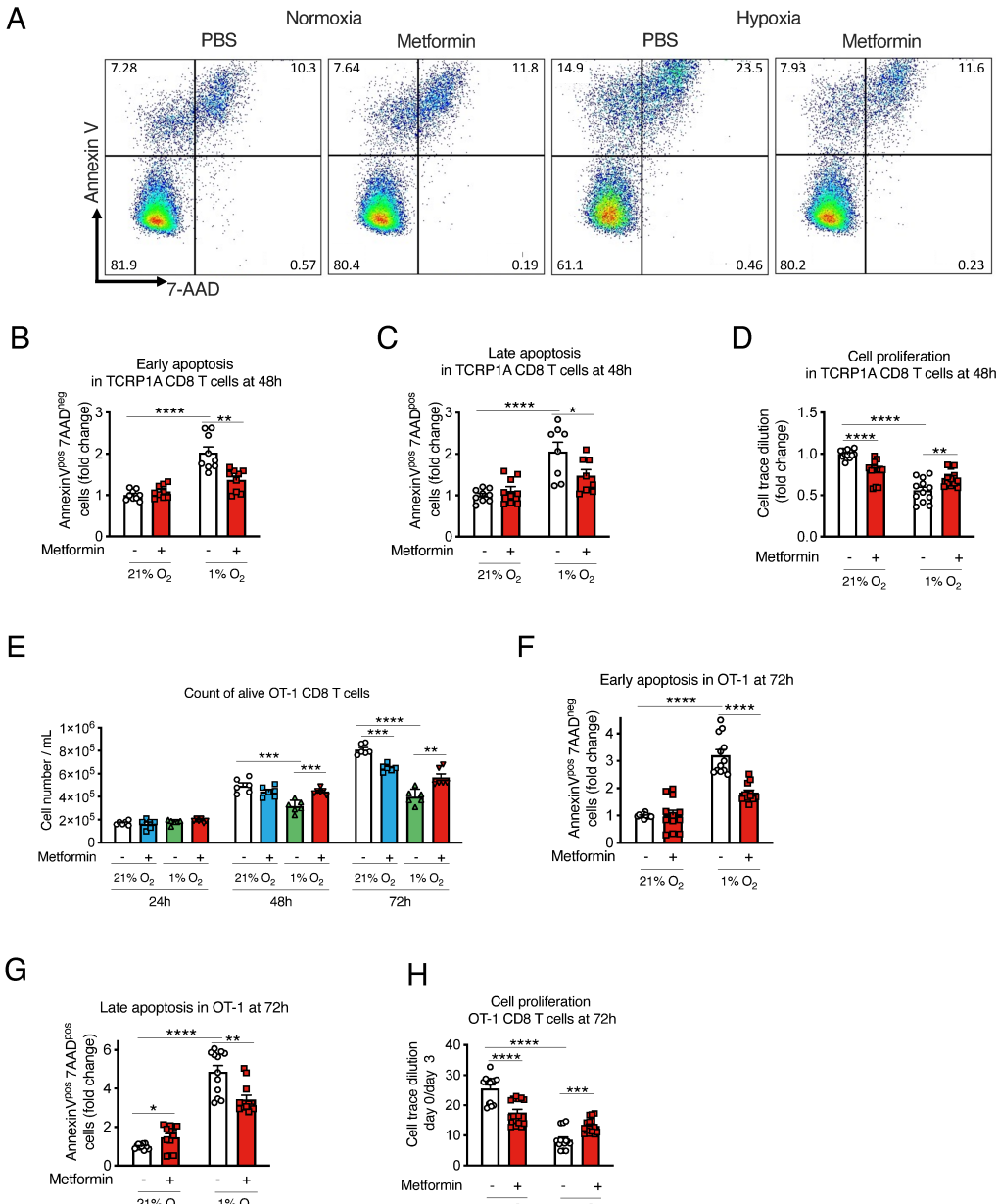
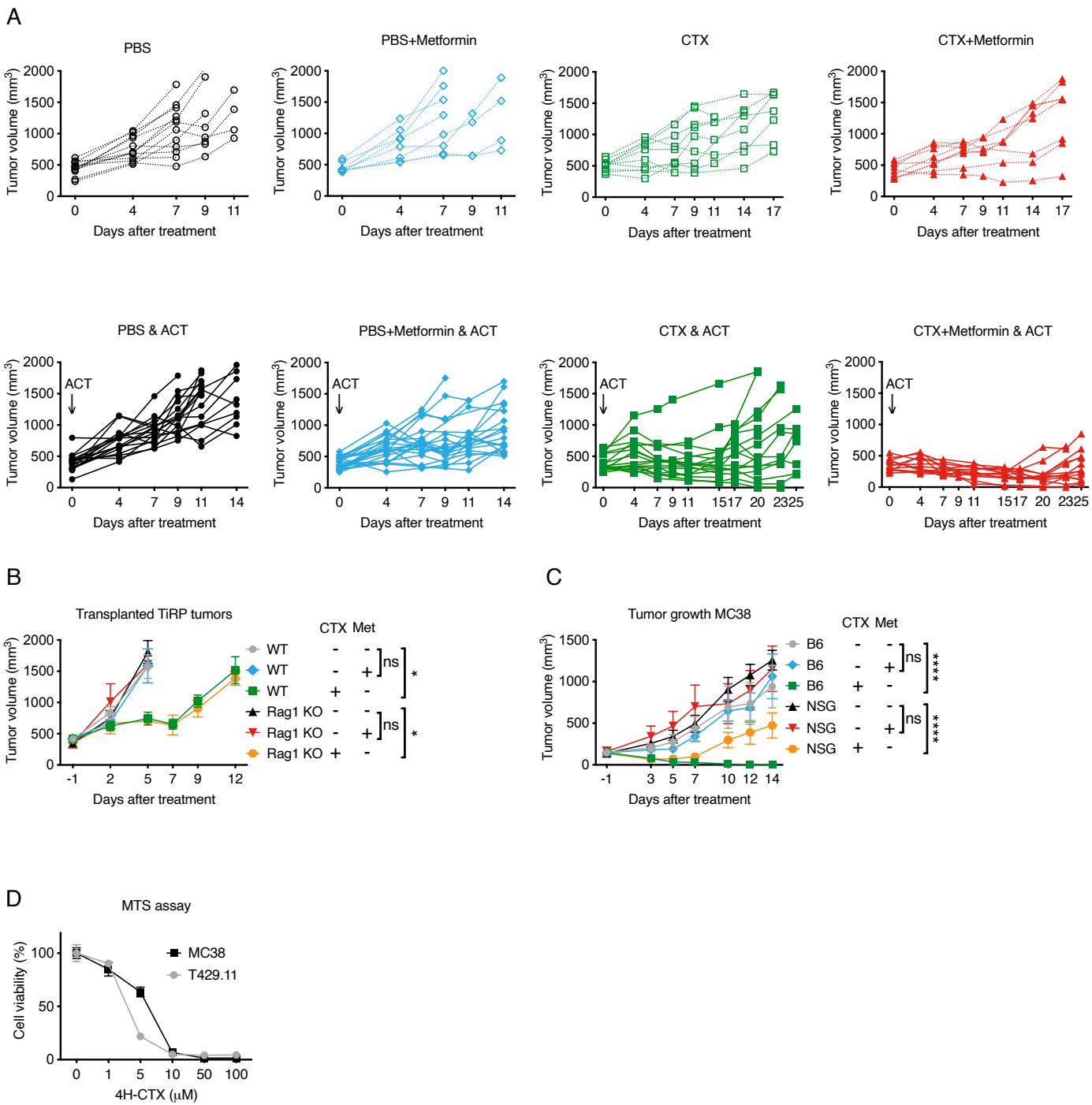
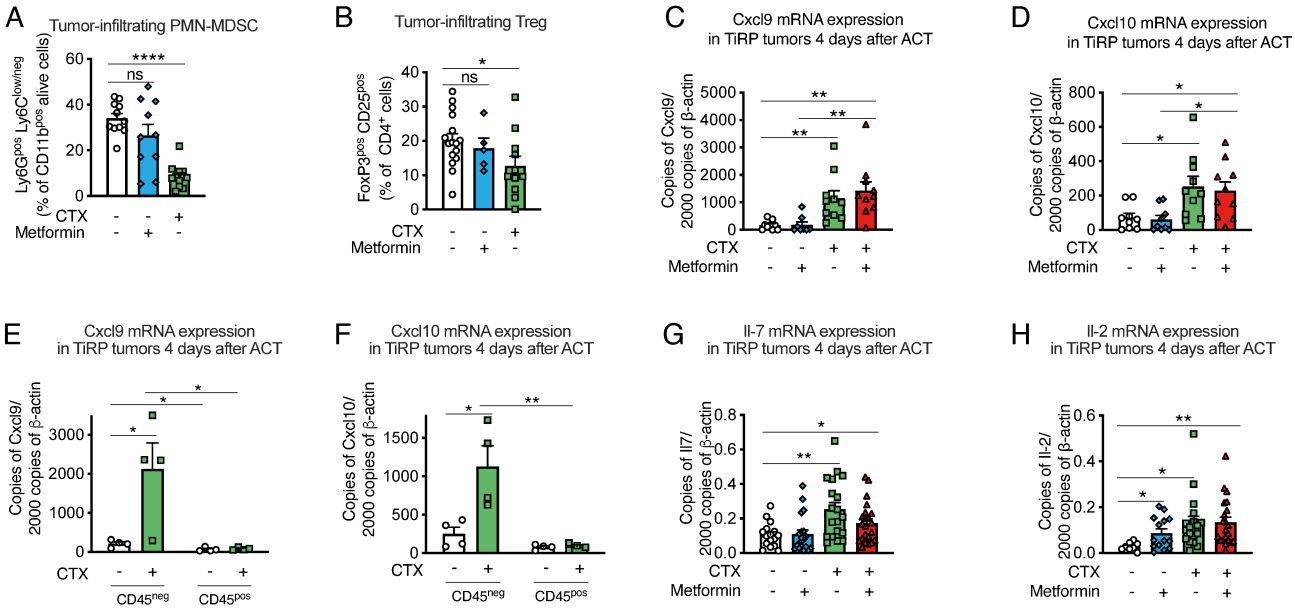
Suppl Fig 1


