Supporting Information

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SI Materials and Methods

Methylchoranthrene-Induced Carcinogenesis Assay. Mice were s.c. injected with methylcholanthrene (200 μg; Sigma-Aldrich) dissolved in peanut oil. Mice with tumor masses that exceeded 10 mm in diameter were recorded as tumor positive.

Antibody Administration. The mice were anesthetized with ether, after which a 50-μL volume of anti-L3T4 ascites (CD4) or anti–Lyt-2.2 (CD8) mAbs diluted in PBS to a total dose of 200 μL per mouse was injected through the retrobulbar venous plexus.

Induction of CTLs and Functional Analysis. Splenocytes (1×10^7) from mice that had once rejected RLmale1 following metformin administration and were resistant to secondary tumor challenge were obtained on day 45 and cultured with 1 μM pRL1a (IPGLPLSL) for 5 d. The resulting $CD8^+$ T cells were isolated from the splenocytes via magnetic separation (Miltenyi Biotec), and the cells (5×10^4) were stimulated with 0.1% paraformaldehyde-fixed cells from the mouse lymphoblast-like mastocytoma cell line P815 (5×10^3) prepulsed with graded doses of peptide in a 96-well round-bottom culture plate for 24 h at 37 °C and 5% CO_2. The stimulated culture supernatants were collected, and the amount of secreted IFNγ was measured using ELISA.

Isolation of TILs. BALB/c or C57BL/6 mice were intradermally inoculated with 2×10^5 RLmale1 or MO5 cells on the right dorsal side. The tumor tissues were dissected from the mice and minced into small pieces in RPMI medium 1640 (Life Technologies). TILs were harvested from the minced tumor tissues using the Medimachine system (AS ONE). Lymphocytes (TILs) could be distinguished from tumor cells as their size is less than a half of tumor cells under microscope. Then, we stained all cells including TILs and tumor cells with indicated fluorescence-labeled antibodies and subjected them onto Flow cytometric analysis.

Flow Cytometric Analysis. Cells were washed and incubated with mAbs for 30 min at 4 °C in 5 mM EDTA and PBS containing 2% FCS (FACS staining buffer). The following mAbs were used: APC-Cy7–conjugated anti-mouse CD8α (BD Biosciences), PE-Cy7-conjugated anti-mouse CD4 (eBioscience), PE-conjugated anti-mouse PD-1 (eBioscience), Alexa Fluor 647-conjugated anti-mouse Tim-3 (BioLegend), PE-conjugated anti-mouse CD62L (eBioscience), FITC-conjugated anti-mouse CD44 (eBioscience), and APC-conjugated anti-mouse KLRG1 (eBioscience) for cell surface marker staining as well as PE-Cy7-conjugated anti-mouse IL-2 (BD Biosciences), PerCP/Cy5.5-conjugated anti-mouse TNFα (BD Biosciences), and FITC-conjugated anti-mouse IFNγ (eBioscience) for intracellular cytokine staining. PE-labeled anti-cleaved caspase-3 (Asp175) and Alexa Fluor 647-conjugated anti-pS6 (S235/236) (Cell Signaling Technology) were used for the detection of early apoptosis and mTOR downstream signaling, respectively. Intracellular cytokine staining was performed with a Fixation/Permeabilization kit (BD Biosciences). Early apoptosis was detected using Annexin-V-FITC (Sigma-Aldrich). After treatment, the cells were washed, suspended in FACS staining buffer, and analyzed on a FACSCanto II flow cytometer (BD Biosciences). We determined a suitable gate for lymphocytes (TILs), compared with that of spleen cells. Tumor cells are always larger than TILs, as determined by FSC-A and SSC-A. The gated populations for lymphocytes (TILs) were identified as $CD4^+$ $CD8^-$, $CD4^-CD8^+$, and double negative cells. On the other hand, the gate for RLmale1 tumor cells identified only $CD4^+CD8^+$ population, a unique phenotype of RLmale1 cells as thymus-derived radiation-induced leukemia cells (they also express CD3). The numbers of total TILs, $CD8^+$TILs and $CD4^+$TILs were calculated by the % populations of those cells and microscopically counted numbers of lymphocytes.

Multifunctional Analysis of Peptide-Stimulated TILs. $CD8^+$ T cells were isolated from TILs via magnetic separation. The purified $CD8^+$ T cells (1×10^5 per well) were cultured with $OVA_{257–264}$ peptide (SIINFEKL; 1 μM) or control peptide (NY-ESO-$1_{91–98}$: YLAMPFAT; 1 μM)-pulsed DC2.4 cells (5×10^5 per well) in a total volume of 200 μL per well of a 96-well plate for 8 h in the presence of a Golgi stop solution containing Monensin (BD Biosciences) at 37 °C in a 5% CO_2 atmosphere.

After the stimulation culture, the cells were harvested and labeled with APC-Cy7-conjugated anti-mouse CD8, PE-conjugated anti-mouse PD-1, and Alexa Fluor 647-conjugated anti-mouse Tim-3, followed by intracellular cytokine staining for IL-2, TNFα, and IFNγ and flow cytometric analysis on a FACSCanto II.

