Immune-mediated antitumor effect by type 2 diabetes drug, metformin

Shingo Elkawa¹,¹, Mikako Nishida¹,¹, Shusaku Mizukami¹, Chihiro Yamazaki², Eiichi Nakayama³, and Heiichiro Udono¹,²

¹Department of Immunology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700-8558, Japan; and ²Faculty of Health and Welfare, Kawasaki University of Medical Welfare, Okayama 701-0193, Japan

Metformin, a prescribed drug for type 2 diabetes, has been reported to have anti-cancer effects; however, the underlying mechanism is poorly understood. Here we show that this mechanism may be immune-mediated. Metformin enabled normal but not T-cell-deficient SCID mice to reject solid tumors. In addition, it increased the number of CD8⁺ tumor-infiltrating lymphocytes (TILs) and protected them from apoptosis and exhaustion characterized by decreased production of IL-2, TNFα, and IFNγ. CD8⁺ TILs capable of producing multiple cytokines were mainly PD-1⁺Tim-3⁺, an effector memory subset responsible for tumor rejection. Combined use of metformin and cancer vaccine improved CD8⁺ TIL multifunctionality. The adoptive transfer of antigen-specific CD8⁺ T cells treated with metformin concentrations as low as 10 μM showed efficient migration into tumors while maintaining multifunctionality in a manner sensitive to the AMP-activated protein kinase (AMPK) inhibitor compound C. Therefore, a direct effect of metformin on CD8⁺ T cells is critical for protection against the inevitable functional exhaustion in the tumor microenvironment.

Immune exhaustion | CD8T cells | antitumor immunity | tumor microenvironment | multifunctionality

In chronic infectious diseases and cancer, CD8⁺ T cells specific for viral and/or tumor antigens undergo repeated TCR stimulation because of persistent pathogens or cancer cells and gradually lose their ability to secrete IL-2, TNFα, and IFNγ, eventually undergoing apoptotic elimination in a process known as immune exhaustion (1). This worsening immune function is accompanied by phenotypic changes in CD8⁺ T cells, including the expression of exhaustion markers such as PD-1 and Tim-3 (2). Antitumor immunity is enhanced in mice deficient in PD-1 or its ligands PDL-1 and PDL-2 (2-4). Galectin 9, a Tim-3 ligand, is secreted by many tumor cells as well as by FoxP3-expressing regulatory T-cell (Treg) and inhibits Tim-3-expressing Th1 cells (5). An anti-Tim-3 antibody that blocks the galectin 9-Tim-3 pathway was found to accelerate antitumor immunity (6). Furthermore, the administration of blocking antibodies against both PD-1 and Tim-3 induced a more profound tumor rejection in comparison with that achieved with either antibody alone (7). The management of functional T-cell exhaustion within tumor tissues is currently an extensive focus in tumor immunotherapy (8, 9), together with efforts to neutralize immune-inhibitory Treg and myeloid-derived suppressor cell (MDSC).

Metformin (dimethylbiguanide) has been widely prescribed for type 2 diabetes. Its unique pharmacological features include its antihyperglycemic efficacy, which counters insulin resistance (10, 11). Early metformin use increases the survival of patients with obesity-involved type 2 diabetes and/or cardiovascular disease (12). In addition, recent reports have described the unexpected anticancer effects of metformin in patients with type 2 diabetes (13). Insulin-based diabetes treatment is associated with an increased cancer risk (14–17), whereas metformin use has been shown to decrease the frequency of specific cancers (18–21). Two independent metaanalyses of epidemiological studies concluded that compared with other treatments, metformin is associated with a 30–40% reduction in the incidence of cancer among patients with type 2 diabetes, indicating the need to investigate the anticancer mechanisms of metformin and conduct long-term randomized controlled trials (RCTs) (22, 23).

In the HER-2/neu transgenic mouse breast cancer model, metformin treatment decreased the tumor burden and was associated with an increased life span (24). Combined use of metformin with chemotherapeutic agents such as cisplatin has also yielded clinical benefits (25, 26). Regarding the anticancer mechanism, metformin appears to preferentially kill cancer-initiating/stem cells from glioblastoma (27), breast (28) and ovarian cancers (29) via AMP-activated protein kinase (AMPK) activation.