A
Normoxia
Hypoxia
PBS
Metformin
PBS
Metformin
7.28
10.3
81.9
0.57
7.64
11.8
80.4
0.19
14.9
23.5
61.1
0.46
7.93
11.6
80.2
0.23
Annexin V
7-AAD
B
Early apoptosis
in TCRP1A CD8 T cells at 48h
AnnexinVpos 7AADneg cells (fold change)
Metformin
21% O2
1% O2
C
Late apoptosis
in TCRP1A CD8 T cells at 48h
AnnexinVpos 7AADpos cells (fold change)
D
Cell proliferation
in TCRP1A CD8 T cells at 48h
Cell trace dilution (fold change)
E
Count of alive OT-1 CD8 T cells
Cell number / mL
24h
48h
72h
F
Early apoptosis in OT-1 at 72h
G
Late apoptosis in OT-1 at 72h
H
Cell proliferation
OT-1 CD8 T cells at 72h
Cell trace dilution day 0/day 3

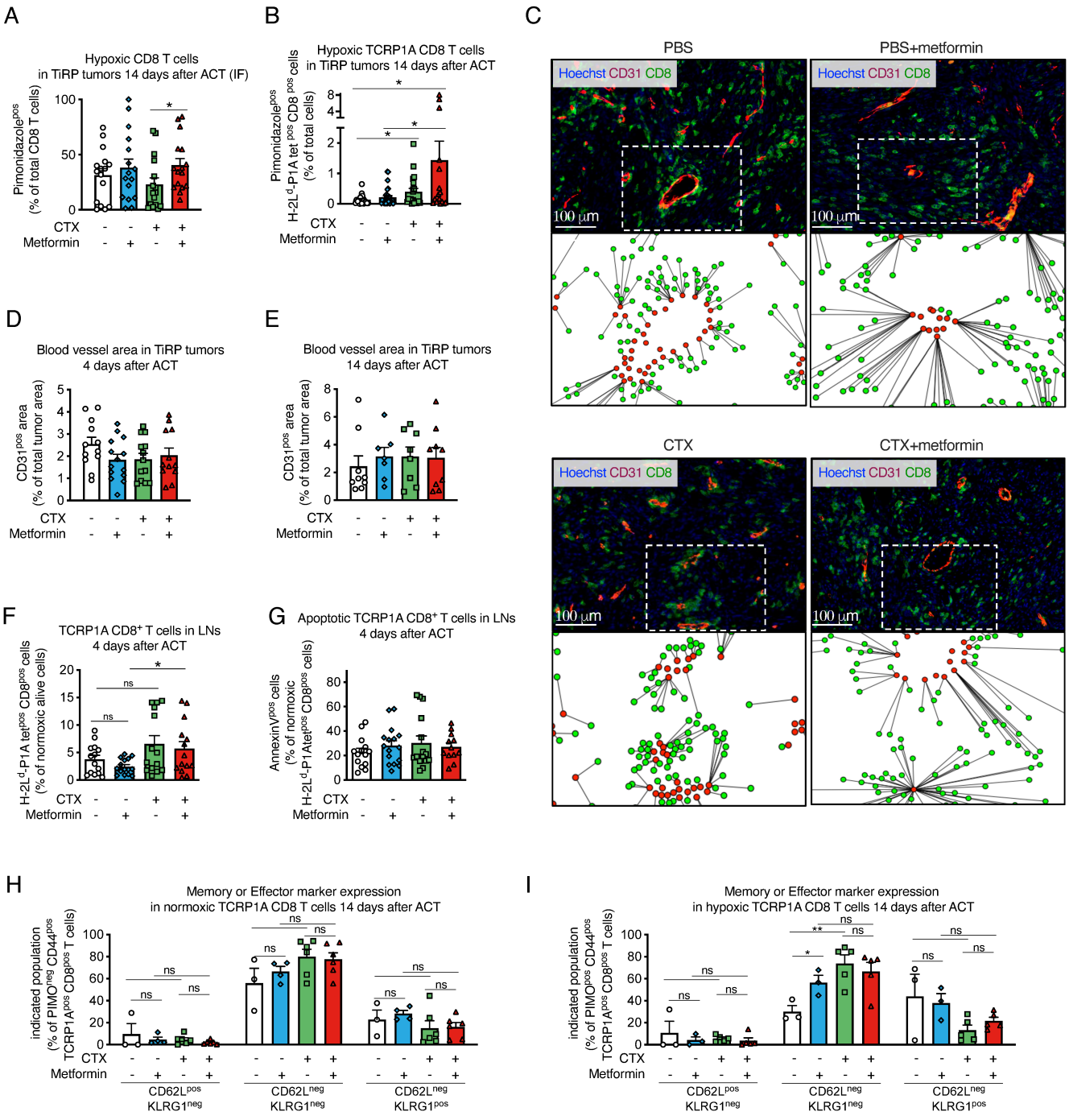
Suppl Fig 2


A
PBS
PBS+Metformin
CTX
CTX+Metformin
PBS & ACT
PBS+Metformin & ACT
CTX & ACT
CTX+Metformin & ACT
Tumor volume (mm3)
Days after treatment
ACT
B
Transplanted TiRP tumors
CTX Met
WT
Rag1 KO
ns
*
C
Tumor growth MC38
B6
NSG
****
D
MTS assay
MC38
T429.11
Cell viability (%)
4H-CTX (μM)

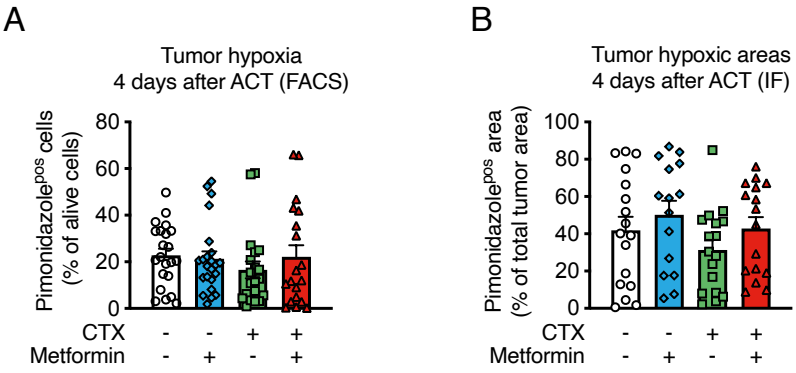
Suppl Fig 3


A
Tumor-infiltrating PMN-MDSC
Ly6G^pos Ly6C^low/neg (% of CD11b^pos alive cells)
****
ns
CTX
Metformin
B
Tumor-infiltrating Treg
FoxP3^pos CD25^pos (% of CD4+ cells)
*
ns
C
Cxcl9 mRNA expression in TiRP tumors 4 days after ACT
Copies of Cxcl9/ 2000 copies of β-actin
D
Cxcl10 mRNA expression in TiRP tumors 4 days after ACT
Copies of Cxcl10/ 2000 copies of β-actin
E
Cxcl9 mRNA expression in TiRP tumors 4 days after ACT
Copies of Cxcl9/ 2000 copies of β-actin
CD45^neg
CD45^pos
F
Cxcl10 mRNA expression in TiRP tumors 4 days after ACT
Copies of Cxcl10/ 2000 copies of β-actin
G
Il-7 mRNA expression in TiRP tumors 4 days after ACT
Copies of Il7/ 2000 copies of β-actin
H
Il-2 mRNA expression in TiRP tumors 4 days after ACT
Copies of Il-2/ 2000 copies of β-actin