Tetramer Staining. TILs were harvested as described above and incubated with PE-conjugated H-$2K^b$ OVA (SIINFEKL) and H-$2K^b$ $TRP2_{180–188}$ tetramers (Medical and Biological Laboratories) for 30 min at 4 °C, followed by incubation with APC-Cy7-conjugated anti-mouse CD8α (Alpha Biomedical; APC-Cy7 conjugation was performed in our laboratory) for 30 min at 4 °C and analysis on a FACSCanto II.

Adoptive Cell Transfer Experiment. CD45.1/OT-I $CD8^+$ T cells were isolated from the splenocytes of OT-I transgenic mice via magnetic separation and were adoptively transferred (2×10^6) on day 7 into B6 mice (CD45.2) that had been inoculated with 3×10^5 MO5 cells on the right dorsal side. Simultaneously, 10 μg of the OVA-mHSP70 fusion protein was i.v. injected and 5 mg/mL of metformin was orally administered per mouse. Three days later, each right inguinal lymph node and each tumor tissue was resected, processed into single-cell suspensions, and pooled. These cells were stimulated with 1.25 ng/mL of PMA and 50 nM ionomycin for 6 h at 37 °C and 5% CO_2 in the presence of Golgi stop, followed by staining with APC-Cy7-conjugated anti-mouse CD8, PE-conjugated anti-mouse CD45.1 (BD Biosciences), and Alexa Fluor 647-conjugated anti-mouse Tim-3 and intracellular staining for IL-2, TNFα, and IFNγ. In another adoptive transfer experiment, the transferred OT-I $CD8^+$ T cells were pretreated in vitro with metformin (10 μM) for 6 h with or without compound C (5 and 50 μM). Two days after transfer, the cells were recovered from the spleens and tumors and subjected to a multifunctionality evaluation.

Intracellular FoxP3 Staining. Intracellular FoxP3 staining was performed using an anti-mouse FoxP3-FITC (eBioscience) staining buffer set (eBioscience) according to the manufacturer's instructions.

Immunoblot Analysis. $CD8^+$ T cells were purified from tumor tissues via a magnet-based purification system (Miltenyi Biotec), lysed, and subjected to immunoblot analysis. Lysates were prepared by suspending cells in lysis buffer (PBS, 1% Nonidet P-40, 1 mM PMSF). The lysates were cleared by centrifugation and subjected to electrophoresis on a SDS (SDS)–polyacrylamide gel. The proteins were then transferred to nitrocellulose membranes, blocked with 1–10% dry milk in TBS-T [TBS (10 mM Tris·HCl pH 7.5, 135 mM NaCl) + 0.05% Tween-20]. Antigen–antibody

complexes were visualized by chemiluminescence (ECL). Anti-AMPKα (1:1,000 dilution), anti-pAMPKα (Thr172; 1:1,000), anti-AMPKβ (1:1,000), anti-pAMPKβ (Ser108; 1:1,000), and anti–p-ACC (S79; 1:500) antibodies were purchased from Cell Signaling Technology. Anti-Bat3 (1:1,000; Abcam), anti-Bag1 (1:1,000; Medical and Biological Laboratories), anti-Bcl2 (1:1,000; Gene Tex), and anti-Bax (1:1,000; Gene Tex) were also used for Western blot analysis. Immunoblot images were cut and rearranged to remove irrelevant information; however, all lanes were obtained from the same blot with the same levels of exposure and contrast.

Measurement of Caspase-3 Activation. Caspase-3 activity was measured in a colorimetric assay using tetrapeptide methyl cumaryl amide (MCA) substrates. $CD8^+$ T cells were purified from tumor tissues via a magnet-based purification system (Miltenyi Biotec), lysed, and subjected to an immunoblot analysis. Lysates were also prepared by suspending cells in lysis buffer (25 mM Tris·HCl pH 7.5, 250 mM sucrose, 1 mM PMSF, 1 mM DTT, 1% Nonidet P-40). The assays were performed in 96-well black plates by incubating 10 μL (1×10^5) of cell lysate with 89 μL of reaction buffer (25 mM Tris·HCl pH 7.5, 250 mM sucrose, 1 mM PMSF, 1 mM DTT) containing 1 μL of peptide substrate (10 mM). The assays incorporated Acetyl-Asp-Asn-Leu-Asp-MCA (Peptide Institute). Absorbance at OD360/450 was monitored at 37 °C for 3 h using a Powerscan HT (DS Pharma Biomedical). For certain experiments, the cell lysate was diluted in the assay buffer as indicated.

Immunohistochemistry. Tumor tissues were removed and fixed by 4% PFA for 48 h. Consecutive 4 μm-thick sections were cut from each trimmed paraffin block. In brief, following deparaffinization, sections were rehydrated, treated with citrate buffer at 96 °C for 30 min, and treated with 3% H_2O_2. The sections were incubated for overnight at 4 °C with specific antibodies to Ki67 antigen (clone D3B5; Cell Signaling Technology(CST) Japan, Tokyo, Japan; 1:200) and cleaved caspase-3(Asp175) (clone 5A1E; CST Japan; 1:2,000). The slides were incubated with secondary antibodies (goat anti-rabbit HRP conjugated, CST Japan). The immunostaining was visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB) for 1–3 min and counterstained with hematoxylin for 5 min. Staining controls were prepared by replacing the primary antibody to buffer solution. All analyses were performed under a light microscope (4× or 40×).

