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

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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 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.

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the drug could protect mice from methylchoranthrene-induced skin carcinogenesis. BALB/c mice were injected with 200 μg of methylchoranthrene 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. S1*A*). 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. 1*A*, *Left*), suggesting the generation of an immunologic memory response. Moreover, the antitumor effect was completely abrogated in SCID mice (Fig. 1*A*, *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. S1*B*). Growth inhibition was observed with a metformin dose as low as 0.2 mg/mL (Fig. S1*C*). 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. S1*D*). 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. 1*B*). 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. 1 *C–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. S1*B*). The number of $PD\text{-}1^-Tim\text{-}3^-$ $CD8^+$ TILs decreased from day 7–10, irrespective of metformin use (Fig. S2*B*). The $PD\text{-}1^-Tim\text{-}3^+CD8^+$ TIL population increased progressively, whereas $PD\text{-}1^+Tim\text{-}3^-$ and $PD\text{-}1^+Tim\text{-}3^+CD8^+$ 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

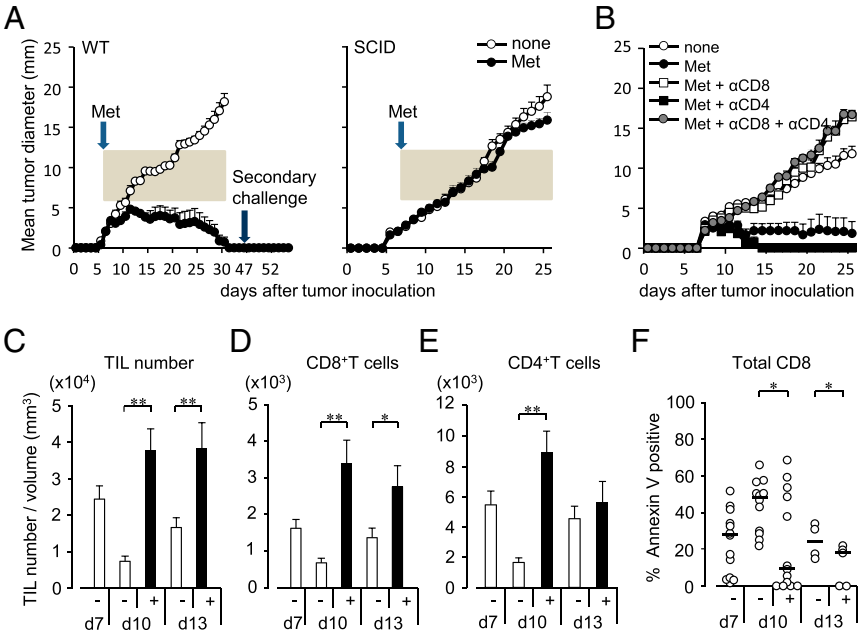

Fig. 1. Metformin suppressed tumor growth in vivo, depending on $CD8^+$ T cells. (*A*) On day 0, BALB/c WT or SCID mice were intradermally inoculated with 2×10^5 RLmale1 cells on the right back. The mice received 5 mg/mL metformin (Met) or not (none) dissolved in the drinking water. The duration of Met 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 5×10^5 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. $n = 5$ in each group. (*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 TIL (*C*), $CD8^+$ (*D*), or $CD4^+$ (*E*) 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 $^*P < 0.05$, $^{**}P < 0.01$ $n = 5$–14 in each group. Each symbol represents an individual mouse. The results depicted are a summary of three independent experiments.