In contrast to the inhibitory action of metformin on tumor cells, here we demonstrate the direct effects of metformin on CD8⁺ T cells, which eventually results in tumor growth inhibition. Metformin protects CD8⁺ tumor-infiltrating lymphocytes (TILs) from apoptosis, and the multifunctionality of exhausted PD-1⁺Tim-3⁺CD8⁺ TILs is restored via a shift from a central memory (TCM) to an effector memory T-cell (TEM) phenotype. This metformin-induced antitumor mechanism is therefore linked to marked changes in the characteristics of CD8⁺ TILs within the tumor microenvironment.

Results

Metformin-Induced Tumor Rejection Depends on CD8⁺ T Cells. As metformin has been reported to decrease the rate of cancer incidence in type 2 diabetic patients, we at first examined whether

Significance

The multifunctional ability of CTLs is downregulated by interaction between immune-checkpoint molecules expressed on CTLs and their ligands expressed on cancer cells, referred to as immune exhaustion. The antibody-mediated, immune-checkpoint blockade turned out to be a promising method for immunotherapy against advanced melanoma. Metformin, a drug prescribed for patients with type 2 diabetes, has been recognized to have anti-cancer effect. We found that CD8⁺ tumor infiltrating lymphocytes (TILs) is a target of metformin. CD8⁺ TILs inevitably undergo immune exhaustion, characterized by diminished production of multiple cytokines such as IL-2, TNFα, and IFNγ, followed by elimination with apoptosis. Metformin is able to counter the state. Along with conventional therapy, treatment of cancer patients with metformin may have a great advantage for cancer therapy.

Author contributions: H.U. designed research; S.E. and M.N. performed research; S.M., C.Y., E.N., and H.U. analyzed data; and H.U. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor. Freely available online through the PNAS open access option.

†S.E. and M.N. contributed equally to this work.

‡To whom correspondence should be addressed. Email: udono@cc.okayama-u.ac.jp.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1417636112/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1417636112
the drug could protect mice from methylcholanthrene-induced skin carcinogenesis. BALB/c mice were injected with 200 μg of methylcholanthrene on the right back and given 5 mg/mL metformin dissolved in the drinking water throughout the experiment. Significant inhibition of tumor development was observed in metformin-treated nondiabetic mice (Fig. S1A). We next attempted to determine whether metformin would be effective against an established solid tumor. Mice were intradermally injected with X-ray-induced RLmale1 leukemia cells and were provided oral metformin beginning on day 7. The tumors were gradually and completely rejected with no reappearance after metformin withdrawal. A rechallenge with more than twice the original number of the same tumor cells did not yield mass formation (Fig. 1A, Left), suggesting the generation of an immunologic memory response. Moreover, the antitumor effect was completely abrogated in SCID mice (Fig. 1A, Right), clearly demonstrating the necessity of T and/or B cells. Cytotoxic T lymphocytes (CTLs) specific for the tumor antigen peptide pRL1a (30) were generated in mice that rejected the tumor (Fig. S1B). Growth inhibition was observed with a metformin dose as low as 0.2 mg/mL (Fig. S1C). Of note, a previous report identified the achievement of plasma metformin concentrations of 0.45 and 1.7 μg/mL using 1 and 5 mg/mL of metformin, respectively, in drinking water (31); these plasma concentrations are similar to those in patients with diabetes treated using metformin (0.5–2 μg/mL). Administration of metformin beginning on day 0, the time point of tumor inoculation, resulted in more effective rejection than on day 7. Beginning treatment on day 10 and 13 was also effective, although the effect was less than on day 0 (Fig. S1D). Finally, as expected, CD8+ but not CD4+ T cells were proven to be responsible for the antitumor effect, because their depletion by mAb completely abrogated the response (Fig. 1B). Complete rejection by metformin was also observed with Renca (renal cell carcinoma), although partial but significant growth inhibition was observed with other tumors, 3LL (non small cell lung carcinoma), Colon 26 (intestinal carcinoma), and 4T1 (breast cancer) (Fig. S1 E–H).