Statistical Evaluation. Student's *t* test was used for statistical evaluations of normally distributed data.

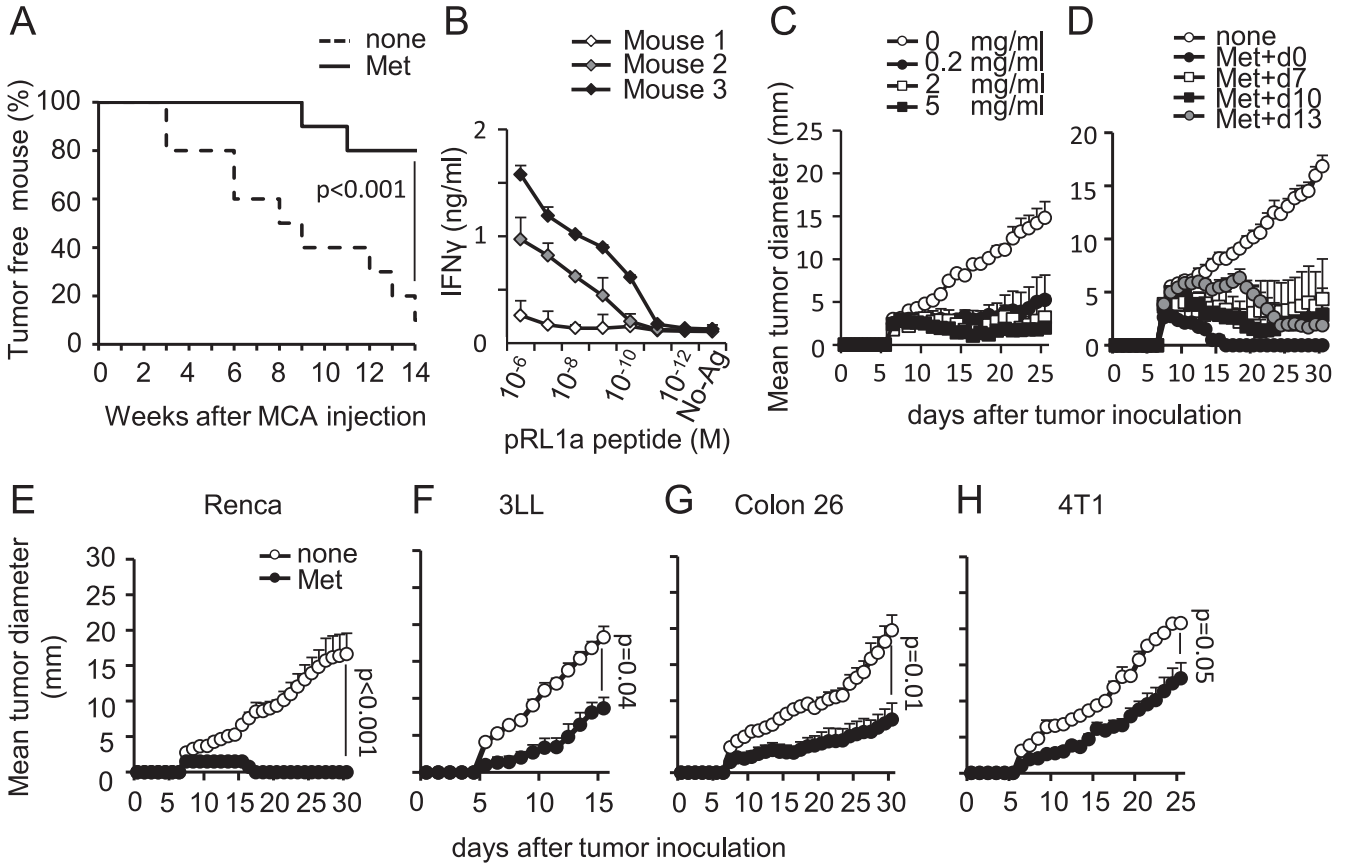

Fig. S1. Metformin prevented methylchoranthrene-induced carcinogenesis and suppressed growth of variety of tumor cells in vivo. (*A*) BALB/c mice were s.c. injected methylchoranthrene (200 μg per head) solved in peanut oil. At the same time, the mice were orally administrated metformin (Met) (5 mg/mL) solved in free drinking water, throughout entire period of monitoring tumor incidence. Mice with a tumor mass whose diameter reached ≥ 10 mm were recorded as tumor positive. $n = 10$ in each group. (*B*) Three of the mice that had rejected the tumor as in Fig.1A were killed and their spleen cells were in vitro stimulated with pRL1a peptide (10^{-6} M) for five days. The resulting effector cells were purified as $CD8^+$T cells by magnet and restimulated with graded doses of pRL1a peptide pulsed P815 cells in triplicates, as indicated, for 24 h and IFNγ secreted into the culture supernatant was measured by ELISA. The data were plotted with SD. Effector to target cell ratio was 10. (*C*) Mice inoculated with 2×10^5 RLmale1 cells were treated with metformin at different concentrations (0, 0.2, 1, 5 mg/mL) as indicated from day 7. Average tumor diameters are plotted with SE. $n = 4$ in each group. (*D*) Mice inoculated with RLmale1 cells were treated with metformin (Met) or not (none), starting at day 0, 7, 10, and 13 as indicated. Average tumor diameters are plotted with SE. $n = 5$ in each group. (*E–H*) Renca (renal cell carcinoma, BALB/c) cells (*E*), 3LL (non small cell lung carcinoma, B6) cells (*F*), Colon 26 (intestinal carcinoma, BALB/c) cells (*G*), and 4T1 (breast cancer, B6) cells (*H*) were intradermally injected onto syngeneic mice, and their average tumor diameters were monitored and plotted with SE. Free drinking water with or without metformin (5 mg/mL) was given to those mice. $n = 5$ in each group in *E–H*. The results are representative of two independent experiments.

