A new aspect of an old friend: the beneficial effect of metformin on anti-tumor immunity

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INTRODUCTION

Metformin is the first-line oral medication for type 2 diabetes (T2D), approved by the FDA in 1994. It has been used for 1.5 million T2D patients worldwide and has been recognized as a safe and well-tolerated drug over the past several decades of clinical experience (1). Interestingly, several case-control studies for T2D patients showed a reduced incidence of various cancers, suggesting an anti-tumor effect of metformin beyond that of T2D patients. In previous studies on diabetes, cardiovascular disease, and autoimmunity, metformin has been shown to improve the prognosis of each disease by affecting inflammation, hypoxia, and cell survival (5). Metformin directly inhibits complex I of the mitochondrial electron-transport chain (ETC), leading to an overall reduction in various intracellular processes that consume ATP (6). Further, metformin treatment induces mitochondria-mediated apoptosis by increasing mitochondrial reactive oxygen species (ROS) (7). In addition, metformin can suppress the activation and expression of the signal transducer and activator of transcription 3 (STAT3) important for cancer-cell survival (8, 9). These studies provide mechanisms by which metformin directly inhibits cell growth by targeting the intrinsic pathway inside cancer cells.

Recent studies in the field of immuno-oncology reported the systemic effect of metformin associated with immunity. Interestingly, the anti-tumor effect of metformin is much higher in immunocompetent mouse models than in immunodeficient ones under the same conditions (10, 11), implying that the effect of metformin can be primarily mediated by anti-tumor immunity in physiological and clinical conditions. The immunomodulatory activity of metformin was already reported seventy years ago as an anti-viral and anti-malarial effect (12). This finding is supported by several recent findings in which metformin increases CD8+ T-cell activity (10, 13).

Since T-cells are the main effector of anti-tumor immunity, T-cell activity has been the final target for most FDA-approved cancer immunotherapies [e.g., ICB (Ipilimumab, Nivolumab, Pembrolizumab, and Atezolizumab), dendritic-cell vaccine (Sipuleucel-T), and ACT (Axicabtageneicoleucel, and Tsagenlecleucel)] (14). However, since TIL activity is effectively inhibited by protumoral TME, the efficacy of current immunotherapies is limited in most solid tumors. In previous studies on diabetes, cardiovascular disease, and autoimmunity, metformin has been shown to improve the prognosis of each disease by affecting inflammation, hypoxia, angiogenesis, and T-cell activity (3, 15), major components of TME as well. These results imply that the anti-tumor effect of metformin can occur via these TME components. Because these benefits suppress multiple TME components, metformin is worth considering as a potential adjuvant for T-cell-based cancer immunotherapy.

Keywords: Adjuvant, Cancer immunotherapy, Cold tumor and tumor microenvironment, Metformin
**TUMOR DEVELOPMENT AND ANTI-TUMOR IMMUNITY**

Cancer cells and our immunity are engaged in a fierce battle during tumor development, which occurs in three main stages: initiation, promotion, and progression. During the initiation stage, genomic mutations caused by several carcinogens, such as mutagenic chemicals, radiation, and viruses, can activate oncogenes and inhibit tumor suppressor genes (16). Initiated cells are transformed by the accumulation of critical mutations. When initiated cells are exposed to growth factors, the cells accumulate more mutations with high proliferation during the promotion stages (17). However, most transformed cells are removed by both innate immunity and adaptive immunity [the elimination phase of immunoediting] (18). In innate immunity, cancer cells having an abnormal expression of non-classical major histocompatibility complex (MHC) I are recognized by natural killer (NK) cells, and activated NK cells lyse cancer cells by secreting cytotoxic perforin and granzymes (19). M1 macrophages and dendritic cell (DCs) are recruited by inflammatory chemokines secreted from cancer cells. These antigen-presenting cells (APCs) do not only engulf cancer cells by means of phagocytosis but also support adaptive immunity by presenting tumor antigen to T-cells in the drain lymph node. Finally, educated CD8+ cytotoxic T lymphocytes (CTLs) specifically eliminate cancer cells (20). A few cancer cells undergo repeated proliferation and elimination, resulting in their long-term survival without tumor formation [the equilibrium phase of immunoediting] (21). Cancer cells that endured harsh long-term screening obtain mutations to escape from immune surveillance [the escape phase of immunoediting]. Most survived cancer cells down-regulate the level of classic MHC I and the related-antigen processing and presentation machinery (APM), thereby preventing recognition by CD8+ CTL (22). On the other hand, cancer cells increase the level of non-classical MHC I to avoid the threat of NK cells (23, 24). After all this, cancer cells that escape from immune surveillance form tumors during the progression stage.