Metformin Prevents Apoptosis of CD8+ TILs, Irrespective of Expression of PD-1 and Tim-3. Injection of a vaccine consisting of antigen (Ag) and adjuvant primes and generates specific T-cell immunity, mainly in draining lymph nodes near the injection site. However, we did not inject tumor antigens with any kind of adjuvant in Fig. 1. Therefore, it is possible that a unique process occurs at the tumor site and leads to antitumor immunity. Based on this notion, we focused on TILs throughout the experiment to clarify the associated mechanism. We found that total numbers of TILs dramatically increased when metformin administration was started on day 7, and that both CD8+ and CD4+ T cells were involved in the increment (Fig. 1C–E). In particular, the number of CD8+ TILs increased nearly fourfold. We considered the possibility that metformin may suppress expression of the immune exhaustion markers PD-1 and Tim-3 on CD8+ TILs, thus avoiding immune exhaustion. Therefore, we investigated the expression of these markers on CD8+ TILs derived from individual tumor-bearing mice (Fig. S1B). The number of PD-1+ Tim-3+ CD8+ TILs decreased from day 7–10, irrespective of metformin use (Fig. S2B). The PD-1+ Tim-3+ CD8+ TIL population increased progressively, whereas PD-1+ Tim-3– and PD-1– Tim-3+ CD8+ TILs remained stable. Metformin did not affect any subset populations (Fig. S2 B–E). However, we surprisingly found that a significant proportion of CD8+ TILs underwent apoptosis, detected by

Fig. 1. Metformin suppressed tumor growth in vivo, depending on CD8+ T cells. (A) On day 0, BALB/c WT or SCID mice were intradermally inoculated with 2 × 10^6 RLmale1 cells on the right back. The mice received 5 mg/mL metformin (Met) or not (none) dissolved in the drinking water. The duration of Met administration is indicated by the shaded rectangle. The mean diameter of each tumor was measured every day and the data are plotted with SE. On day 45, Met-treated WT mice, all of which had rejected the tumor, were rechallenged with 5 × 10^6 RLmale1 cells. n = 6 in each group. The results are representative of two independent experiments. (B) Mice inoculated with RLmale1 were treated with metformin (Met) or not (none), starting on day 7 and i.v. injected with anti-CD8 mAb and/or anti-CD4 mAb on the same day. Average tumor diameters are plotted with SE. (C–E) Mice inoculated with RLmale1 cells were treated with Met (+) or not (−) from day 7. On day 7, 10 and 13, the tumor mass was isolated and TILs were recovered. The numbers of TILs per tumor volume (mm^3) were calculated. The numbers of TILs, CD8+ T cells, and CD4+ T cells per tumor volume are depicted. Also, the populations of CD8+ TILs stained with Annexin V were plotted (F). All data were with SD (n = 14 on days 7 and 10, n = 5 on day 13). 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."
Annexin V (Fig. 1F and Fig. S3A), and that metformin suppressed apoptosis induction in all subsets, including PD-1+ TILs. The Annexin V-positive populations among CD4+CD8+ thymocytes, which depends on a mitochondrial pathway (32), was not down-regulated by metformin (Fig. S4), suggesting that an apoptotic mechanism unique to the tumor microenvironment is metformin-sensitive.

We next examined the metformin effects in another tumor system. MO5 is a subclone of B16 melanoma cells expressing ovalbumin (OVA) (33). Metformin administration induced significantly enhanced induction of all subsets, including PD-1+ TILs, compared with untreated mice (Fig. 2B). CD8+ TILs were further stained with OVA263-272 tetramer and examined for their cytokine-producing capacity. Only IFN-γ-producing cells or very small populations producing both IFN-γ and TNF-α or IL-2 could be identified in untreated mice, whereas a marked increase in the population producing both IFN-γ and TNF-α was observed with metformin (Fig. 2C).

**Fig. 2.** Metformin improves the multifunctionality of antigen-specific CD8+ TILs in vivo. (A) Mice inoculated with 2 x 10⁷ MO5 cells were treated with or without metformin from day 7, as indicated by the shadowed rectangle, and tumor growth was monitored. The results are representative of two independent experiments. n = 5 per group. (B) On days 7, 10, and 13, TILs were recovered from tumor masses and examined for K562-OVA257-264 and K562-TRP2264 tetramer binding (n = 7–13). (C) TILs recovered on days 7, 10, and 13 from five mice per group [with (+) or without (−) metformin] were pooled and stimulated with DC2.4 cells that had been pre pulses with OVA257-265 peptide (10⁻⁸ M) for 8 h. TIL cytokine-producing ability was later examined.

