293T cells expressing PD-L1-FLAG, with or without ITCH co-transfection, were subjected to anti-FLAG immunoprecipitation and mass spectrometry analysis. Quantification of the indicated ubiquitination sites on PD-L1 is shown (log2).
K, Tandem mass spectra of the ubiquitinated PD-L1-K46 (left) or PD-L1-K162 (right) peptides**.**
L, As in **J**, except quantification of K63 ubiquitination on ubiquitin.
M, Tandem mass spectrum of the ubiquitinated ubiquitin-K63 peptide.
N, Jurkat cell-surface PD-1 expression measured by FACS analysis after indicated treatments or co-culture with M238R1-NYESO/A2 cells.
P value, Student's *t* test, ns, not significant. * $p < 0.05$. ** $p < 0.01$, *** $p < 0.001$.



Supplementary Figure S3. Gating strategy for FACS analysis.
An example shown following tumor dissociation, with percentages at each step of gating the parental populations.

Supplementary Figure S4. Impact of *Itch* knockdown on *in vitro* growth of murine melanoma cell lines.
A, Growth curves of cultured YUMM1.7ER and NILER1-4 cell lines stably expressing control or ITCH-shRNAs (left and middle). Mean ± SEMs (n = 3). WBs of YUMMER1.7 cell lines stably expressing control or ITCH-shRNAs (right).
B, Clonogenic growth (10 days) of YUMM1.7ER and NILER1-4 cell lines stably expressing control and ITCH-shRNAs off or on trametinib (Tram) treatment at indicated concentrations.



Supplementary Figure S5. Effects of ITCH or PD-L1 expression on immune infiltration or immune cell gene expression.

A, Tumor growth curves of shCONTROL (shCtr) and ITCH-knockdown (shITCH-3) YUMM1.7ER melanoma under no treatment or on trametinib (Tram, 1 mg/kg/d) treatment in C57BL/6 mice. shCONTROL and ITCH-knockdown tumors (n = 3) after 14 days of trametinib treatment were dissociated into single cells followed by CyTOF and scRNA-seq analysis. Mean ± SEMs (n = 8 for NT groups and n = 10 for Tram groups).

B, Fraction of $CD4^+$ or $CD8^+$ cells in total live cells from shCONTROL and ITCH-knockdown YUMM1.7ER tumors. Mean ± SEMs (n = 3). P value, Student's *t* test, ** $p < 0.01$, ns: not significant.