As tumors grow, most solid tumors establish TMEs composed of cellular and non-cellular constituents, including immune cells, fibroblasts, blood vessels, inflammatory signals, hypoxia, and glucose deprivation (25-27). The malignancy of cancer is closely related to the interaction between cancer cells and TME. In particular, pro-tumoral TME, such as hypoxia, chronic inflammation, and immune suppressors [e.g., inhibitory immune checkpoints, M2-like tumor-associated macrophages (TAMs), regulatory T-cells (Tregs), and myeloid-derived suppressor cells (MDSCs)], inhibit T-cell surveillance and facilitate metastasis (28).

**THE EFFECT OF METFORMIN ON ANTI-TUMOR IMMUNITY**

TME components are entangled in very complex associations, and the malfunction of anti-tumor immunity in TME results from the accumulation of multiple immunosuppressive effects at different stages. Therefore, a comprehensive approach against multiple targets can be effective in improving the efficacy of cancer immunotherapy. The following are TME components targeted by metformin (Fig. 1 and Table 1). We want to understand the effect of metformin in terms of anti-tumor immunity.

**The effect of metformin in chronic inflammation**

Inflammation is like a double-edged sword in cancer progression. As an integral part of innate immunity, inflammation plays important roles in removing transformed cells and subsequently presenting cancer antigens for adaptive immunity (29). Nevertheless, chronic inflammation is known to increase the incidence and malignancy in various types of cancer (30, 31). Activation of various inflammatory cells, such as macrophages, neutrophils, and mast cells, causes a cytokine storm, including tumor necrosis factor (TNF-α), interleukin (IL)-1β, IL-6, transforming growth factor (TGF)-β, and ROS (32). Chronic exposure to these inflammatory signals increases the population of M2-like TAM in TME (33), and not only induces metastasis by activating angiogenesis and epithelial-mesenchymal transition (EMT) (26, 34), but also suppresses T-cell immunity (35). Further, chronic inflammation can differentiate bone-marrow myeloid precursor cells into MDSCs, a major type of immune suppressive cells (36), which deplete T-cells by expressing inhibitory checkpoints and interfering with T-cell trafficking into tumors (37).

Pharmacological and genetic inhibition of these inflammatory factors has reduced tumor growth in animal models, and several control-case studies have shown that using non-steroidal anti-inflammatory drugs (NSAIDs) reduces incidence and mortality in various types of cancer (38-40). Recently, an anti-inflammatory effect of metformin has been reported in several different disease models (Fig. 1A and Table 1). Metformin attenuated the induction of multiple sclerosis, a central nervous system (CNS) autoimmune disease. In this study, metformin inhibited the infiltration of monocytes into the CNS by down-regulating the level of chemokines and pro-inflammatory cytokines like IFN-γ, TNF-α, IL-6, and IL-17 (41). Control-case studies on T2D patients indicated that metformin reduces cardiovascular complications (42, 43), and related mechanism studies suggested that metformin decreases the level of inflammatory cytokines, including TNF-α and IL-6, by inactivating NF-κB signaling via the AMPK pathway (44, 45). Further, metformin also inhibited generation of ROS and IL-1β from LPS-activated macrophages by inhibiting the mitochondrial complex I (46). Consistently, Wang et al. showed that metformin inhibits both tumor growth and angiogenesis by tilting TAM polarization from an M2- to an M1-like phenotype (47). Moreover, Qin et al. reported that metformin has an anti-tumor effect by reducing MDSC accumulation in the TME via the AMPK/DACH1/CXCL1 axis (48). Collectively, metformin has a beneficial effect of alleviating pro-tumoral inflammation, which is expected to prevent cancer malignancy and T-cell exhaustion in the TME.
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The effect of metformin in intratumoral hypoxia

Hypoxia is an important malignant TME component (49). As a tumor grows, cancer cells rapidly consume oxygen, which induces hypoxia in the tumor (50). Cancer cells significantly alter their genetic profiles to overcome the harsh condition and maintain proliferation (51). Hypoxia-inducible factor (HIF-1) accumulated by hypoxia is the major transcription factor that activates genes involved in glucose absorption, glycolysis, and angiogenesis (51, 52). Hypoxia and HIF signaling also induce metastasis by increasing expression of EMT-associated genes and by increasing dissemination of cancer cells by means of angiogenesis (53). Furthermore, hypoxia and HIF signaling help cancer cells escape from immune surveillance. Hypoxia and HIF signaling are known to increase the population of immunosuppressive cells, such as MDSCs, M2-like TAM, and Treg, in the tumor (54, 55). In addition, hypoxia may interfere with adaptive immunity by inhibiting the stimulatory capacity of DC to activate T-cells (56). Hypoxia also limits the number of TILs by reducing proliferation and viability in the TME (57). Consequently, hypoxia increases the level of PD-L1, a major inhibitory immune checkpoint molecule that induces T-cell exhaustion in the TME.