Metformin Induced Multifunctional CD8+ TEM Expressing the Exhaustion Marker Tim-3. We next investigated the capacity for triple cytokine (IL-2, TNF-α, IFN-γ) production or the multifunctionality of CD8+ TILs in the context of TCM/TEM classification. CD8+ TILs recovered from RLmale1 tumor masses were stimulated with PMA/ionomycin for 6 h in vitro and monitored for cytokine production. Without metformin, the cytokine-producing cells on day 10 were mainly identified as TCM (Fig. 4B). In contrast, with metformin, triple cytokine-producing cells appeared in correlation with the increased population of TEM (Fig. 4C). The populations with various cytokine-producing patterns in the presence and absence of metformin are summarized in Fig. 4D. Metformin markedly changed the multifunctionality of CD8+ TILs. Taking these results together, we concluded that metformin-induced TEM capable of producing...
multiple (triple and double) cytokines are most important for tumor rejection. We next classified CD8+ TILs on the basis of their expression of PD-1 and Tim-3, followed by intracellular cytokine staining. We found that CD8+ TILs with triple cytokine-producing abilities belonged exclusively to the PD-1+Tim-3+ subset, which was the supposedly exhausted population in the RLmale1 tumor model (Fig. S6). We further confirmed this notion using adoptive transfer experiments. MO5-inoculated mice were adoptively transferred with OT-I CD8+ T cells. The transferred T cells had been previously shown to undergo vigorous division and were thus cross-primed in vivo via the adjuvant-free administration of a fusion protein comprising OVA and Mycobacterium heat shock protein 70 (OVA-mHSP70) as a vaccine (36, 37). OVA-mHSP70 injection significantly enhanced the migration of the transferred CD45.1+OT-I CD8+ T cells into the tumor tissues; however, the cytokine-producing abilities of these cells were poor (Fig. 5A). In contrast, injection of the fusion protein together with oral metformin administration apparently improved the multifunctionality of the migrated T cells, which were classified as the ‘Tim-3’ population (Fig. 5A).

Metformin-Treated Antigen-Specific Naïve CD8 T Cells Migrate into Tumors and Exert Antitumor Immunity Following Adoptive Transfer. It is unknown whether plasma metformin concentrations as low as 10 μM (1.6 μg/mL) would directly influence the fate of T cells. To address this important question, we incubated CD8+ T cells isolated from naïve OT-I mice with 10 μM metformin for 6 hr in the presence or absence of different doses of the AMPK inhibitor compound C (38) as indicated (Fig. 5B). After extensive washing, the cells were transferred into MO5-bearing mice. Two days later, splenic T cells and TILs were recovered and investigated for the presence and multifunctionality of donor-derived CD8+ T cells. Metformin-treated CD8+ TILs comprised up to 9.9% of all CD8+ T cells and were identified as triple cytokine-producing cells (Fig. 5B). However, compound C treatment abrogated the migration, although donor CD8+ T cells were present in the spleens of all groups (Fig. 5B). Accordingly, tumor growth inhibition was apparent in the metformin-treated group, although this effect was blocked by compound C (Fig. 5C). The weak but significant metformin-mediated increase in the phosphorylation of AMPK and its downstream target acetyl-CoA carboxylase (ACC) and the abrogation of this effect by compound C were observed by Western blot analysis (Fig. 5D).

The results led us to conclude that the direct action of metformin on CD8+ T cells, at least partly, reduced their exhaustion within the tumor microenvironment in a manner sensitive to the AMPK inhibitor compound C.

AMPK Phosphorylation, Enhanced Bat3 Expression, and Caspase-3 Inhibition Mediated by Metformin. Finally, we examined the expression of CD8+ TIL molecules that may possibly be influenced by metformin administration. After CD8+ TIL purification on day 10, cell lysates were immediately prepared for candidate molecule detection via Western blot analysis and for caspase-3 activity measurement using a fluorescent substrate. The levels of phosphorylated AMPKα and β were increased; a twofold increase in Bat3 expression was also observed, whereas Bcl2 and Bax expression were unaltered (Fig. S7A). As expected, caspase-3 activity was prominent without metformin but was completely abrogated in CD8+ TILs from metformin-treated mice (Fig. S7B), which offers a plausible explanation for apoptosis inhibition. To further examine the apoptotic cell populations, we evaluated the expression of active caspase-3 in TCM, TEM, and TE. Without metformin, TCM, TEM, and TE all expressed active caspase-3 whereas with metformin, primarily TCM expressed this activated enzyme (Fig. S7C). These results may explain the dominance of TCM over TEM in the absence of metformin and the dominance of TEM and TE in the presence of metformin. pS6, a downstream target of mTOR, was positive in TCM, TEM, and TE without metformin but negative with metformin (Fig. S7D), indicating that metformin inhibits mTOR, possibly via AMPK activation.