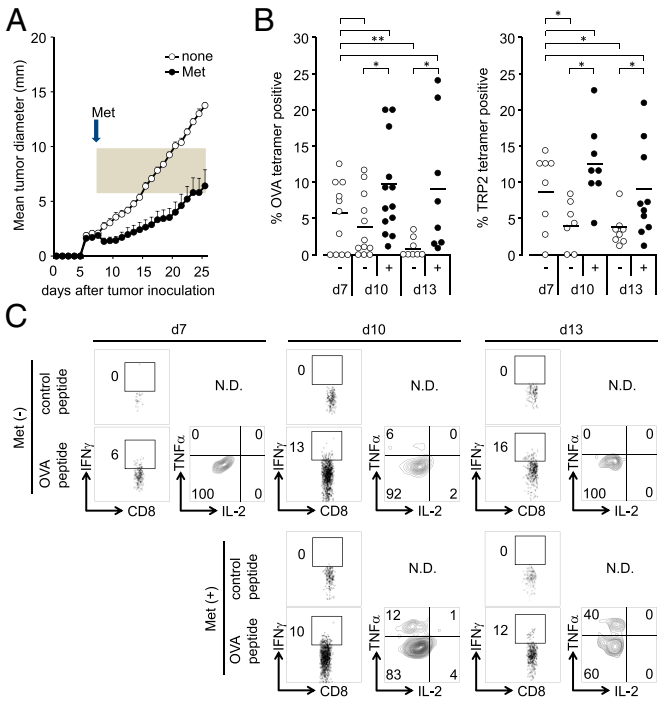

Fig. 2. Metformin improves the multifunctionality of antigen-specific CD8$^+$ TILs in vivo. (*A*) Mice inoculated with 2×10^5 MO5 cells were treated with or without metformin from day 7, as indicated by the shadowed rectangle, and tumor growth was monitored. The results are representative of two independent experiments. $n = 5$ per group. (*B*) On days 7, 10, and 13, TILs were recovered from tumor masses and examined for K^b-OVA$_{257–264}$ and K^b-TRP2$_{180–188}$ 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 prepulsed with OVA$_{257–265}$ peptide (10^{-6} M) for 8 h; TIL cytokine-producing ability was later examined.

Annexin V (Fig. 1*F* and Fig. S3*A*), and that metformin suppressed apoptosis induction in all subsets, including PD-1$^-$Tim-3$^+$CD8$^+$TILs (Fig. S3 *B–E*). Of note, the physiologically essential apoptotic process of 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 significant antitumor activity (Fig. 2*A*). OVA- and TRP2-specific CD8$^+$ TILs were identified by specific tetramers. Both TIL populations in untreated mice decreased gradually from day 7–13; in contrast, metformin administration maintained or increased these populations (Fig. 2*B*). CD8$^+$ TILs again underwent apoptosis, which was suppressed by metformin administration (Fig. S5 *A* and *B*). The Annexin V-positive populations among OVA tetramer-positive and -negative (includes TRP-2–positive population) CD8$^+$ TILs were near 80% at day 10; however, metformin suppressed this rate to <20–40% (Fig. S5 *C* and *D*). These results are consistent with those observed in the RLmale1 model. Next, to examine the functional state of antigen-specific TILs, magnet-purified CD8$^+$ TILs isolated from tumor tissues were incubated with DC-like DC2.4 cells that had been pulsed with an epitope peptide (OVA$_{257–264}$); TILs were later examined for their cytokine production capacity. Only IFNγ-producing cells or very small populations producing both IFNγ and TNFα or IL-2 could be identified in untreated mice, whereas a marked increase in the population producing both IFNγ and TNFα was observed with metformin (Fig. 2*C*).

Influence of Metformin on the TCM/TEM Ratio of CD8$^+$TILs. CD8$^+$ TILs in the context of memory T cells are poorly understood. Elegant studies with an acute viral infection model have proposed classification of memory T cells into central memory (TCM; CD44$^+$, CD62L^{high}) and effector memory (TEM; CD44$^+$, CD62L^{low}) (34, 35). TCM were shown to mediate viral-specific recall responses. Based on this model, we investigated TCM and TEM CD8$^+$ TILs. Without metformin, the staining of CD8$^+$ TILs from an RLmale1 tumor using antibodies against CD62L and CD44 revealed that proportions of TCM and TEM were nearly equal on day 7 and 10 but shifted to TCM dominance on day 13. In contrast, metformin maintained TEM dominance from day 10 to day 13 (Fig. 3*A*). Further dissection of the TIL compartment based on CD62L and KLRG1 expression revealed that short-lived effector T cells (TE; CD62L^{low}KLRG1^{high}) were visible on day 7 but gradually decreased by day 13. In contrast, metformin yielded increases in both TEM and TE populations on day 13 (Fig. 3*B*), coinciding with tumor regression (Fig. 1*A*). In the MO5 model, metformin again caused TEM dominant over TCM (Fig. 3 *C* and *D*). At this stage, we concluded that TEM and/or TE are more responsible than TCM for tumor rejection.