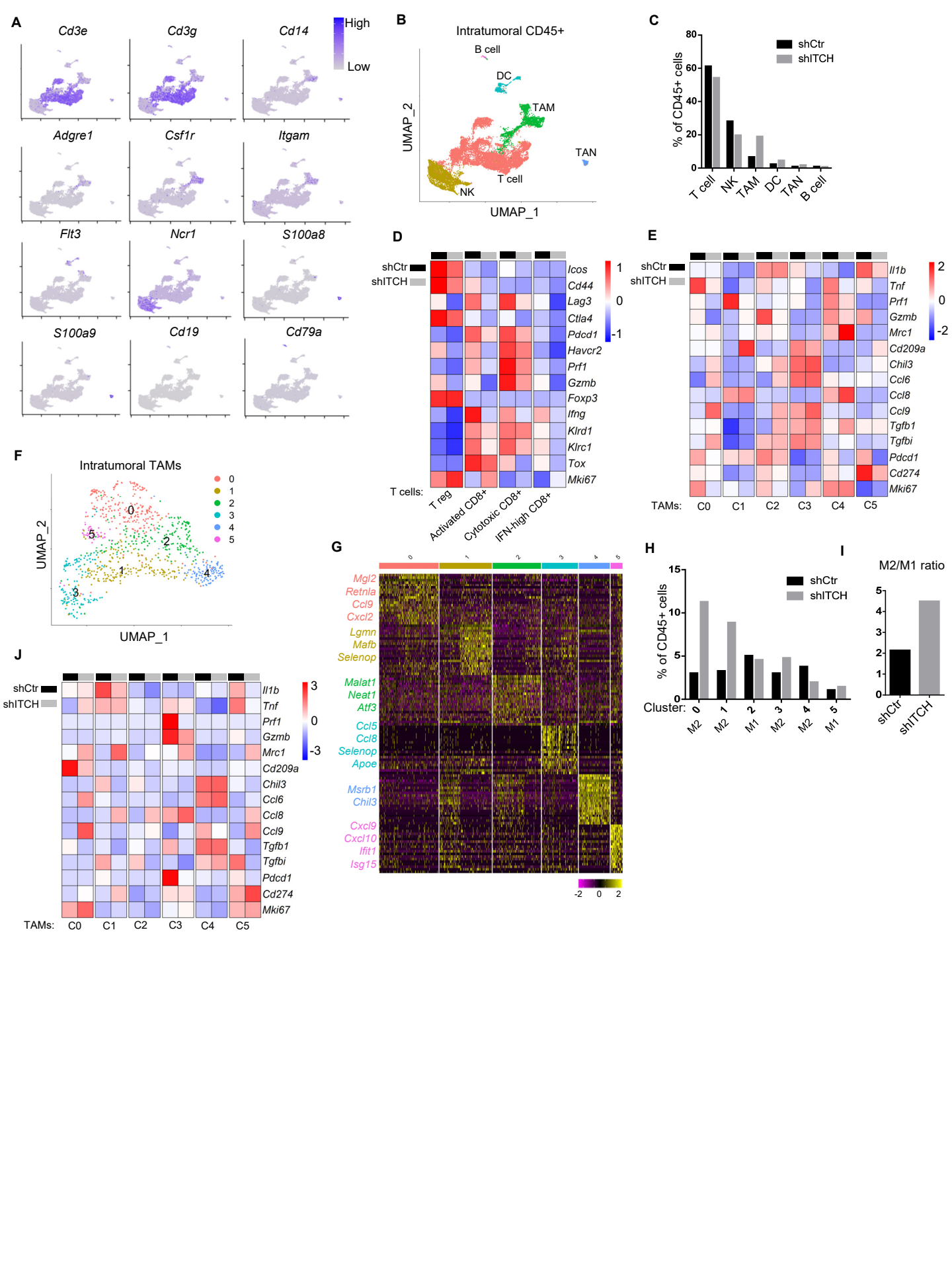
C, Heatmap showing scaled mean expression levels of indicated markers in different cell clusters of $CD4^+$ T cells (YUMM1.7ER).

D, Heatmap showing scaled mean expression levels of indicated markers in different cell clusters of $CD8^+$ T cells (YUMM1.7ER).