Discussion

In this report, we showed that established solid tumors are regressed by oral administration of metformin, and that CD8+ T cell mediate this effect. The number of FoxP3 expressing CD4+ regulatory T cells (Treg) has been implicated as a critical component in suppressing tumor immunity (39). However, their numbers were not decreased, rather, transiently increased by metformin administration in RLmale 1 tumor model (Fig. S8). Upon tumor rejection, the treated mice became resistant to rechallenge with the same tumor, providing proof of memory T-cell generation. Because no protective effect was observed in SCID mice, the direct killing of tumor cells by metformin is negligible. It was also confirmed by immunohistochemistry (IHC) of tumors. Tumors of mice treated with metformin showed decreased expression of Ki67 as a proliferation marker, accordingly, increased expression of active caspase 3 as an

---

**Fig. 4.** Metformin-induced CD8+ TILs with multifunctionality are TEM rather than TCM. (A) TILs were isolated on the indicated days from five mice per group inoculated with 2 × 10^6 RLmale1. Met treatment was started (+) or not (−) from day 7. TILs were then pooled on indicated days and stimulated with PMA/Alanomycin for 6 hr stained for surface molecules including CD8, CD44, CD62L, followed by intracellular staining for IL-2, TNFα, and IFNγ. CD8+ TILs producing TNFα were further analyzed for expression of CD62L and CD44 to identify TCM and TEM. Also, to investigate multifunctionality, cytokine-producing CD8+ TILs were further examined for production of IFNγ and IL-2. (B) Summary of the populations of cytokine producing CD8+ TILs on day 10 is shown. Gated populations for CD8+IFNγ+, CD8+TNFα+, or CD8+IL-2+ were further analyzed for their production of TNFα and IL-2, IFNγ and IL-2, or IFNγ and TNFα. The gating strategy gives rise to some ranges for % populations of double and triple cytokine producing TILs. The numbers within parenthesis indicate numbers of corresponding CD8+ TILs per tumor volume (mm³).
apoptosis marker; however, the effect was abrogated by CD8 T-cell depletion (Fig. S9). Our used model systems comprised highly immunogenic tumors, and it is unclear whether metformin would have the same effect on less immunogenic tumors. Demonstration of a similar effect in an autochthonous tumor model would be required in the future. Nonetheless, metformin countered apoptotic induction and reduced cytokine production in CD8^+^ TILs and thus blocked immune exhaustion within the tumor tissues we tested. The adoptive transfer experiment shown in Fig. 5 further demonstrated that the direct effect of metformin on CD8^+^ T cells, even at a physiologically relevant low concentration, markedly altered the cells’ multifunctionality following migration into the tumor. Experiments with a genetic approach will be required to fully demonstrate whether this effect is mediated via AMPK activation in CD8^+^ T cells, because compound C is not highly specific for AMPK.

Dissection of TILs from the point of view of memory T cells in the context of multifunctionality provides mechanistic insight into metformin-induced antitumor immunity. Memory T cells have been classified as TCM, migrating between lymphoid organs, and TEM, circulating principally in the blood, spleen and peripheral tissues (34, 35, 40). In acute virus infection models, as the virus is cleared, the population of TCM progressively increase, whereas the total numbers of TEM rapidly decrease (41). The naturally occurring proportional shift from TEM to TCM, however, was not associated with metformin-induced rejection in the tumor models. For example, in the absence of metformin in the RLMale1 model, the TCM population gradually increased to exceed the TEM population by day 13 (Fig. 3 A and B); however, this proportional shift to TCM was associated with progressive tumor growth rather than tumor regression. Metformin possibly affects the TCM/TEM ratio by regulating TEM apoptosis (Fig. S7C). The consequent decreased TCM/TEM ratio was apparently associated with antitumor activity in both the RLMale1 and MOS models.