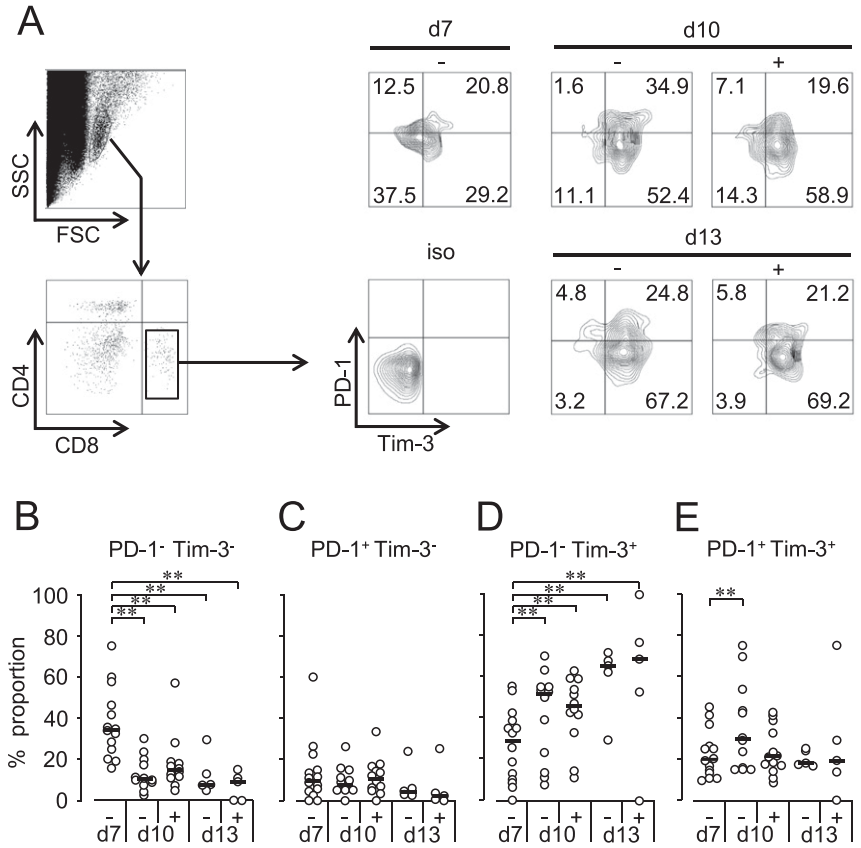

Fig. S2. FACS analysis of CD8$^+$TILs on expressions of PD-1/Tim-3. TILs were recovered on day 7,10 and 13 from metformin-treated or not treated mice inoculated with 2×10^5 RLmale1 cells and labeled with antibodies to CD8, CD4, PD-1, Tim-3. Lymphocytes gated by FSC and SSC were analyzed by CD4 and CD8 expressions at first. Then, expressions of PD-1/Tim-3 (*A*) were investigated. The populations of PD-1$^-$Tim-3$^-$ (*B*), PD-1$^+$Tim-3$^-$ (*C*), PD-1$^-$Tim-3$^+$ (*D*), and PD-1$^+$ Tim-3$^+$ (*E*) out of CD8$^+$TILs were plotted. The horizontal bars indicate median values, and *P* values obtained by two-tailed Student's *t* test are shown as $*P < 0.05$, $**P < 0.01$. $n = 5$–14 in each group on day 7, 10 and 13 with (+) or without (–) metforimin.