Metformin Induced Multifunctional CD8$^+$ TEM Expressing the Exhaustion Marker Tim-3. We next investigated the capacity for triple cytokine (IL-2, TNFα, IFNγ) 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. 4*A*). In contrast, with metformin, triple cytokine-producing cells appeared in correlation with the increased population of TEM (Fig. 4*A*). The populations with various cytokine producing patterns in the presence and absence of metformin are summarized in Fig. 4*B*. Metformin markedly changed the multifunctionality of CD8$^+$ TILs. Taking these results together, we concluded that metformin-induced TEM capable of producing

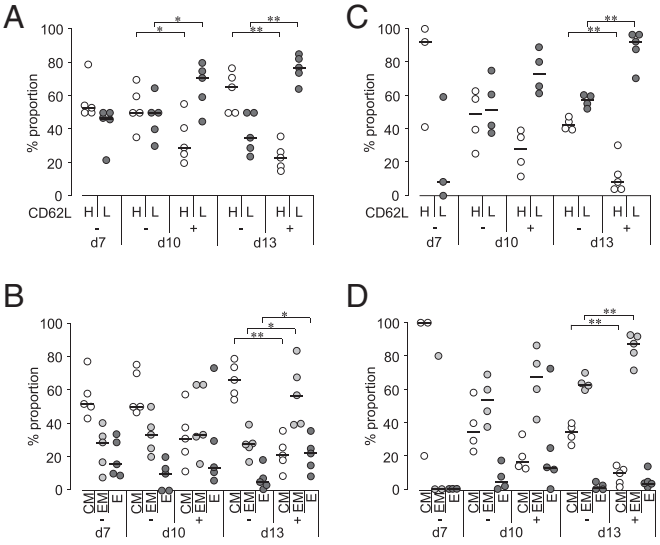

Fig. 3. Influence of metformin on the TCM/TEM ratio of CD8$^+$ TILs. TILs were isolated on days 7, 10, and 13 from mice inoculated with RLmale1 (*A* and *B*, $n = 5$) or MO5 (*C* and *D*, $n = 3–5$) with (+) or without (–) metformin, and analyzed for CD8 and memory markers including CD44, CD62L, KLRG1. The proportion (%) of CD62L^{high} (H) and CD62L^{low} (L) among CD44$^+$ cells in RLmale1 and MO5 models are shown in *A* and *C*, respectively. The proportion (%) of CD62L^{high}, KLRG1^{low} (central memory; CM) and CD62L^{low}, KLRG1 ^{low} (effector memory; EM) and CD62L^{low}, KLRG1 ^{high} (effector; E) in RLmale1 and MO5 are shown in *B* and *D*, respectively. $^*P < 0.05$, $^{**}P < 0.01$.