Analysis of the cytokine-producing capacities of CD8^+^ TILs also revealed the importance of TEM over TCM. A significant proportion of the CD8^+^ TIL population was maintained by metformin and produced IL-2, TNFα, and IFNγ. These triple cytokine-producing CD8^+^ TILs were exclusively of the PD1^+^Tim3^+^ phenotype (Fig. S6), which is committed to a TEM rather than a TCM fate (Fig. 4). Moreover, although therapeutic vaccination with OVA-mHSP70 stimulated the migration of adoptively transferred OT-I CD8^+^ T cells into tumor tissues, these TILs lost multifunctionality (Fig. 5A). Possibly, the cells were exhausted from the tumor microenvironment. Co-administration of metformin, however, led to the activation of the migrated Tim-3^+^ OT-I CD8^+^ T cells and the production of multiple cytokines (Fig. 5A4). Therefore, combined use of metformin and cancer vaccines may improve the efficacy of the vaccine. These findings provide novel insights into anticancer immunity. It is possible that tumor persistence stimulates the development of CD8^+^ TILs into TCM cells, which will immediately become useless against tumor growth because of immune exhaustion, and that metformin counters this situation, leading to the conversion of TCM to activated-state TEM that are fully active against tumors, despite exhibiting the surface phenotype of an exhausted cell (e.g., Tim-3 expression).

A previous report found that metformin treatment following vaccination with attenuated *Listeria monocytogenes* expressing OVA (LmOVA) protected mice from challenge by tumor cells expressing OVA (42). This effect was caused by metformin-induced expansion of memory T cells after vaccination. As the tumor challenge occurred after metformin withdrawal, it is a matter of a prophylactic vaccination effect, which is different from the effects on immune exhaustion states in the tumor microenvironment.
mTOR inhibition is among the downstream consequences of AMPK signaling, which is activated by metformin. Therefore, rapamycin, an inhibitor of mTORC1, may share mechanistic effects with metformin. Rapamycin has been shown to promote the generation of memory T cells (42–44) particularly in viral infection models. A common feature in the results was the increased population of TCM over TEM consequent to rapamycin treatment (45). In our tumor models, however, metformin treatment preferentially increased the TEM population. It remains to be determined whether this result can be reproduced in other tumor models. A common feature in the results was the increased memory population of T cells. To confirm this, we performed flow cytometry to measure the percentage of memory T cells. In our tumor models, however, metformin treatment preferentially increased the TEM population. It remains to be determined whether this result can be reproduced in other tumor models.

Materials and Methods

Mice. BALB/c and C57BL/6 (B6) mice were purchased from CLEA Japan and SLC. Breeding pairs of C57-B10 Scid mice were provided by K. Kuribayashi, Mie University School of Medicine, Mie, Japan.

Eikawa et al. 10.1073/pnas.1417636112

SI Materials and Methods

Methylcholanthrene-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-LT4 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 × 10^6) were stimulated with 0.1% paraformaldehyde-fixed cells from the mouse lymphoblast-like masto cytoma cell line P815 (5 × 10^5) prepsolved with graded doses of peptide in a 96-well bottom culture plate for 24 h at 37 °C and 5% CO₂. The stimulated culture supernatants were collected, and the amount of secreted IFNy 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 OVA257-264 peptide (SIINFEKL: 1 μM) or control peptide (NY-ESO-1a1-20; YLAMPFAK; 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 containing Monensin (BD Biosciences) at 37 °C in a 5% CO₂ 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-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.

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% CO₂ in the presence of Golgi stop, followed by staining with APC-Cy7-conjugated anti-mouse 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-FTTC (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–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

Supporting Information

Eikawa et al. www.pnas.org/cgi/content/short/1417636112
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 re-
arranged to remove irrelevant information; however, all lanes
were obtained from the same blot with the same levels of ex-
posure and contrast.

Measurement of Caspase-3 Activation. Caspase-3 activity was mea-
sured 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 in-
cubating 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 deparaffiniza-
tion, sections were rehydrated, treated with citrate buffer at
96 °C for 30 min, and treated with 3% H2O2. 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′-dia-
minobenzidine tetrahydrochloride (DAB) for 1–3 min and
counterstained with hematoxylin for 5 min. Staining controls
were prepared by replacing the primary antibody to buffer so-
lution. 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.
Metformin prevented methylcholanthrene-induced carcinogenesis and suppressed growth of variety of tumor cells in vivo. (A) BALB/c mice were s.c. injected methylcholanthrene (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 IFN\(\gamma\) 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 \(2 \times 10^5\) 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 $2 \times 10^5$ 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 (−) metformin.

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.
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.

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, OVA257-264-Kb 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 RLmae1 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 $1 \times 10^5$ CD8+ TILs and x1/2 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 $1 \times 10^5$ 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 $2 \times 10^5$ 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 (mm³) 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 tumor, which was completely abrogated by CD8 T 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×).