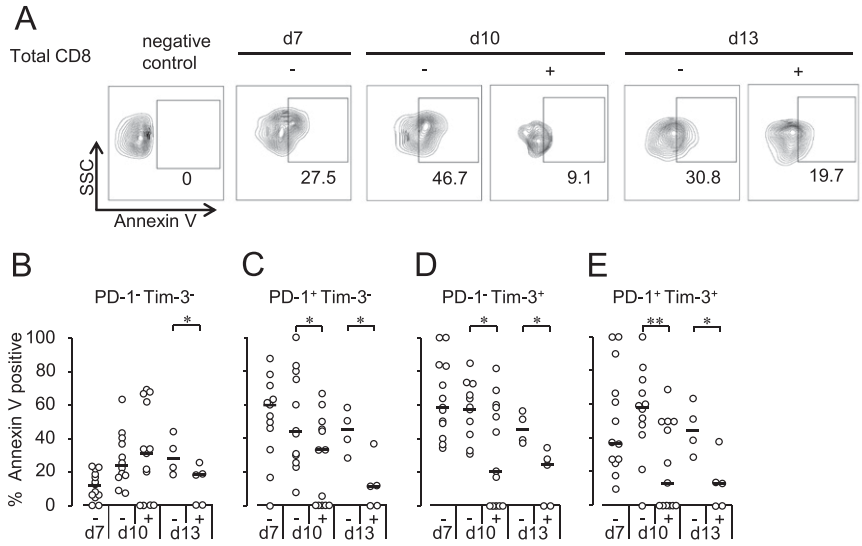

Fig. S3. FACS analysis of CD8$^+$TILs on expressions of PD-1/Tim-3 and Annexin V binding. TILs shown in Fig.S2 were examined for Annexin V binding. Lymphocytes gated as CD8$^+$T cells were analyzed for their binding of Annexin V (*A*). The Annexin V (+) populations of PD-1$^-$Tim-3$^-$ (*B*), PD-1$^+$Tim-3$^-$ (*C*), PD-1$^-$Tim-3$^+$ (*D*), and PD-1$^+$Tim-3$^+$ (*E*) among CD8$^+$TILs were plotted. The horizontal bars indicate median values, and *P* values obtained by two-tailed Student's *t* test are shown as $*P < 0.05$, $**P < 0.01$ $n = 5$–14 in each group. Each symbol represents an individual mouse. The results depicted are a summary of three independent experiments.

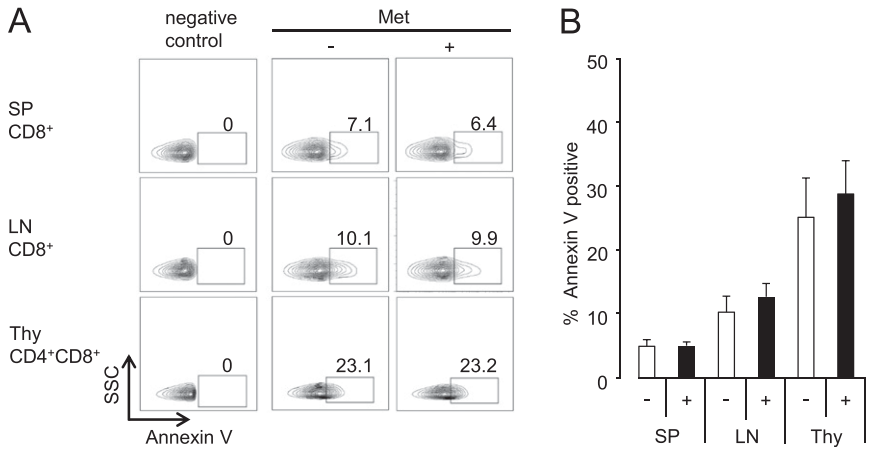

Fig. S4. Physiologically essential apoptosis of $CD4^+CD8^+$ thymocytes are not inhibited by metformin administration. BALB/c mice were orally administrated metformin (5 mg/mL) and three days later, spleen cells (SP), lymph node cells (LN) and thymocytes (Thy) were obtained. The cells were stained with antibodies to CD4 and CD8, or Annexin V. $CD8^+$T cells of SP and LN, and $CD4^+CD8^+$ thymocytes were investigated on binding to Annexin V. The FACS data are representative of three mice (*A*) and the results are shown as bar graphs (*B*), with the mean ± SD, $n = 3$ in each group.

