Suppl Fig 2

A

PBS

PBS+Metformin

CTX

CTX+Metformin

Days after treatment

Tumor volume (mm$^3$)

0 4 7 9 11 1517 20 23 25

Days after treatment

Tumor volume (mm$^3$)

0 4 7 9 11 1517 20 23 25

Days after treatment

Tumor volume (mm$^3$)

0 4 7 9 11 1517 20 23 25

Days after treatment

Tumor volume (mm$^3$)

0 4 7 9 11 1517 20 23 25

Days after treatment

B

Transplanted TiRP tumors

WT

WT

WT

Rag1 KO

Rag1 KO

Rag1 KO

ACT

ACT

ACT

ACT

Days after treatment

Tumor volume (mm$^3$)

0 2 5 7 9 11 12

Days after treatment

Tumor volume (mm$^3$)

0 2 5 7 9 11 12

Days after treatment

Tumor volume (mm$^3$)

0 2 5 7 9 11 12

Days after treatment

Tumor volume (mm$^3$)

0 2 5 7 9 11 12

Days after treatment

C

Tumor growth MC38

B6

B6

B6

NSG

NSG

NSG

Days after treatment

Tumor volume (mm$^3$)

- - + ns

- - + ns

- - + ns

Days after treatment

Tumor volume (mm$^3$)

- - + ns

- - + ns

- - + ns

Days after treatment

Tumor volume (mm$^3$)

- - + ns

- - + ns

- - + ns

Days after treatment

Tumor volume (mm$^3$)

- - + ns

- - + ns

- - + ns

Days after treatment

D

MTS assay

Cell viability (%)

0 1 5 10 50 100

4H-CTX (µM)

MC38

T429.11
Suppl Fig 3

A. Tumor-infiltrating PMN-MDSC

B. Tumor-infiltrating Treg

C. Cxcl9 mRNA expression in TiRP tumors 4 days after ACT

D. Cxcl10 mRNA expression in TiRP tumors 4 days after ACT

E. Cxcl9 mRNA expression in TiRP tumors 4 days after ACT

F. Cxcl10 mRNA expression in TiRP tumors 4 days after ACT

G. Il-7 mRNA expression in TiRP tumors 4 days after ACT

H. Il-2 mRNA expression in TiRP tumors 4 days after ACT
Suppl Fig 5

A  Tumor hypoxia 4 days after ACT (FACS)

B  Tumor hypoxic areas 4 days after ACT (IF)
Suppl Fig 6

A

B

C

Tumor hypoxia in LLC-OVA

Hypoxic CD8 TILs in LLC-OVA

Metformin - +

Metformin - +

100 µm

100 µm

Hoechst Pimonidazole CD8

H2O

Hoechst Pimonidazole CD8

Metformin

**Suppl Fig 7**

A) Cell proliferation in TCRP1A CD8 T cells after 72h

B) Cellular ROS in TCRP1A CD8 T cells at 24h

C) Early apoptosis in TCRP1A CD8 T cells at 24h

D) Cell proliferation in TCRP1A CD8 T cells at 72h

E) Cellular ROS in TCRP1A CD8 T cells at 72h

F) Mitochondrial ROS in TCRP1A CD8 T cells at 72h

G) Western blot analysis of pAMPK alpha, AMPK alpha, Tubulin, Prkka1gRNA, Metformin, HIF-1α, HDAC1

H) Early apoptosis in TCRP1A CD8 T cells after 72h at 1% O2

I) Late apoptosis in TCRP1A CD8 T cells after 72h at 1% O2

J) Cell proliferation in TCRP1A CD8 T cells after 72h at 1% O2

K) ROS in TCRP1A CD8 T cells after 72h at 1% O2

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Suppl Fig 8

A

B16F1 Tumor growth

- CTX Met α-PD-1
- - - - - - - - - - - ns
- - - - - - - - - - - ns

Days after treatment

1 3 5 7 10

Tumor volume (mm³)

1500

500

0

B

B16F1: PBS + Isotype

B16F1: PBS + Metformin + Isotype

B16F1: PBS + anti-PD-1

B16F1: PBS + Metformin + anti-PD-1

Days after treatment

1 3 5 7 10

Tumor volume (mm³)

2000

1000

0

C

Tumor-infiltrating CD8 T cells in hypoxic areas in B16

% of alive pimonidazole+ CD8+ cells

25

20

15

10

5

0

p=0.06

CTX

PD-1

Metformin

- - - - - - - - - - -

D

Apoptosis of tumor-infiltrating CD8 T cells in B16 tumors

Annexin

Pimonidazole

Pimonidazole

Pimonidazole

E

MC38 Tumor growth

10⁶ MC38 cells (1st injection)

Day 0

Day 7

anti-PD-1

Day 34

Metformin in drinking water

Day 64

10⁶ MC38 cells (2nd injection)

MC38 Tumor growth

0 3 5 7 10 13 16

Days after MC38 injection

0

50

100

150

200

250

Nave

Rechallenged
Suppl Fig 9

A. Early apoptosis of human CD8 T cells at 72h

B. Mitochondrial ROS in human CD8 T cells at 72h

C. IFNγ mRNA expression

D. TNFα mRNA expression
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% O2) or hypoxia (1% O2) 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% O2) or hypoxia (1% O2) in absence 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 fluorescence 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 mm³, 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<sup>flox/flox</sup> (WT) mice or in TiRP Rag1 KO mice; when the tumor size was around 400 mm³, 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 mm³, 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
Supplementary material

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 mm³ 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 mm³, 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 mm³, 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-way analysis of variance with Tukey’s multiple comparison correction.

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 mm³, 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;
Supplemental material

Supplemental 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 mm³, 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.

Supplemental 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.

Supplemental 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% O₂) or hypoxia (1% O₂) 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% O₂) or hypoxia (1% O₂) 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% O₂) 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 10⁶ MC38 cells in right flank (1st 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 10⁶ MC38 cells injected in left flank. Naïve mice were used as control. (Naïve: 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% O₂) or hypoxia (1% O₂) 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-2Ld P1A35-43 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% O2) or hypoxia (1% O2), 5 000 CD8 T cells were seeded in fresh medium pre-incubated at 21% O2 or 1% O2 and co-cultured at 21% O2 or 1% O2, in presence or absence of metformin, with 3 000 P511 cells or with P204.1 cells that were pulsed or not with P1A35-43 (LPYLGLWLVF) 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-γ (Hs.PT.58.3781960), human TNFα (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-CTG-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 rehydration, 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$_2$O$_2$) 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$^{+}$ 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 pO$_2$ 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 over night 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. 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. 10⁷ 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'CAGATGAGTACTGAACC)
- 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 (Thermo Fisher 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 cocultured 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

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### Supplementary Table S2

#### Immunohistochemistry reagents

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### Supplementary Table S3

#### Western blot reagents

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REFERENCES