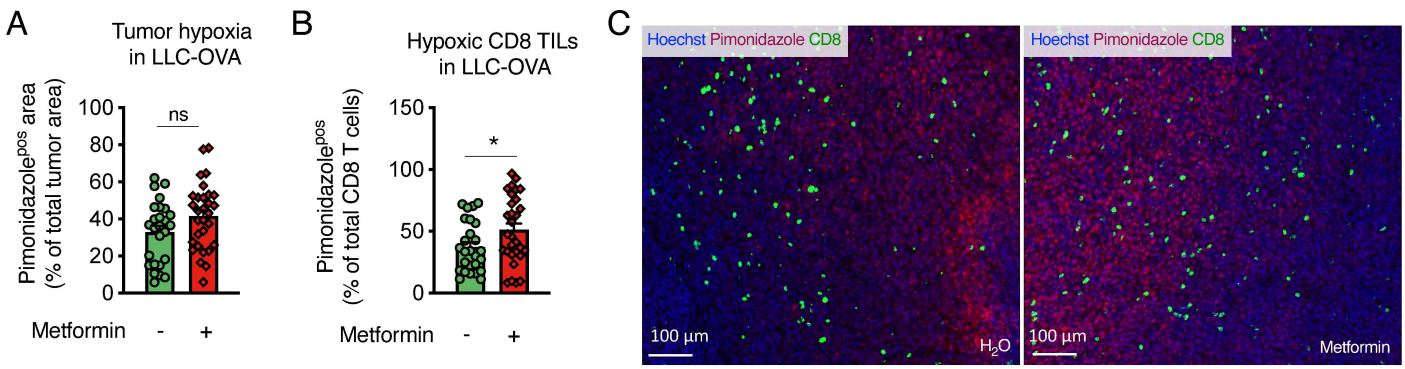
Suppl Fig 4


A
Hypoxic CD8 T cells in TiRP tumors 14 days after ACT (IF)
Pimonidazole^pos (% of total CD8 T cells)
CTX
Metformin
B
Hypoxic TCRP1A CD8 T cells in TiRP tumors 14 days after ACT
Pimonidazole^pos H-2L^d-P1A tet^pos CD8^pos cells (% of total cells)
C
PBS
PBS+metformin
CTX
CTX+metformin
Hoechst CD31 CD8
100 µm
D
Blood vessel area in TiRP tumors 4 days after ACT
CD31^pos area (% of total tumor area)
E
Blood vessel area in TiRP tumors 14 days after ACT
F
TCRP1A CD8+ T cells in LNs 4 days after ACT
H-2L^d-P1A tet^pos CD8^pos cells (% of normoxic alive cells)
ns
G
Apoptotic TCRP1A CD8+ T cells in LNs 4 days after ACT
AnnexinV^pos cells (% of normoxic H-2L^d-P1Atet^pos CD8^pos cells)
H
Memory or Effector marker expression in normoxic TCRP1A CD8 T cells 14 days after ACT
indicated population (% of PIMO^neg CD44^pos TCRP1A^pos CD8^pos T cells)
CD62L^pos KLRG1^neg
CD62L^neg KLRG1^neg
CD62L^neg KLRG1^pos
I
Memory or Effector marker expression in hypoxic TCRP1A CD8 T cells 14 days after ACT

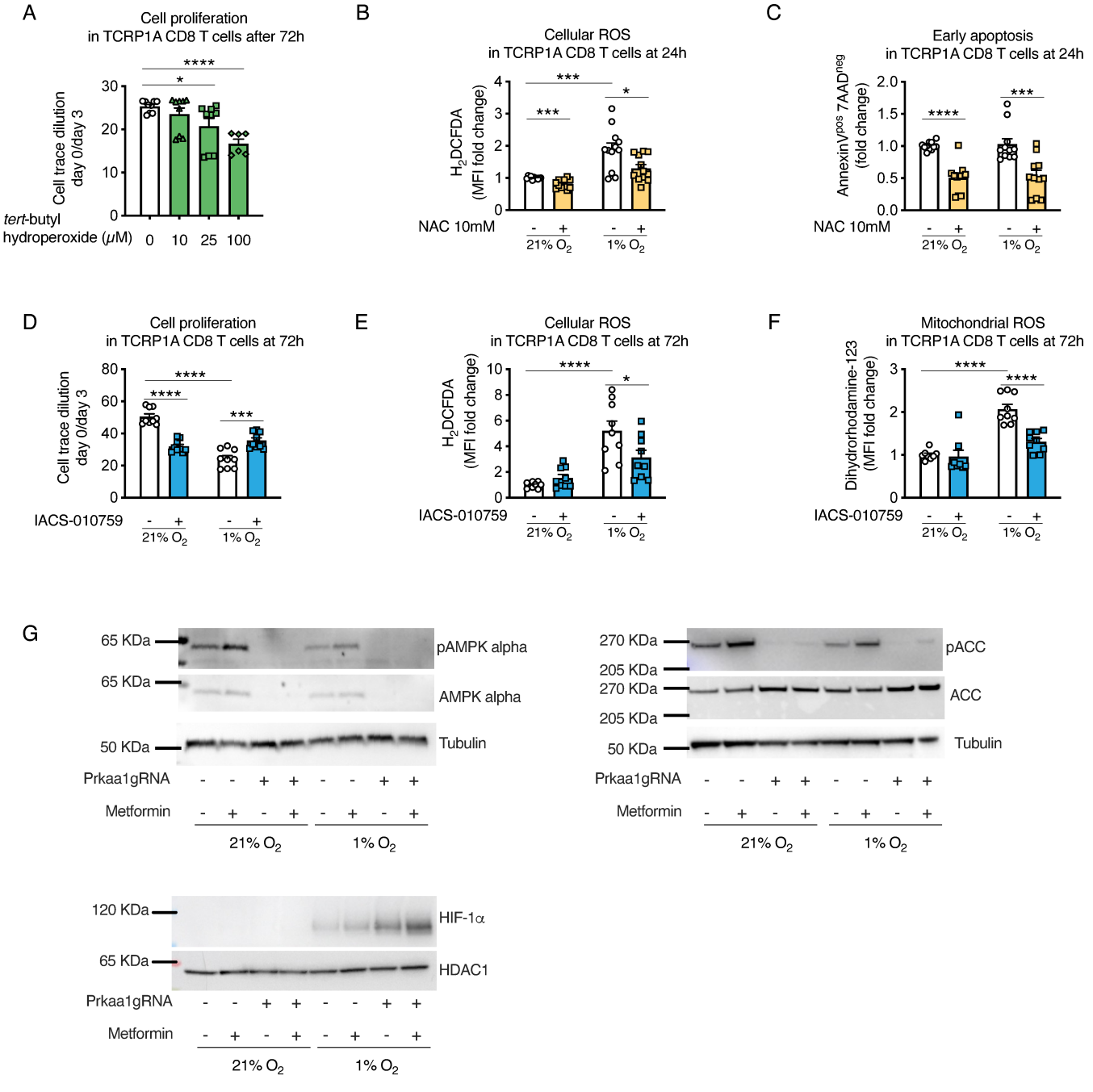
Suppl Fig 5


A
Tumor hypoxia
4 days after ACT (FACS)
Pimonidazole^pos cells
(% of alive cells)
80
60
40
20
0
CTX - - + +
Metformin - + - +
B
Tumor hypoxic areas
4 days after ACT (IF)
Pimonidazole^pos area
(% of total tumor area)
100
80
60
40
20
0
CTX - - + +
Metformin - + - +