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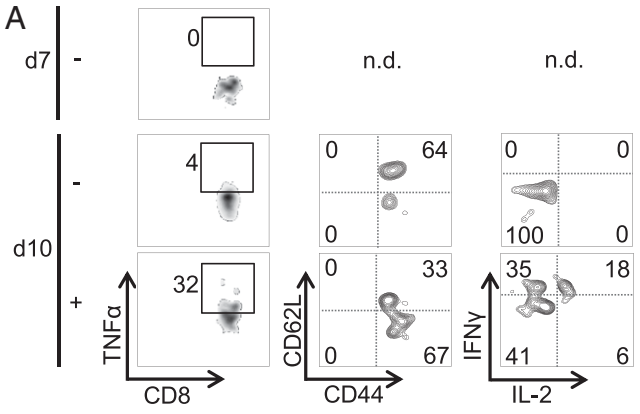
multiple (triple and double) cytokines are most important for tumor rejection. We next classified $CD8^+$ TILs on the basis of their expression of PD-1 and Tim-3, followed by intracellular cytokine staining. We found that $CD8^+$ TILs with triple cytokine-producing abilities belonged exclusively to the $PD\text{-}1^-Tim\text{-}3^+$ 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. 5*A*). 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 $Tim\text{-}3^+$ population (Fig. 5*A*).



B

cytokine	Met (-)	Met (+)
$IL\text{-}2^+ TNF\alpha^+ IFN\gamma^+$	-	4.6-7.2% (169-264)
$IL\text{-}2^+ TNF\alpha^+ IFN\gamma^-$	-	1.3-1.9% (48-69)
$IL\text{-}2^+ TNF\alpha^- IFN\gamma^+$	-	5.3-7.2% (194-264)
$IL\text{-}2^- TNF\alpha^+ IFN\gamma^+$	-	7.2-11.2% (264-411)
$IL\text{-}2^+ TNF\alpha^- IFN\gamma^-$	2.0% (15)	1.7% (62)
$IL\text{-}2^- TNF\alpha^+ IFN\gamma^-$	4.0% (30)	13.1% (480)
$IL\text{-}2^- TNF\alpha^- IFN\gamma^+$	9.0% (67)	36.0% (1320)

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 2×10^5 RLmale1. Met treatment was started (+) or not (–) from day 7. TILs were then pooled on indicated days and stimulated with PMA/ionomycin for 6 h, 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 $CD8^+IFN\gamma^+$, $CD8^+TNF\alpha^+$, or $CD8^+IL\text{-}2^+$ 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 (mm^3).

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 h in the presence or absence of different doses of the AMPK inhibitor compound C (38) as indicated (Fig. 5*B*). 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. 5*B*). However, compound C treatment abrogated the migration, although donor $CD8^+$ T cells were present in the spleens of all groups (Fig. 5*B*). Accordingly, tumor growth inhibition was apparent in the metformin-treated group, although this effect was blocked by compound C (Fig. 5*C*). 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. 5*D*). 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. S7*A*). As expected, caspase-3 activity was prominent without metformin but was completely abrogated in $CD8^+$ TILs from metformin-treated mice (Fig. S7*B*), 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. S7*C*). 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. S7*D*), 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 cells 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