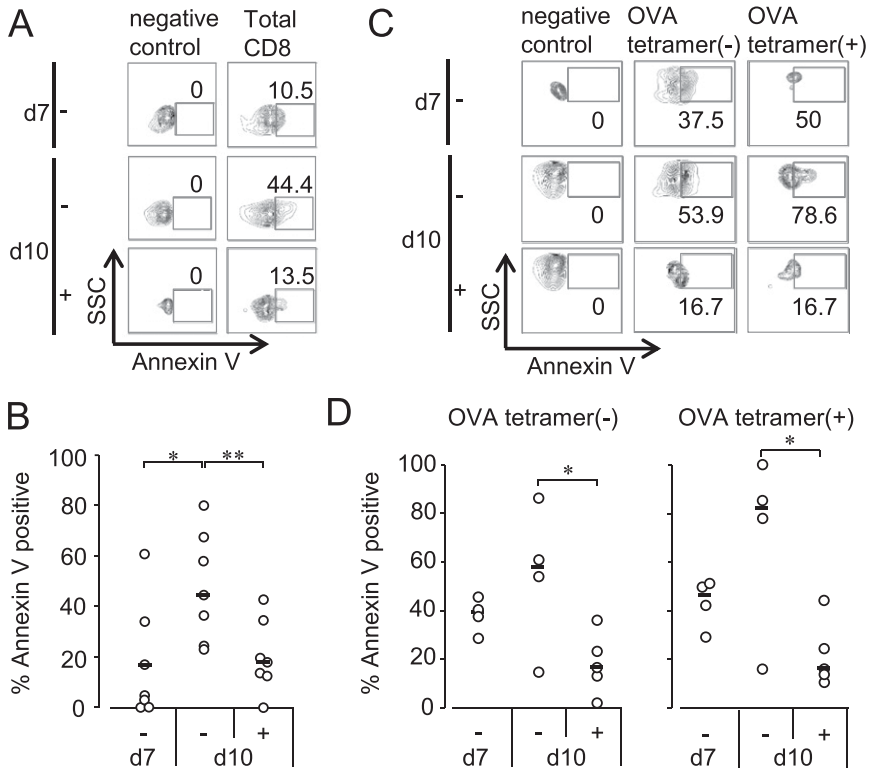

Fig. S5. Metformin prevents antigen-specific $CD8^+$ TILs from apoptosis. Mice inoculated with 2×10^5 MO5 cells were treated with (+) or without (–) metformin from day 7. (*A*) On days 7 and 10, TILs were recovered from the tumor masses, and $CD8^+$ TILs were examined for Annexin V binding. The results are representative of seven individual mice. (*B*) The percentages of Annexin V-positive cells among the $CD8^+$ TILs isolated from the seven mice, as indicated in *A*, were plotted. (*C* and *D*) TILs recovered on days 7 and 10 were examined for anti-CD8 mAbs, $OVA_{257–264}$-K^b tetramer, and Annexin V binding. The percentages of Annexin V-positive cells among the $CD8^+$ TILs from each group [$n = 4$ for day 7 metformin (–), $n = 4$ for day 10 metformin (–), and $n = 5$ for day 10 metformin (+)] were plotted in *D*. The FACS data in *C* are representative of *D*. $^*P < 0.05$, $^{**}P < 0.01$.

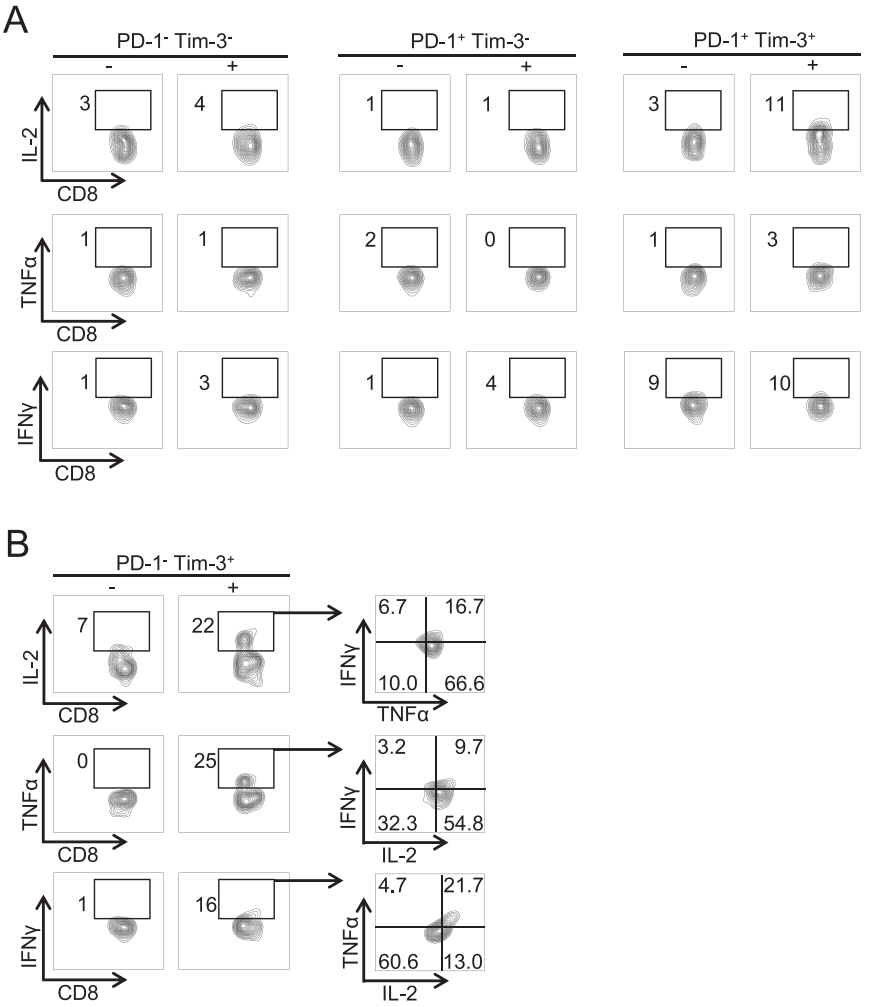

Fig. S6. Metformin-induced multifunctional CD8$^+$ TILs are PD-1$^-$Tim-3$^+$. TILs were isolated on day 10 from 5 to 6 mice per group that had been inoculated with RLmale1 cells on day 0 and treated with (+) or without (–) metformin beginning on day 7; the cells were pooled and stimulated with PMA/ionomycin for 6 h, followed by staining for surface molecules such as CD8, PD-1, and Tim-3 and intracellular staining for IL-2, TNFα, and IFNγ. (*A*) FACS data indicating the cytokine production patterns of PD-1$^-$Tim-3$^-$ (*Left*), PD-1$^+$Tim-3$^-$ (*Center*), and PD-1$^+$Tim-3$^+$ (*Right*) CD8$^+$TILs are shown in the context of metformin (+) or (–). The numbers within the small squares indicate the percentage of positive cells producing the indicated cytokines. (*B*) PD-1$^-$Tim-3$^+$ CD8$^+$ TILs producing IL-2 (*Top*), TNFα (*Middle*), or IFNγ (*Bottom*) were further analyzed for the production of TNFα and IFNγ, IL-2 and IFNγ, and IL-2 and TNFα, respectively. Of note, cells capable of producing triple cytokines (IL-2, TNFα, and IFNγ) belonged exclusively to the PD1$^-$Tim3$^+$ CD8$^+$ TIL population.