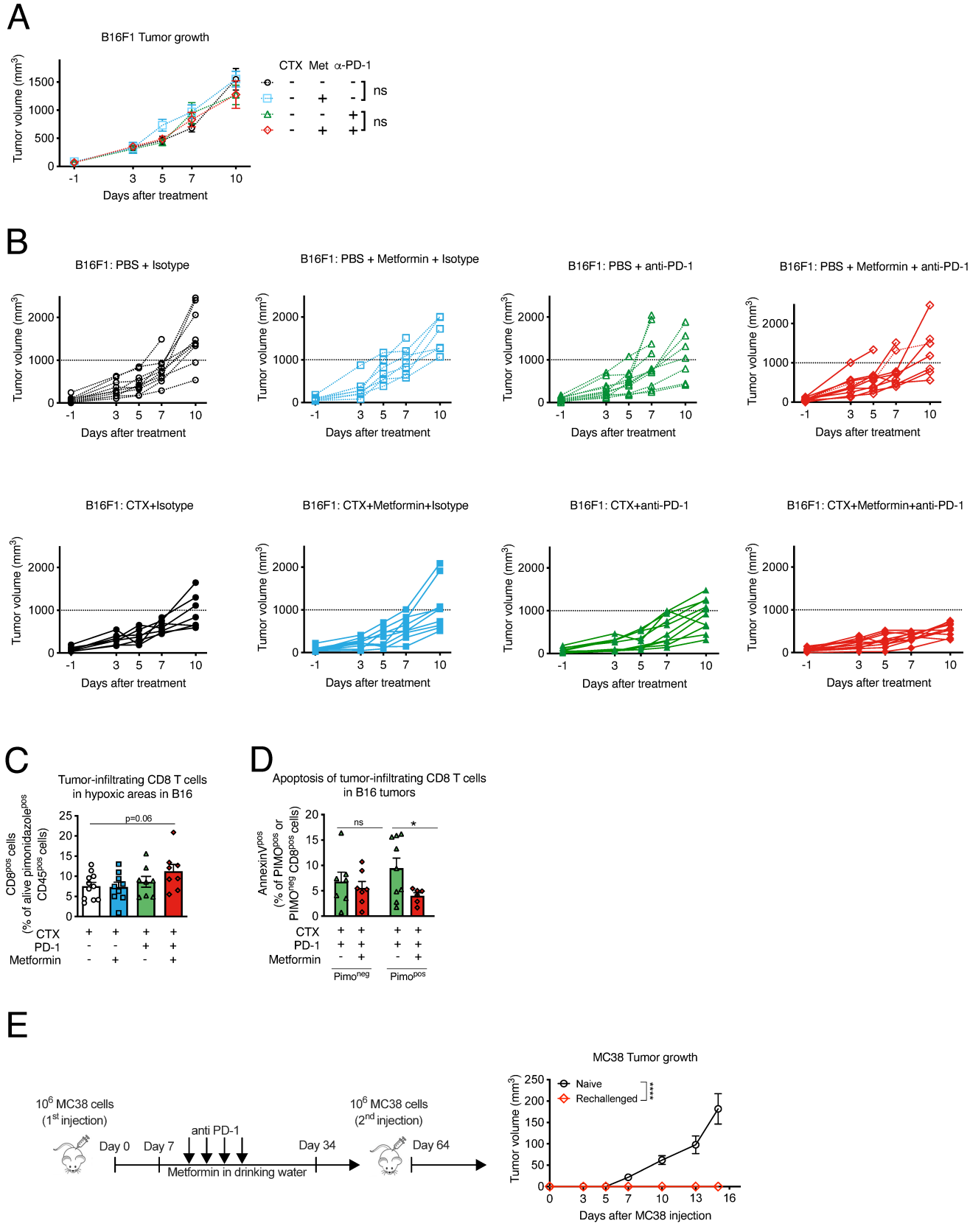
Suppl Fig 6


A
Tumor hypoxia in LLC-OVA
Pimonidazole^pos area (% of total tumor area)
100
80
60
40
20
0
ns
Metformin - +
B
Hypoxic CD8 TILs in LLC-OVA
Pimonidazole^pos (% of total CD8 T cells)
150
100
50
0
*
Metformin - +
C
Hoechst Pimonidazole CD8
100 µm
H2O
Hoechst Pimonidazole CD8
100 µm
Metformin

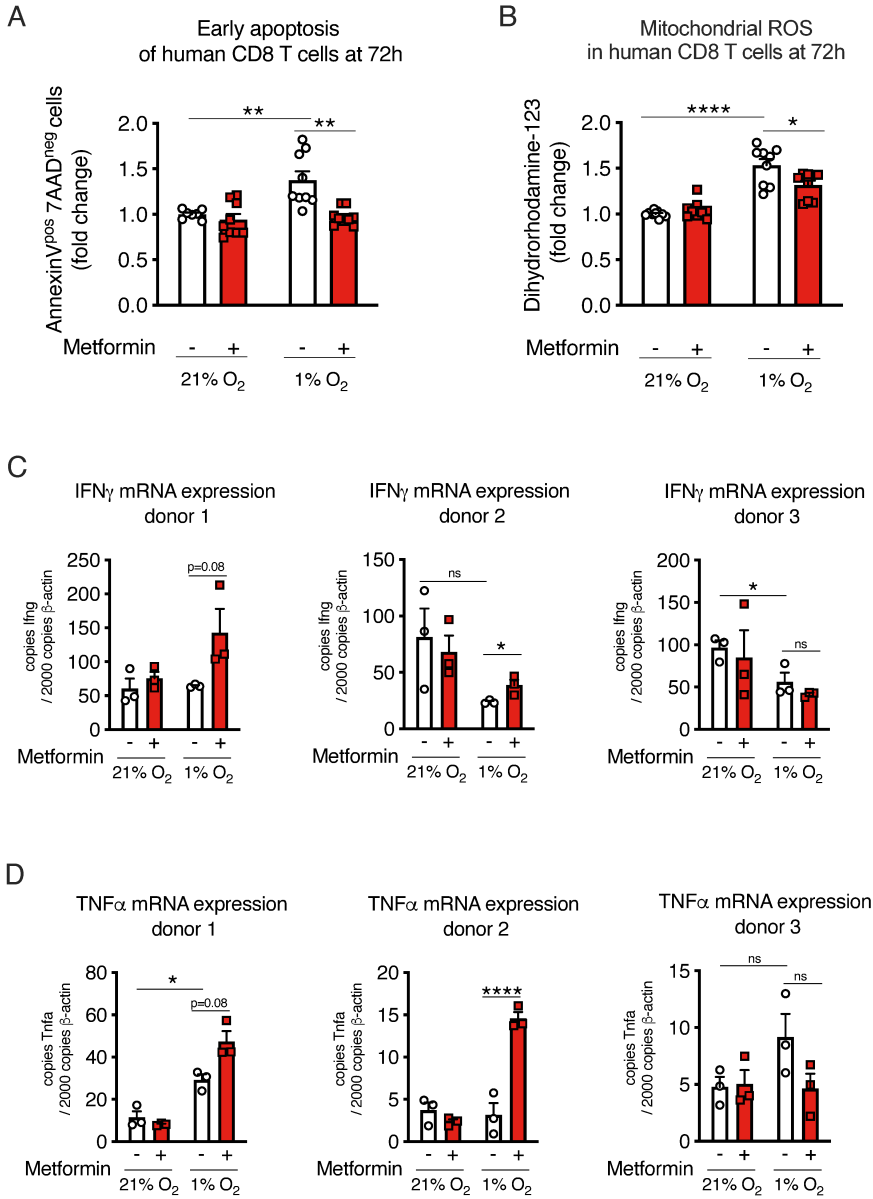
Suppl Fig 7


A
Cell proliferation in TCRP1A CD8 T cells after 72h
Cell trace dilution day 0/day 3
tert-butyl hydroperoxide (μM) 0 10 25 100
B
Cellular ROS in TCRP1A CD8 T cells at 24h
H2DCFDA (MFI fold change)
NAC 10mM
21% O2
1% O2
C
Early apoptosis in TCRP1A CD8 T cells at 24h
AnnexinVpos 7AADneg (fold change)
NAC 10mM
21% O2
1% O2
D
Cell proliferation in TCRP1A CD8 T cells at 72h
Cell trace dilution day 0/day 3
IACS-010759
21% O2
1% O2
E
Cellular ROS in TCRP1A CD8 T cells at 72h
H2DCFDA (MFI fold change)
IACS-010759
21% O2
1% O2
F
Mitochondrial ROS in TCRP1A CD8 T cells at 72h
Dihydrorhodamine-123 (MFI fold change)
IACS-010759
21% O2
1% O2
G
65 KDa
pAMPK alpha
65 KDa
AMPK alpha
50 KDa
Tubulin
Prkaa1gRNA
Metformin
21% O2
1% O2
270 KDa
pACC
205 KDa
270 KDa
ACC
205 KDa
50 KDa
Tubulin
Prkaa1gRNA
Metformin
21% O2
1% O2
120 KDa
HIF-1α
65 KDa
HDAC1
Prkaa1gRNA
Metformin
21% O2
1% O2
H
Early apoptosis in TCRP1A CD8 T cells after 72h at 1% O2
AnnexinVpos 7AADneg (fold change to control)
Metformin
Scramble gRNA
Prkaa1 gRNA
I
Late apoptosis in TCRP1A CD8 T cells after 72h at 1% O2
AnnexinVpos 7AADpos (fold change to control)
ns
Metformin
Scramble gRNA
Prkaa1 gRNA
J
Cell proliferation in TCRP1A CD8 T cells after 72h at 1% O2
Cell trace dilution day 0/day 3
Metformin
Scramble gRNA
Prkaa1 gRNA
K
ROS in TCRP1A CD8 T cells after 72h at 1% O2
H2DCFDA (MFI fold change)
Metformin
Scramble gRNA
Prkaa1 gRNA

Suppl Fig 8


A
B16F1 Tumor growth
Tumor volume (mm3)
Days after treatment
CTX Met α-PD-1
ns
ns
B
B16F1: PBS + Isotype
B16F1: PBS + Metformin + Isotype
B16F1: PBS + anti-PD-1
B16F1: PBS + Metformin + anti-PD-1
B16F1: CTX+Isotype
B16F1: CTX+Metformin+Isotype
B16F1: CTX+anti-PD-1
B16F1: CTX+Metformin+anti-PD-1
C
Tumor-infiltrating CD8 T cells in hypoxic areas in B16
p=0.06
CD8pos cells (% of alive pimonidazolepos CD45pos cells)
CTX
PD-1
Metformin
D
Apoptosis of tumor-infiltrating CD8 T cells in B16 tumors
ns
*
AnnexinVpos (% of PIMOpos or PIMOneg CD8pos cells)
Pimoneg
Pimopos
E
10^6 MC38 cells (1st injection)
Day 0
Day 7
anti PD-1
Metformin in drinking water
Day 34
10^6 MC38 cells (2nd injection)
Day 64
MC38 Tumor growth
Naive
Rechallenged
****
Days after MC38 injection