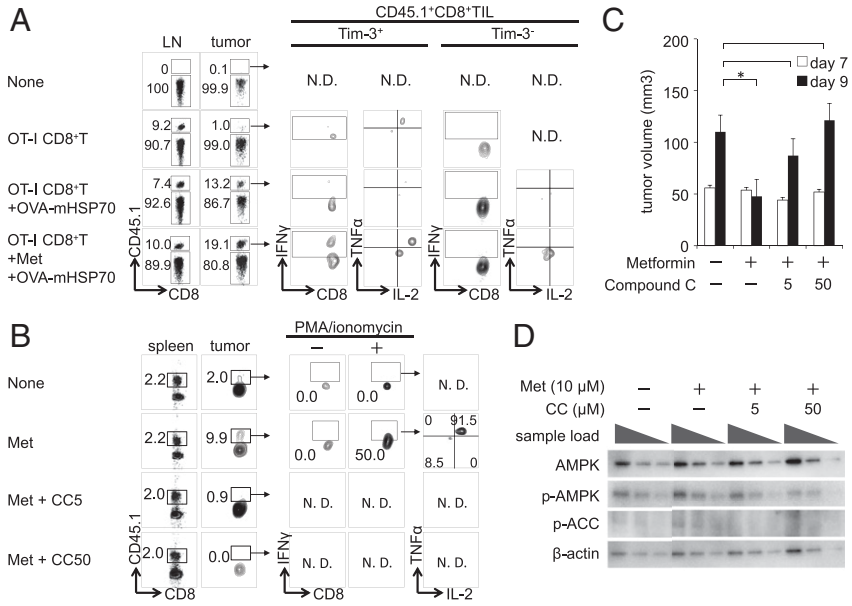


Fig. 5. Metformin use for combination with vaccine or cell therapy using CD8T cells. (*A*) Combined use of cancer vaccines and metformin improves $CD8^+$ TIL multifunctionality. B6 mice (CD45.2) inoculated with 3×10^5 MO5 cells were adoptively transferred or not with 2×10^6 CD45.1/OT-I $CD8^+$ T cells on day 7 ($n = 5$). Simultaneously, 10 μg of the OVA-mHSP70 fusion protein were i.v. injected along with or without the oral administration of 5 mg/mL metformin as indicated. Three days later, the right inguinal lymph nodes (LNs) and tumor masses were removed, prepared as single-cell suspensions. The cells were stimulated with PMA/ionomycin, followed by labeling with antibodies and were subjected to flow cytometric analysis. (*B*) Metformin-treated antigen-specific naïve CD8 T cells acquire multifunctionality within the tumor. B6 mice (CD45.2) inoculated with MO5 cells were adoptively transferred or not with 3×10^6 CD45.1/OT-I CD8 T cells on day 7 ($n = 5$). The cells to be transferred were isolated from CD45.1 OT-1 mice and precultured with 10 μM metformin with or without compound C (5, 50 μM) for 6 h before transfer. Two days later, the spleen and tumor tissues were removed and prepared as single-cell suspensions. The cells were then investigated for migration and multifunctionality. (*C*) The mean tumor diameters were measured on days 7 and 9 after MO5 inoculation and were plotted with SE. (*D*) The Western blot detection of AMPK, p-AMPK, and p-ACC in $CD8^+$ T cells treated with metformin in vitro. Anti-actin was used as a loading control. OT-1 CD8 T cells treated in *A* were lysed, titrated 1-, 1/2-, and 1/4-fold, and subjected to the assay.

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. S7*C*). The consequent decreased TCM/TEM ratio was apparently associated with antitumor activity in both the RLmale1 and MO5 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. 5*A*). Possibly, the cells were exhausted from the tumor microenvironment. Coadministration of metformin, however, led to the activation of the migrated $Tim\text{-}3^+$ OT-I $CD8^+$ T cells and the production of multiple cytokines (Fig. 5*A*). 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 possible that additional pharmacological effects are involved in response to metformin versus rapamycin treatment. Further experiments will be required to elucidate cellular and molecular mechanism underlying metformin-induced reversion of exhausted $CD8^+$TILs.

Materials and Methods

Mice. BALB/c and C57BL/6 (B6) mice were purchased from CLEA Japan and SLC. Breeding pairs of CB-17 SCID mice were provided by K. Kuribayashi, Mie University School of Medicine, Mie, Japan.

Tumor Cell Lines. BALB/c radiation leukemia RLmale1, B6 OVA-gene introduced B16 melanoma MO5, B6 nonsmall cell lung carcinoma 3LL, BALB/c intestinal carcinoma Colon 26, BALB/c renal cell carcinoma Renca, and BALB/c breast cancer cell 4T1 were used for the tumor assay. 3LL, Colon 26, Renca, and 4T1 were kindly provided by H. Yagita, Juntendo University School of Medicine, Tokyo, Japan.

Tumor Growth Assay. Mice were intradermally inoculated with 2×10^5 tumor cells (in 0.2 mL) on the right back with a 27-gauge needle. Before inoculation of tumor cells, the hair was cut with clippers. Mice were orally administrated metformin hydrochloride (Wako) (5 mg/mL) or as indicated dissolved the drinking water. The diameter of the tumors was measured with Vernier calipers twice at right angles to calculate the mean diameter.

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