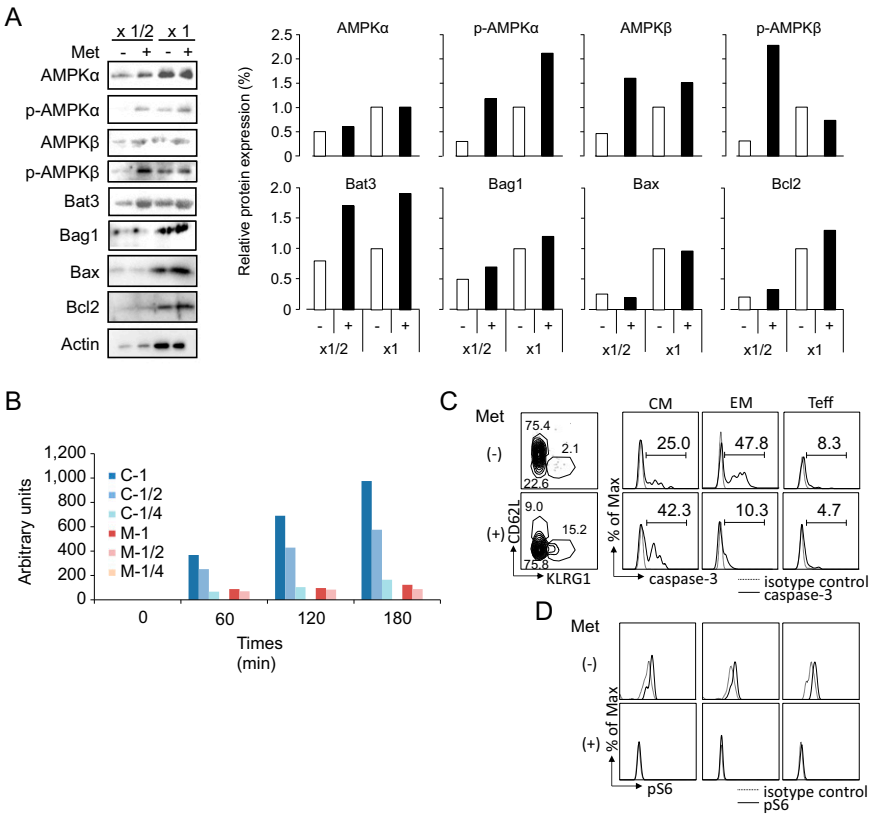

Fig. S7. AMPK phosphorylation, increased Bat3 expression, and down-regulated caspase-3 activity in $CD8^+$ TILs isolated from mice following metformin administration. (*A*) $CD8^+$ TILs were isolated on day 10 from 10 mice that had been inoculated with RLmale1 cells and treated with (+) or without (–) metformin beginning on day 7; the cells were pooled, lysed, and subjected to Western blot analysis with the indicated antibodies. x1 is undiluted lysate from 1×10^5 $CD8^+$ TILs and ×1/2 is a 50% dilution. The band intensities in the left panels are shown as bar graphs in the right panels, as indicated. (*B*) Caspase-3 activity against a fluorescent substrate was investigated. Graduated amounts of cell lysates from $CD8^+$ TILs generated as in *A* were examined for their caspase-3 activity using Powerscan HT. C (control)-1 and M (metformin)-1 indicate lysates from 1×10^5 $CD8^+$ TILs isolated from mice without or with metformin, respectively. C-1/2 and M-1/2 indicate half amounts of C-1 and M-1, respectively, whereas C-1/4 and M-1/4 indicate quarter amounts, respectively. Expression levels of cleaved caspase-3 (*C*) and pS6 (*D*) in $CD8^+$ TIL isolated on day 13 from RLmale1 tumor-bearing mice that had been treated with or without metformin. The recovered TILs were surface stained with antibodies against CD8, CD62L, CD44, and KLRG1 and intracellularly stained with antibodies against cleaved caspase-3 and phosphorylated S6, followed by flow cytometric analysis.

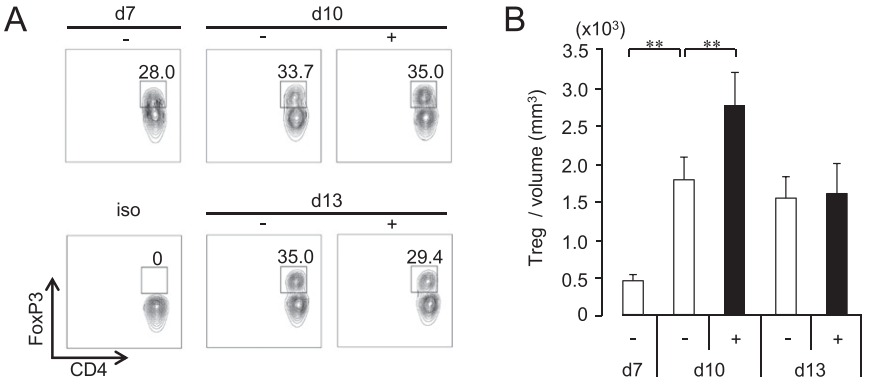

Fig. S8. The population of $Foxp3^+CD4^+$T cells (Treg) within tumor tissues is not suppressed by metformin administration. On day 7, 10, and 13, TILs were recovered from mice inoculated with 2×10^5 RLmale1 cells and stained for CD4 and FoxP3. (*A*) Representative contour plots showing FoxP3 expression on gated $CD4^+$ T cells. (*B*) Numbers of Treg per volume (mm^3) of tumor. All data are expressed as the mean ± SD, $n = 5$–14 in each group. *P* values were obtained by two-tailed Student's *t* test. $*P < 0.05$, $**P < 0.01$.