Suppl Fig 9


A
Early apoptosis
of human CD8 T cells at 72h
AnnexinV^pos 7AAD^neg cells
(fold change)
**
**
Metformin
21% O2
1% O2
B
Mitochondrial ROS
in human CD8 T cells at 72h
Dihydrorhodamine-123
(fold change)
****
*
Metformin
21% O2
1% O2
C
IFNγ mRNA expression
donor 1
copies Ifng
/ 2000 copies β-actin
p=0.08
Metformin
21% O2
1% O2
IFNγ mRNA expression
donor 2
ns
*
IFNγ mRNA expression
donor 3
*
ns
D
TNFα mRNA expression
donor 1
copies Tnfa
/ 2000 copies β-actin
*
p=0.08
TNFα mRNA expression
donor 2
****
TNFα mRNA expression
donor 3
ns
ns

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Metformin rescues CD8 T-cell fitness in hypoxia in vitro. A-C. Representative FACS plots (**A**) and quantification (**B**,**C**) of early (**B**) and late (**C**) apoptosis measured by FACS staining for AnnexinV and 7-Aminoactinomycin D (7-AAD) in activated TCRP1A CD8 T cells incubated in normoxia (21% O_2) or hypoxia (1% O_2) treated or not with metformin (2 mM) for 48h; results are expressed in fold changes compared to normoxia control. **D**. Activated TCRP1A CD8 T cells were labeled with CellTrace Violet Cell Proliferation Kit before being treated as in (**A**) to assess cell proliferation calculated as dilution of median fluorescence intensity of the violet dye between day 0 (before treatments) and 48h of treatments; results are expressed in fold changes compared to normoxia control. **E**. Viable cell count of activated OT-1 CD8 T cells incubated in normoxia (21% O_2) or hypoxia (1% O_2) in presence or not of metformin (2 mM). **F**,**G**. Quantification of early (**F**) and late (**G**) apoptosis measured by FACS staining for AnnexinV and 7-Aminoactinomycin D (7-AAD) in activated OT-1 CD8 T cells treated as in **E**, at 72h; results are expressed in fold changes compared to normoxia control. **H**. Activated OT-1 CD8 T cells were labeled with CellTrace Violet Cell Proliferation Kit before being treated as in (**E**) to assess cell proliferation calculated as dilution of median fluoresce intensity of the violet dye between day 0 (before treatments) and 72h of treatments. **B,C** is a pool 3 independent experiments; **E** is a pool of 2 independent experiments. **D**,**F-H** is a pool of 4 independent experiments. All data are mean ± SEM. * p values <0.05; ** p values <0.01; *** p values <0.001; **** p values <0.0001, calculated by two-tailed unpaired t-test.

Supplementary Figure 2. Metformin improves tumor response to adoptive cell therapy (ACT) and CD8 T-cell infiltration in hypoxic TiRP tumor areas. A. Single curves of tumor growth in TiRP mice that have received a single injection of CTX (100 mg/kg) or PBS when the tumor size was around 400 mm^3, followed or not by ACT of 10 million activated TCRP1A CD8 T cells 24 hours later and treated or not with metformin (0.5 mg/mL) in drinking water (**A**, PBS no ACT: *n*=12; PBS+Metformin no ACT: *n*=8; CTX no ACT: *n*=10; CTX+Metformin no ACT: *n*=9; PBS & ACT: *n*=25; PBS+Metformin & ACT: *n*=23; CTX+ & ACT: *n*=17; CTX+Metformin & ACT: *n*=13). **B**. Growth curves of TiRP tumors transplanted in B10.D2;Ink4a/$Arf^{flox/flox}$ (WT) mice or in TiRP Rag1 KO mice; when the tumor size was around 400 mm^3, mice received a single injection of CTX (100 mg/kg) or PBS, followed the day after by metformin (0.5 mg/mL) in drinking water, in absence of ACT (**B**, WT PBS: *n*=6; WT CTX: *n*=5; WT PBS+Metformin: *n*=5; TiRP Rag1 KO PBS: *n*=4; TiRP Rag1 KO CTX: *n*=4; TiRP Rag1 KO PBS+ Metformin: *n*=3). **C**. Growth curves of MC38 tumors in B6 or NSG mice; when the tumor size was around 150 mm^3, mice received a single injection of CTX (100 mg/kg) or PBS, followed the day after by metformin (0.5 mg/mL) in drinking water, in absence of ACT (**C**, B6 PBS: *n*=8; B6 CTX: *n*=10; B6 PBS+Metformin: *n*=7; NSG PBS: *n*=7; NSG CTX: *n*=7; NSG PBS+ Metformin: *n*=5). **D.** MC38 colon carcinoma cell line and T429.11 (TiRP-derived melanoma) cell line were treated with 4-hydroperoxycyclophosphamide (4H-CTX, 1µM, 5µM, 10µM, 50µM or 100µM) for 24h

and cell viability was assessed by MTS assay. **D** is representative of 2 independent experiments. Data in **B-D** are mean ± SEM. ns, not significant; * p values <0.05; **** p values <0.0001, calculated by two-way analysis of variance with Tukey's multiple comparison correction.

Supplementary Figure 3. Metformin improves tumor response to adoptive cell therapy (ACT) and CD8 T-cell infiltration in hypoxic TiRP tumor areas. A,**B**. FACS analysis of tumor infiltrating PMN-MDSCs (**A**) or CD4 Treg cells (**B**) in TiRP mice 5 days after a single injection of CTX (100 mg/kg) or PBS when the tumor size was around 400 mm^3 followed 24 hours later by metformin (0.5 mg/mL) in drinking water (**A**, PBS: *n*=11; PBS+Metformin: *n*=10; CTX: *n*=12; **B**, PBS: *n*=16; PBS+Metformin: *n*=5; CTX: *n*=11). **C,D**. qPCR analysis for Cxcl9 (**C**) or Cxcl10 (**D**), from whole tumors of TiRP mice that have received a single injection of CTX (100 mg/kg) or PBS when the tumor size was around 400 mm^3, followed by ACT of 10 million activated TCRP1A CD8 T cells 24 hours later and treated or not with metformin (0.5 mg/mL) in drinking water, 4 days after ACT. (**C**, PBS: *n*=9; PBS+Metformin: *n*=8; CTX *n*=10; CTX+Metformin: *n*=10; **D**, PBS: *n*=9; PBS+Metformin: n=9; CTX+: *n*=10; CTX+Metformin: *n*=10). **E,F**. qPCR analysis for Cxcl9 (**E**) and Cxcl10 (**F**) from CD45-negative and CD45-positive cells FACS sorted from tumors of TiRP mice that have received a single injection of CTX (100 mg/kg) or PBS when the tumor size was around 400 mm^3, followed by ACT of 10 million activated TCRP1A CD8 T cells 24 hours later (**E**,**F** *n*=4). **G**,**H**. qPCR analysis for Il7 (**G**) or Il2 (**H**) from whole TiRP tumors treated as in **C**,**D**. (**G**, PBS: *n*=16; PBS+Metformin: *n*=20; CTX: *n*=19; CTX+Metformin: *n*=22; **H**, PBS: n=8; PBS+Metformin: *n*=14; CTX: *n*=18; CTX+Metformin: *n*=21). All data are mean ± SEM. ns, not significant; * p values <0.05; ** p values <0.01; **** p values <0.0001, calculated by two-tailed unpaired t-test.

Supplementary Figure 4. Metformin improves tumor response to adoptive cell therapy (ACT) and CD8 T-cell infiltration in hypoxic TiRP tumor areas.
A. IF quantification of hypoxic tumor-infiltrating CD8 T cells in TiRP mice that have received a single injection of CTX (100 mg/kg) or PBS when the tumor size was around 400 mm^3, followed by ACT of 10 million activated TCRP1A CD8 T cells 24 hours later and treated or not with metformin (0.5 mg/mL) in drinking water, 14 days after ACT (**A**, *n*=16). **B**. FACS analysis of TCRP1A CD8 T cells among hypoxic cells in TiRP tumors treated as in (**A**) 14 days after ACT (**B**, PBS+: *n*=19; PBS+Metformin: *n*=28; CTX+: *n*=21; CTX+Metformin: *n*=17). **C**. Representative images (top) of CD8 and CD31 IF staining in TiRP tumors treated as in **A**, 4 days after ACT, and illustrative reconstruction (bottom) of the distance (black line) of CD8 cells (green circles) and the nearest CD31 cell (red circles) of the selected regions on the panel on top (complete total image quantification in Fig. 2H). **D,E**. IF quantification of CD31-positive areas in TiRP tumors treated as in **A** , 4 days (**D**) or 14 days (**E**) after ACT (**D**, PBS: *n*=12; PBS+Metformin: *n*=14; CTX: *n*=14; CTX+Metformin: *n*=12; **E**, PBS: *n*=9; PBS+Metformin: *n*=7; CTX: *n*=8; CTX+Metformin: *n*=9). **F,G.** FACS analysis of TCRP1A infiltration (**F**) and apoptosis (**G**) 4 days after ACT in tumor-draining lymph nodes of TiRP mice treated as in **A** (PBS: *n*=14; PBS+Metformin: *n*=15; CTX: *n*=14;

CTX+Metformin: *n*=14). **H,I.** FACS analysis 14 days after ACT of CD62L and KLRG1 markers in normoxic (**H**) and hypoxic (**I**) tumor infiltrating TCRP1A CD8 T cells in TiRP mice treated as in (**A**) (**H,I**, PBS: *n*=3; PBS+Metformin: *n*=3; CTX: *n*=6; CTX+Metformin: *n*=6). All data are mean ± SEM. ns, not significant; * p values <0.05; ** p values <0.01; **** p values <0.0001, calculated by two-tailed unpaired t-test.