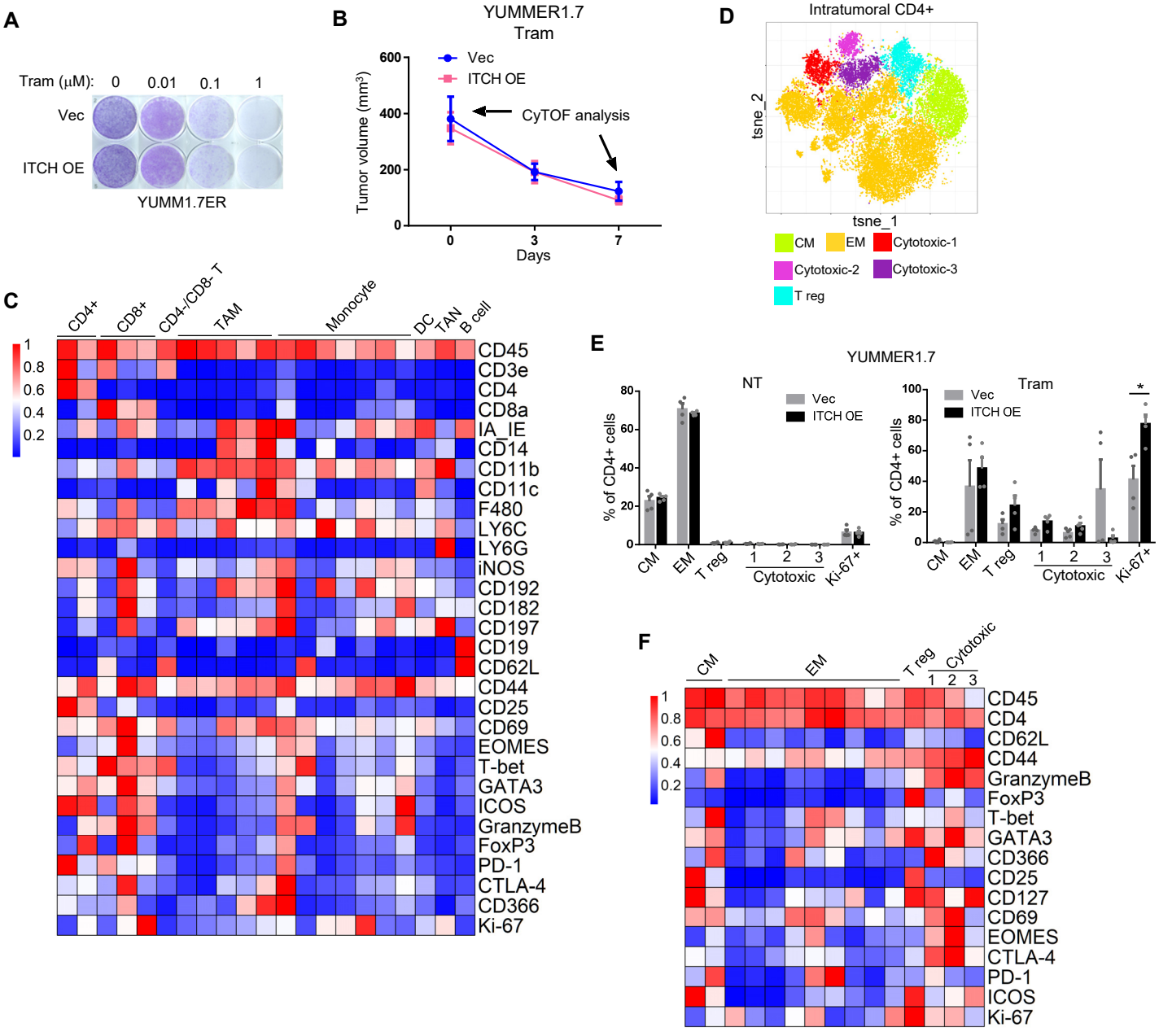
E, Tumor growth curves of shCONTROL (shCtr) and ITCH-knockdown (shITCH mix) NILER1-4 melanoma under trametinib (Tram, 3 mg/kg/d) treatment in C57BL/6 mice. shCONTROL and ITCH-knockdown tumors (n = 4) after 5 days of trametinib treatment were dissociated into single cells followed by CyTOF and scRNA-seq analysis. Mean ± SEMs (n = 8 for NT groups and n = 10 for Tram groups).

F, Heatmap showing scaled mean expression levels of indicated markers in different cell clusters of $CD4^+$ T cells (NILER1-4).

G, Heatmap showing scaled mean expression levels of indicated markers in different cell clusters of $CD8^+$ T cells (NILER1-4).


A
Cd3e
Cd3g
Cd14
High
Low
Adgre1
Csf1r
Itgam
Flt3
Ncr1
S100a8
S100a9
Cd19
Cd79a
B
Intratumoral CD45+
B cell
DC
TAM
TAN
T cell
NK
UMAP_2
UMAP_1
C
% of CD45+ cells
shCtr
shITCH
T cell
NK
TAM
DC
TAN
B cell
D
shCtr
shITCH
Icos
Cd44
Lag3
Ctla4
Pdcd1
Havcr2
Prf1
Gzmb
Foxp3
Ifng
Klrd1
Klrc1
Tox
Mki67
T cells:
T reg
Activated CD8+
Cytotoxic CD8+
IFN-high CD8+
E
Il1b
Tnf
Prf1
Gzmb
Mrc1
Cd209a
Chil3
Ccl6
Ccl8
Ccl9
Tgfb1
Tgfbi
Pdcd1
Cd274
Mki67
TAMs:
C0
C1
C2
C3
C4
C5
F
Intratumoral TAMs
UMAP_2
UMAP_1
G
Mgl2
Retnla
Ccl9
Cxcl2
Lgmn
Mafb
Selenop
Malat1
Neat1
Atf3
Ccl5
Ccl8
Selenop
Apoe
Msrb1
Chil3
Cxcl9
Cxcl10
Ifit1
Isg15
H
% of CD45+ cells
shCtr
shITCH
Cluster:
M2
M2
M1
M2
M2
M1
I
M2/M1 ratio
shCtr
shITCH
J
shCtr
shITCH
Il1b
Tnf
Prf1
Gzmb
Mrc1
Cd209a
Chil3
Ccl6
Ccl8
Ccl9
Tgfb1
Tgfbi
Pdcd1
Cd274
Mki67
TAMs:
C0
C1
C2
C3
C4
C5