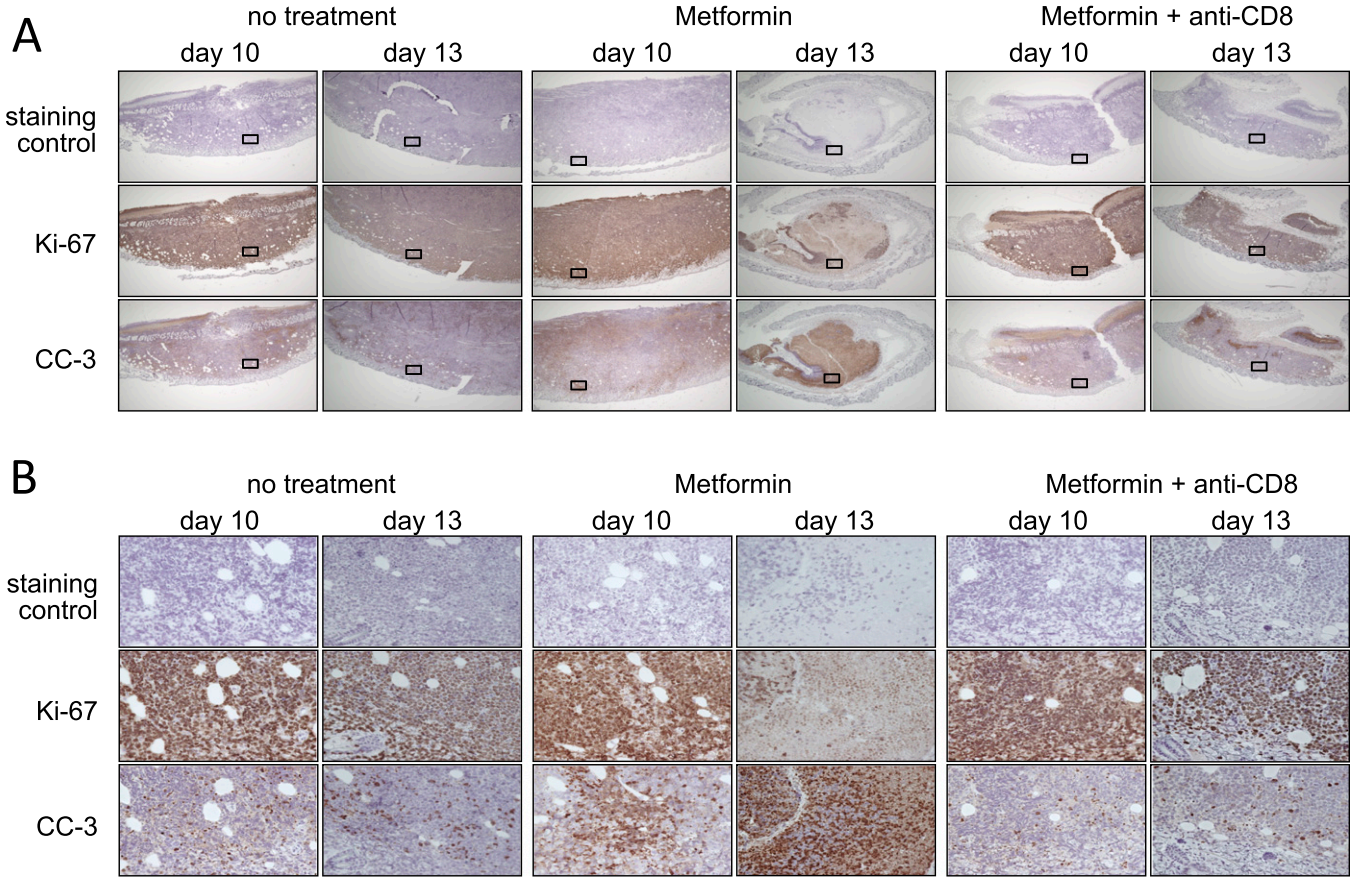

Fig. S9. Metformin administration decreased expression of Ki67 and increased active caspase 3 of RL male 1tumor, which was completely abrogated by CD8T cell depletion. Tumors were isolated at day 10 and 13 from mice treated with metformin or not, with or without anti-CD8 mAb injection. Analysis of immunohistochemistry was performed by KEYENCE BZ-9000 microscopy. (*A*) Ki67 and cleaved caspase 3 (CC-3) were immunostained as indicated, and shown along with negative staining control. (Magnification: 4×.) (*B*) The surrounded area by a rectangle in each panel of *A* as shown in larger magnification (40×).