Supplementary Figure 5. Metformin does not affect tumor hypoxia in TiRP mice. **A**,**B**. FACS (**A**) or IF (**B**) quantification of pimonidazole staining in TiRP mice 4 days after ACT, that have received a single injection of CTX (100 mg/kg) or PBS when the tumor size was around 400 mm^3, followed by ACT of 10 million activated TCRP1A CD8 T cells 24 hours later and treated or not with metformin (0.5 mg/mL) in drinking water. (**A**, PBS: *n*=22; PBS+Metformin: *n*=23; CTX: *n*=20; CTX+Metformin: *n*=19; **B**, PBS: *n*=17; PBS+Metformin: *n*=15; CTX: *n*=17; CTX+Metformin: *n*=16). All data are mean ± SEM.

Supplementary Figure 6. Metformin improves tumor response to ACT in different models. IF quantification (**A,B**) and representative images (**C**) of hypoxia (**A**) and hypoxic CD8 T cells (**B**) in LLC-OVA tumors receiving ACT of 2 million activated OT-1 CD8 T cells and treated or not with metformin at 0.5 mg/mL in drinking water (**A**,**B**, pool of 3 independent experiments, PBS: *n*=26; Metformin: *n*=30). All data are mean ± SEM. ns, not significant; * p values <0.05, calculated by two-tailed unpaired t-test.

Supplementary Figure 7. Metformin prevents hypoxia-induced apoptosis by reducing ROS production. A. FACS quantification of proliferation assessed by CellTrace Violet dilution from day 0 (before treatments) to day 3 in activated TCRP1A CD8 T cells treated with different doses of *tert*-butyl hydroperoxide for 72h. **B-C**. FACS quantification of cellular ROS (**B**) and early apoptosis (**C**) in TCRP1A CD8 treated with 10 mM N-acetyl-L-cysteine (NAC) for 24h **D**-**F**. FACS quantification of proliferation (**D**) assessed by CellTrace Violet dilution from day 0 to day 3, cellular ROS (**E**) and mitochondrial ROS (**F**) in activated TCRP1A CD8 T cells incubated in normoxia (21% O_2) or hypoxia (1% O_2) in presence or not of IACS-010759 (100 nM). **G**. Representative Western blot analysis of AMPK (top left), ACC (top right) and HIF-1α (bottom left, serving as a control of hypoxia) in WT (scramble gRNA) or AMPK alpha1 KO (Prkaa1 gRNA) TCRP1A CD8 T cells incubated in normoxia (21% O_2) or hypoxia (1% O_2) in presence or not of metformin (2 mM) for 48h. **H**-**K**. FACS quantification of early (**H**) and late (**I**) apoptosis, proliferation (**J**) assessed by CellTrace Violet dilution from day 0 to day 3, and cellular ROS (**K**) in WT (scramble gRNA) or AMPK alpha1 KO (Prkaa1 gRNA) TCRP1A CD8 T cells incubated in hypoxia (1% O_2) in presence or not of metformin (2 mM). **A** is a pool of 3 independent experiments (100 µM dose was assessed in 2 independent experiments). **B**-**C** is a pool of 4 independent experiments; **D-F,J** is a pool of 3 independent experiments. **H**,**I** is a pool of 6 independent experiments; **K** is a pool of 5 independent experiments. All the conditions are assessed in triplicate in each individual experiment. **B,C,E,F,H,I,K** Data are expressed as fold change to control. MFI, median fluorescence intensity. All data

are mean ± SEM. ns, not significant; * p values <0.05; ** p values <0.01; *** p values <0.001; **** p values <0.0001, calculated by two-tailed unpaired t-test.

Supplementary Figure 8. Metformin improves tumor response to anti-PD-1 treatment. **A**. Growth curves of B16F1 tumors treated once with PBS (day -1), one week after tumor cell injection, followed by metformin (0.5 mg/mL) in drinking water in combination with i.p. injection of anti-PD1 antibody (100 μg/mouse) or isotype every 3-days starting from day 0. (**A**, pool of 2 independent experiments; PBS + Isotype: *n*=10; PBS + Metformin + Isotype: *n*=7; PBS + anti-PD-1: *n*=11; PBS + Metformin + anti-PD-1: *n*=10). **B**. Single growth curves of B16F1 tumor growth from **A** and Fig. 6A. **C,D**. FACS analysis of CD8 T cell infiltration (**C**) and apoptosis (**D**) in B16F1 tumors treated once with 100 mg/kg CTX (day –1) one week after tumor cell injection, followed or not by metformin (0.5 mg/mL) treatment in drinking water in combination with i.p. injection of anti-PD-1 antibody (100 μg/mouse) or isotype every 3-days starting from day 0 for total of 3 injections. Mice were sacrificed 7 days after start of treatment. (**C**, pool of 2 independent experiments: CTX + Isotype: n=11; CTX + Isotype + Metformin: n=9; CTX + anti-PD-1: n=8; CTX + anti-PD-1 + Metformin: n=8; **D**, pool of 2 independent experiments: CTX + anti-PD-1: n=9; CTX + anti-PD-1 + Metformin: n=7). **E**. C57BL/6 mice were injected with 10^6 MC38 cells in right flank (1^{st} injection) and injected i.p. with anti-PD-1 antibody (200 μg/mouse) every 3-days for total of 4 injections and treated with metformin (0.5 mg/mL) in drinking water for 27 days. 30 days after total regression of tumors, mice were rechallenged with 10^6 MC38 cells injected in left flank. Naïve mice were used as control. (Naive: *n*=9; Rechallenged: *n*=10). Data in **A,C-E** are mean ± SEM. ns, not significant, * p values <0.05 calculated by two-tailed unpaired t-test, **** p value <0.0001 by two-way analysis of variance.

Supplementary Figure 9. Metformin reduces hypoxia-dependent apoptosis in human CD8 T cells. **A**. Quantification of early apoptosis in human CD8 T cells activated with anti-CD3/CD28 beads and feeder cells and incubated in normoxia (21% O_2) or hypoxia (1% O_2) and treated or not with metformin (2 mM). **B**. FACS quantification of mitochondrial ROS in human CD8 T cells treated as in (**A**). **C,D.** qPCR analysis for IFN-γ (**C**) and TNF-α (**D**), from activated human CD8 T cells isolated from 3 donors and treated as in (**A**). **A**,**B**. is a pool of 3 independent experiments. All data are mean ± SEM. ** p values <0.01; *** p values <0.001; **** p values <0.0001, calculated by two-tailed unpaired t-test.

SUPPLEMENTARY METHODS

Flow cytometry staining

For apoptosis quantification, CD8 T cells were washed in annexin V buffer (BD Biosciences, 640941) and stained with annexin V (Biolegend, 640941, 1:40) for 20 min at RT, washed and resuspended in annexin V buffer supplemented with 7AAD (Thermo Fisher Scientific, A1310, 1:1000). For ROS quantification, CD8 T cells were resuspended in HBSS (Sigma, 6648) pre-incubated in normoxic or hypoxic conditions, stained with CM-H2DCFDA (Invitrogen, C6827, 1:1000) or Dihydrorhodamine 123 (Invitrogen, D23806, 1:1000) for 30 min at 37°C, in normoxic or hypoxic conditions, and washed in HBSS. For staining of surface markers, CD8 T cells were resuspended in PBS buffer containing 2% FBS and 2 mM EDTA (FACS buffer) and incubated with CD16/CD32 blocking antibody for 15 min at 4°C followed by incubation with fluorescent anti-PD1 and anti-LAG3 antibodies for 20 min at 4°C. Samples were acquired at BD FACSVerse. Antibodies are listed in Supplementary Table S1.

Tumor single cell suspensions were obtained by tumor dissociation with the gentleMACS Dissociator and digestion in RMPI medium (Thermo Fisher Scientific, 11875093) containing collagenase I (100 U/mL), collagenase II (100 U/mL) and dispase (1 U/mL) for 1 hour at 37°C. 70 µm- and 40µm-cell strainers were used to eliminate cell aggregates. Lymph nodes were mechanically dissociated on a 40µm cell strainer. After red blood cell lysis (Thermo Fisher Scientific, 00-4300-54), cells were resuspended in FACS buffer. Equal number of cells were used for FACS staining. Cells were stained with Fixable Viability dye (15 min, 4°C) and washed twice before being incubated with CD16/CD32 blocking (15 min, 4°C). PE-conjugated H-2L^{d} P1A$_{35\text{-}43}$ tetramer (homemade) was added to the cell suspension for 15 min at 4°C before staining with fluorescent primary antibodies (20 min, 4°C) (Suppl table 1). For apoptosis staining on tumor single cell suspension, cells were washed and resuspended in Annexin buffer (BD Biosciences, 640941) and stained with fluorescent Annexin V (Biolegend, 640941 or 640906) for 20 min at RT. Cells were then fixed and permeabilized using Cytofix/Cytoperm buffer (BD Biosciences, 554714). After washes, cells were incubated with anti-pimonidazole FITC for 1 hour at 37°C. For intranuclear staining, cells were fixed and permeabilized using FoxP3/Transcription factor staining buffer (Thermo Fisher Scientific, 00-5523-00) followed by incubation with fluorescent anti-FoxP3 primary antibody for 1 hour at 37°C. Samples were acquired at BD LRSFortessa and analyzed with FlowJo 10.7.1. Single stained and Fluorescence Minus One (FMO) samples were used as control. Antibodies and reagents are listed in Supplementary Table S1.