Supplementary Figure S6. Impact of tumor cell-intrinsic ITCH deficiency on immune cells and gene expression.
A, UMAP of intra-tumoral $CD45^+$ single cells showing expression levels of indicated cell lineage markers (shCONTROL and ITCH-knockdown NILER1-4 tumors, both on trametinib treatment).
B, UMAP of intra-tumoral $CD45^+$ single cells (in **A**) with indicated cell types denoted by distinct colors. NK (Natural killer cells), TAM (Tumor associated macrophages), DC (Dendritic cells), TAN (Tumor associated neutrophils).
C, Fractions of indicated cell types in total $CD45^+$ cells from NILER1-4 shCONTROL and ITCH-knockdown tumors, both on trametinib treatment.
D, Heatmap showing scaled mean expression levels of indicated genes in indicated T cell clusters from shCONTROL and ITCH-knockdown NILER1-4 tumors, both on trametinib treatment.
E, Heatmap showing scaled mean expression levels of indicated genes in indicated TAM cell clusters from shCONTROL and ITCH-knockdown NILER1-4 tumors, both on trametinib treatment.
F, UMAP of intratumoral TAMs (n = 886) analyzed by scRNA-seq (shCONTROL and ITCH-knockdown YUMM1.7ER tumors, both on trametinib treatment). Different cell clusters denoted by distinct colors.
G, Heatmap showing expression levels of differentially expressed genes (rows) among different TAM subpopulations (columns) in **F**. Representative genes of each cluster are highlighted.
H, Fractions of each TAM subpopulation in total $CD45^+$ cells from shCONTROL and ITCH-knockdown YUMM1.7ER tumors, both on trametinib treatment.
I, The ratio of M2-like TAMs to M1-like TAMs in shCONTROL and ITCH-knockdown YUMM1.7ER tumors, both on trametinib treatment.
J, Heatmap showing scaled mean expression levels of indicated genes in indicated TAM cell clusters from shCONTROL and ITCH-knockdown YUMM1.7ER tumors, both on trametinib treatment.



Supplementary Figure S7. CyTOF analysis of CD45⁺ and CD4⁺ populations in YUMM1.7ER control and ITCH over-expression tumors.

A, Clonogenic growth (7 days) of YUMM1.7ER cell lines stably expressing empty vector (Vec) and over-expressing (OE) ITCH, off or on trametinib (Tram) treatment at indicated concentrations.

B, Tumor growth curves of Vec and ITCH OE YUMM1.7ER tumors on trametinib (Tram, 0.45 mg/kg/d) treatment in C57BL/6 mice. Vec and ITCH OE tumors (n = 4 for each condition) before and after 7 days of trametinib treatment were dissociated into single cells and analyzed by CyTOF. Mean ± SEMs (n = 11-12).

C, Heatmap showing scaled mean expression levels of indicated protein markers in different cell clusters of CD45⁺ cells (YUMM1.7ER, NT and Tram).

D, t-SNE map of intra-tumoral CD4⁺ cells from Vec and ITCH-OE YUMM1.7ER tumors on NT and trametinib treatment, as analyzed by CyTOF. Inferred cell types denoted by distinct colors.

E, Fractions of indicated cell types in CD4⁺ T cells from Vec and ITCH-OE YUMM1.7ER tumors on NT or trametinib treatment. Mean ± SEMs (n = 4). P value, Student's *t* test. * $p < 0.05$.

F, Heatmap showing scaled mean expression levels of indicated protein markers in different cell clusters of CD4⁺ T cells (YUMM1.7ER, NT and Tram).