IFN-γ ELISA

After 48h of incubation with or without metformin, in normoxia (21% O_2) or hypoxia (1% O_2), 5 000 CD8 T cells were seeded in fresh medium pre-incubated at 21% O_2 or 1% O_2 and co-cultured at 21% O_2 or 1% O_2, in presence or absence of metformin, with 3 000 P511 cells or with P204.1 cells that were pulsed or not with P1A$_{35\text{-}43}$ (LPYLGWLVF) antigenic peptide (homemade) in X-vivo medium (Lonza, BE04-380Q) for 45 min at 37°C. After overnight incubation, supernatants were collected and

analyzed by ELISA using the DUOSET Mouse IFN-γ (R&D systems, DY485), according to the manufacturer's instructions.

Cytokine staining

After 48 hours of incubation with or without metformin, in normoxia or hypoxia, an equal number of CD8 T cells were resuspended in X-Vivo medium (Lonza, BE04-380Q) supplemented with L-Arginine (0.55 mM, Merck), L-asparagine (0.24 mM, Merck), Glutamine (1.5 mM, Merck), b-mercaptoethanol (50 µM, Sigma, M3148), and incubated with P511 cells in presence of Brefeldin A (10 µg/mL, Sigma, B7651) and anti-CD107α antibody for 5 hours at 37°C in normoxia or hypoxia. After staining with fixable viability dye and anti-CD8α antibody, cells were fixed and permeabilized (CytoFix/CytoPerm kit) and stained for IFNy and TNFα for 45 min at 4°C. Samples were acquired at BD LRSFortessa. Antibodies and reagents are listed in Supplementary Table S1.

Quantitative RT-PCR

For RNA extraction, tumors were lysed using TissueLyser LT (Qiagen, 85600). RNA from tumor homogenates or from cells was extracted with NucleoSpin RNA kit (Macherey Nagel, 740955) according to the manufacturer's instructions. 1 µg of RNA was retro-transcribed by the RevertAid RT Kit (ThermoFisher Scientific, K1691) according to the manufacturer's instructions. qRT-PCR was performed using Takyon Low ROX Probe 5X MasterMix dTTP (Eurogentec, UF-LP5X-C0501) and QuantStudio 3 Real-time PCR instrument (Thermo Fisher Scientific) using the following program: 3 min at 95°C, then 40 cycles of 10 seconds at 95°C and 1 min at 60°C. Samples were run in technical duplicate. The following premade assays were purchased from Integrated DNA Technologies: mouse CXLC-9 (Mm.PT.47.16060565), mouse CXLC-10 (Mm.PT.58.43575827), mouse IL-2 (Mm.PT.58.11478202), mouse IL-7 (Mm.PT.58.10325839), human IFN-y (Hs.PT.58.3781960), human TNF-α (Hs.PT.58.45380900), human β-actin (Hs.PT.39a.22214847). Mouse β-actin probe and primers were purchased from Eurogene: β-actin: Fw: 5'-CTC-TGG-CTC-CTA-GCA-CCA-TGA-AG-3', Rv: 5'-GCT-GGA-AGG-TGG-ACA-GTG-AG-3', Probe: 5'-FAM-ATC-GGT-GGC-TCC-ATC-CTG-GC-TAMRA-3'.

Immunofluorescence multiplex staining.

Tumors were fixed in 4% formaldehyde overnight at 4°C, dehydrated, embedded in paraffin and cut in 5 µm-thick sections. After deparaffinization with Histo-Clear II (National diagnostic, HS2021GLL) and rehydratation, antigen retrieval was performed with citrate buffer (pH6, 10 mM citric acid, 0.05% Tween-20) in 2100 Antigen Retriever (Aptum). Endogenous peroxidases were blocked with Bloxall (Vector laboratories, SP-6000-100) for 15 min followed by 1 hour incubation with normal goat serum blocking solution 2.5% (Vector lab, S-1012-50) at RT. Sections were stained by sequential multiplex immunostaining. Stainings were performed in Animal-free blocker and diluent (Vector lab, SP-5035-100) for 1 hour with the following primary antibodies: rabbit anti-CD31, rabbit anti-CD8α and rabbit anti-pimonidazole. Tissues were then incubated with ready-to-use EnVision+ system-HRP

Labelled Polymer Anti-Rabbit (Dako, K4003) for 1 hour as a secondary antibody, followed by signal revelation with Tyramide Signal Amplification system (1:100 in buffer containing 1 M borate, 3 M NaCl, 0.1% Tween 20, pH 7.8 and 0.003% H_2O_2) for 10 min. After antibody elution with citrate buffer (pH6) in 2100 Antigen Retriever (Aptum), the procedure was repeated for the following primary antibody starting from the blocking step. Antibodies and reagents are listed in Supplementary Table S2. Nuclei were counterstained with Hoechst 33342 (Invitrogen, H3570). Slides were mounted with HIGHDEF IHC fluoromount (Enzo, ADI-950-260-0025) and scanned with the Pannoramic 250 Flash III tissue scanner (3DHistech). Images were analyzed with HALO image analysis platform (Indica labs) by using Highplex or Cytonuclear FL modules for cell identification combined with classifiers for quantification of pimonidazole positive and negative areas. To calculate the distances between each CD8 T cell and the nearest $CD31^{pos}$ endothelial cell we designed a dedicated R script that used the position and phenotype of each event identified by HALO.

Electron paramagnetic resonance (EPR)

For in vivo EPR experiments, mice bearing tumors of about 260 mm^3 were injected intratumorally with 50 µL of charcoal suspension (100 mg/mL in 0.9% NaCl containing 3% arabic gum), before CTX or PBS treatment. Longitudinal EPR measurements were started the day after (day 0) and repeated on day 3, 7, 10 and 14. On day 0, ACT was performed and metformin treatment was started. Spectra were acquired on an EPR spectrometer equipped with a low-frequency microwave bridge operating at 1.15 GHz and an extended loop resonator (ClinEPR, Lyme, NH) was used for pO2 measurements, as described by Acciardo *et al*[1]. Typical acquisition parameters were as follows: modulation of amplitude 0.4 G, modulation of frequency 21 kHz, sweep time: 3 seconds, number of scans: 10 to 20. During EPR experiments, animals were kept under inhalational anesthesia with isoflurane (2.5% during anesthesia induction and 1.2% during maintenance) in 1.5 L/min airflow. Acquisition was started 5 min after setting isoflurane to 1.2%. Tumor pO_2 values were obtained by measuring the peak-to-peak EPR signal linewidth, which was then converted into pO_2 by means of a calibration curve.

MC38 tumor rechallenge

Gender matched, 7-9 weeks old CD57BL/6 WT female mice were injected subcutaneously into the right flank with 10^6 MC38 colon carcinoma cells. When tumors reached around 100 mm^3 mice, mice were injected with four i.p. injections of anti-PD1 antibody (Bio-X-Cell, RMP1-14) at 200 µg/mouse every three days and treated with metformin (Enzo ALX-270-432, 0.5 mg/mL in drinking water) for 27 days. Mice that rejected first tumor were rechallenged 30 days after with 10^6 MC38 cells injected subcutaneously into the left flank. In parallel, naïve mice were injected with the same cells as control.

8-OHdG staining and quantification

After 48 hours of incubation with or without metformin, in normoxia or hypoxia, equal numbers of CD8 T cells were fixed in cold methanol at -20°C for 10 min, washed in PBS buffer and treated as described by Ohno *et al*[2]. RNA was eliminated by 1 hour

incubation at 37°C with 2.5 mg/mL RNAse A (Sigma, 9001-99-4) in buffer containing 10 mM Tris-HCl (pH 7.5) and 15 mM NaCl. After 3 washes in PBS buffer supplemented with 0.1% Triton (PBS-T), cells were treated with HCl 2N for 5 min, and then incubated in Tris-base (50 mM) for 10 min. After 3 washes in PBS cells were permeabilized in PBS supplemented with 1% Triton X-100 for 10 min and then washed 3 times in PBS. Cells were then resuspended in a saponin buffer consisting of PBS with 0.05% saponin, 0.1% glycine, 1% FBS, 1% bovine serum albumin (BSA), 0.05% sodium azide supplemented with 20% pre-immune donkey serum (Sigma, D9663), and incubated for 30 min at RT. Cells were then incubated overnight at 4°C with rabbit anti-8-OHdG antibody (Bioss, bs-1278R) diluted 1:100 in saponin buffer supplemented with 10% pre-immune donkey serum. After 3 washes in saponin buffer, cells were incubated for 1 hour at RT with donkey anti-rabbit AF647 secondary antibody (1:500, Thermo Fisher Scientific, A31573) and Hoechst 33342 (1:2000, Invitrogen, H3570) in saponin buffer supplemented with 10% pre-immune donkey serum. Cells were then washed once in saponin buffer, twice with PBS containing 0.05% saponin and once in PBS. Cells were transferred in 96 well assay plate (Corning, 3603). Images were acquired on ImageXpress PICO Automated Cell Imaging System (Molecular Devices) and analyzed using the CellReporterXpress Image Acquisition and Analysis Software (version 2.9.1.23580). Analysis was performed by using the Cell Scoring analysis to identify 8-OHdG-positive cells out of total Hoechst-positive cells.

CD8 T-cell nucleofection

PRKAA1 KO CD8 T cells were generated by electroporation of Cas9 ribonucleoprotein complexes as described by Seki *et al*[3]. Briefly, Alt-R crRNA and Alt-tracrRNA (Integrated DNA technologies) were mixed in equimolar concentrations and annealed by heating at 95°C for 5 min and slowly cooled to room temperature. crRNA-tracrRNA duplexes (150 pmol) were incubated with TrueCut Cas9 protein v2 (60 pmol, Thermo Fisher Scientific, A36496) for 10 min. 10^7 activated CD8 T cells were resuspended in 80 µL P4 Primary Cell 4D-Nucleofector X kit kit (Lonza, V4XP-4024) and incubated with 20 µL RNP for 2 min. Electroporation was performed in 4D nucleofector X Unit (Lonza) using CM137 nucleofector program. After electroporation, cells were cultured in complete medium supplemented with rhIL-2 and let resting for 4 days. The following crRNA and tracrRNA were purchased from Integrated DNA technologies.

Alt-R® CRISPR-Cas9 tracrRNA (1072534)
Alt-R® CRISPR-Cas9 Negative Control crRNA #1 (1072544)
Mm.Cas9.PRKAA1.1.AD (5'CAGATGGTGTACTGAACC)
Mm.Cas9.PRKAA1.1.AE (5'GAGGGTGCCTGAACAGCTTC)
Mm.Cas9.PRKAA1.1.AF (5'GGCACATGGTCATCATCAA)

MTS assay

Cell survival was determined by the MTS assay based on reduction of the MTS tetrazolium compound by NAD(P)H-dependent dehydrogenases. The assay was performed according to the specifications of the manufacturer (Promega, G1112).

Briefly, MC38 or T429.11 cells were seeded 96-well plates (5000 cells/well) and cultured for 24h in presence of increasing doses of 4-hydroperoxy-cyclophosphamide (Cayman, 19527). Following treatments, combined MTS and PMS solution was added directly to the culture medium and incubated for 2 hr in a humidified atmosphere. Absorbance of samples was measured at 490 nm (630 nm reference wavelength) with a Microplate Reader (Promega). MTS reduction levels were expressed as a percentage of control.

Western blot

For protein extraction, cells were lysed in cold RIPA buffer supplemented with Halt Phosphatase inhibitor cocktail (ThermoFisher Scientific, 78446) for 20 min on ice. After elimination of cell debris by centrifugation, proteins were quantified by Pierce Protein BCA Assay Kit (ThermoFisher Scientific, #23225). For HIF-1α protein detection, nuclear proteins were extracted using NE-PER nuclear and cytoplasmic extraction reagents (Themo Fisher scientific, 78833) according to manufacturer's instructions. 30 µg of total proteins were denaturated at 95°C for 5 min in NuPAGE LDS sample buffer (Thermo Fisher Scientific, NP0007), loaded on NuPAGE 3% - 8% gels (Thermo Fisher Scientific, EA0378BOX) and separated by gel electrophoresis using NuPAGE Tris-acetate SDS Running Buffer (Thermo Fisher Scientific, LA0041). Dry transfer was performed by iBlot (Thermo Fisher Scientific) using iBlot Gel Transfer Stacks, nitrocellulose (ThermoFisher Scientific, IB23002) and a transfer program P0 (1 min at 20 V, 4 min at 23 V, 2 min at 25 V). After blocking in TBS 0.1% Tween 20 (TBS-T) with 5% nonfat dry milk (Nestlé) for 1 hour at RT, the membranes were incubated overnight at 4°C with primary antibodies (Suppl table 3). After washing in TBS-T, membranes were incubated with HRP-linked secondary antibody diluted in TBS-T containing 5% nonfat dry milk (Nestlé) for 1 hour at RT. HRP signal was revealed using SuperSignal West Pico or Femto (Thermo Fisher Scientific, 34580-34095) and detected with Fusion Solo S (Vilber Lourmat). Antibodies and reagents are listed in Supplementary Table S3.

Human CD8 T-cell activation

Upon approval from the Comité d'Ethique Hospitalo-Facultaire Saint-Luc–UCLouvain, CD8 T cells were isolated from peripheral blood mononuclear cells of hemochromatosis patients with CD8 MicroBeads (Miltenyi, 130-045-201) and co-cultured with irradiated (10.000 rads) Epstein-Barr virus transformed B cells (LG2-EBV) in $3x10^5$ T cells to 10^6 LG2-EBV ratio in presence of $3x10^4$ CD3/CD28 beads (Gibco, 11131D) and 25 U/mL rhIL-2 in complete IMDM medium containing 10% human serum.

SUPPLEMENTARY TABLES

Supplementary Table S1

Flow cytometry reagents

	Reference	Dilution
7AAD	Thermofisher, A1310	1:1000
Annexin V APC	Biolegend, 640941	1:40
Annexin V FITC	Biolegend, 640906	1:40
Annexin V buffer	BD Biosciences, 556454	1:10
Anti-CD4, cl GK1.5	Biolegend, 100438	1:200
Anti-CD8α, cl 53-6.7	Biolegend, 100732, 100738	1:200
Anti-CD11b, cl M1/70	Biolegend, 101222	1:200
Anti-CD16/CD32 blocking antibody, cl 2.4G2	BD Biosciences, 553141	1:100
Anti-CD25, cl 3C7	Biolegend, 101904	1:200
Anti-CD45, cl 30-F11	Biolegend, 103128	1:200
Anti-CD107a, cl 1D4B	Biolegend, 121618	1:200
Anti-FoxP3, cl FJK-16s	eBioscience, 17-5773-82	1:100
Anti-IFNy, cl XMG1.2	Biolegend, 505810	1:20
Anti-Lag3, cl C9B7W	Biolegend, 125221	1:50
Anti-Lag3, cl11C3C65	Biolegend, 369313	1:100
Anti-Ly6C, cl HK1.4	Biolegend, 128027	1:200
Anti-Ly6G, cl 1A8	Biolegend, 127614	1:200
Anti-pimonidazole	Hydroxyprobe, HP-FITC-Mab 4.3.11.3	1:100
Anti-PD-1, cl 29F.1A12	Biolegend, 135214	1:100
Anti-PD-1, cl EH12.2H7	Biolegend, 329907	1:100
Anti-TNFα, cl MP6-XT22	Biolegend, 506349	1:40
PE-conjugated H-2L^{d}/P1A$_{35-43}$ tetramer	Homemade (1μM)	1:20
Viability dye eFluor780	ThermoFisher Scientific, 65-0865	1:1000

Supplementary Table S2

Immunohistochemistry reagents

	Reference	Dilution
Rabbit anti-CD31	Abcam, EPR17259	1:2000
Rabbit anti-CD8a	Cell signaling, D4W2Z	1:400
Rabbit anti-pimonidazole	Hydroxyprobe, HP3-100kit	1:100
Alexa Fluor 488 tyramide reagent	Thermo Fisher, B40953	1:100
Alexa Fluor 594 tyramide reagent	Thermo Fisher, B40957	1:100
Alexa Fluor 647 tyramide reagent	Thermo Fisher, B40958	1:100

Supplementary Table S3

Western blot reagents

	Reference	Dilution
Halt Phosphatase inhibitor cocktail	Thermo Fisher Scientific, 78446	1:200
Pierce protein BCA assay kit	Thermo Fisher Scientific, 23225	
NuPage Tris-acetate SDS Running buffer	Thermo Fisher Scientific, LA0041	
Rabbit anti-AMPKα	Cell Signaling, 2532	1:1000
Rabbit anti-phospho AMPKα	Abcam, ab133448	1:1000
Rabbit anti-Acetyl-CoA Carboxylase	Cell Signaling, 3676	1:1000
Rabbit anti-phospho Acetyl-CoA Carboxylase	Cell Signaling, 11818	1:1000
Rabbit anti-HIF-1α	Cayman chemicals, 10006421	1:3000
HRP-anti-HDAC1	Cell Signaling, 59581	1:5000
HRP anti-βtubulin	Abcam, ab21058	1:10000
Goat HRP-anti-rabbit IgG	Cell signaling, cl 7074S	1:5000

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