A recent study reported the anti-hypoxic effect of metformin (Fig. 1B and Table 1). In this study, metformin treatment reduced a hypoxic probe-positive area, which was accompanied by a reduced level of HIF-1α by hypoxia in hepatocellular carcinoma (59) and oral squamous-cell carcinoma (60). Importantly, Scharping et al. reported that metformin can relieve intratumoral hypoxia by reducing oxygen consumption, resulting in improving T-cell immunity in the TME. Consequently, metformin had a significant synergetic anti-tumor effect with ICB targeting PD-1 (61). Such evidence suggests that metformin treatment can improve T-cell immunity by alleviating intratumoral hypoxia.

The effect of metformin in the expression of PD-L1

Programmed death-ligand 1 (PD-L1) is a main immune checkpoint molecule that negatively regulates T-cell activity. In various cancer types, such as renal-cell carcinoma (RCC) breast cancer, colorectal cancer, gastric cancer, non-small-cell lung cancer (NSCLC), papillary thyroid, and testicular cancers (62, 63), a high level of PD-L1 expression is observed, which is associated with poor prognosis (63-65). The binding of PD-L1 with programmed cell death protein 1 (PD-1) receptor on activated CD8+ CTL inhibits a T-cell receptor (TCR) signaling cascade and suppresses the activity of CTLs (66, 67). Therefore, there have been active attempts to restore CTL activity by blocking PD-L1/PD-1 signaling. FDA approved-ICBs, such as nivolumab, pembrolizumab, and atezolizumab, target PD-1 or PD-L1 (14).

Recently, our group reported that metformin can increase
Table 1. The effect of metformin and its downstream factors on TME components

| TME components               | Key regulator / region | Regulatory mechanism                                                                 | Disease                        | References |
|------------------------------|------------------------|--------------------------------------------------------------------------------------|-------------------------------|------------|
| Chronic inflammation         | IFN-γ, TNF-α, IL-6, and IL-17 | Metformin down-regulates the level of chemokines and pro-inflammatory cytokines.     | Multiple sclerosis             | (41)       |
|                              | TNF-α and IL-6         | Metformin decreases the level of inflammatory cytokines by suppressing NF-κB signaling. | T2D, cardiovascular complications | (42, 43)   |
|                              | ROS and IL-1β          | Metformin decreases the level of inflammatory cytokines from LPS-activated macrophages by means of inhibition of mitochondrial complex I. | Inflammation                  | (44, 45)   |
| TAM polarization             |                        | Metformin inhibit tumor growth by promoting M2-TAM polarization                      | Cancer                        | (47)       |
| Hypoxia                      |                        | Metformin reduces intratumoral hypoxia.                                              | Cancer                        | (58)       |
|                              | HIF-1α                 | Metformin suppresses accumulation of HIF-1α.                                         | Cancer                        | (59, 60)   |
| Oxygen consumption           |                        | Metformin relieves intratumoral hypoxia, resulting in improving T-cell immunity in the TME. | Cancer                        | (61)       |
| PD-L1 expression             | PD-L1                  | Metformin-activated AMPK induces ERAD of PD-L1, resulting in enhanced CD8+ TIL activity. | Cancer                        | (11)       |
|                              | PD-L1                  | Metformin improves cytotoxicity of CD8+ T-cell by reducing membrane-bound PD-L1 level. | Cancer                        | (68)       |
| Metabolic reprogramming of T- cell | AMPK                  | Metformin does not only increase the multi-functionality of CD8+ CTL but also improve long-term memory immunity. | Cancer                        | (10)       |
|                              | Oxidative metabolism   | AMPK maintain consistent activity of CTL under glucose depletion.                    | Cancer                        | (76)       |
|                              | Protein phosphatases    | Genetic ablation of AMPKα1 in T-cell cause apoptosis of CD8+ CTL.                    | Cancer                        | (77)       |
|                              | mTORC1                 | TSC2 KO mice generate highly glycolytic and potent effector CTL.                     | Cancer                        | (78)       |
|                              | mTOR                   | Rapamycin treatment increases the population of CD8+ memory T-cells.                | Virus infection               | (82)       |

Anti-tumor immunity by improving CD8+ TIL activity (Fig. 1C and Table 1) (11). As the underlying mechanism, metformin-activated AMPK directly phosphorylates at Ser195 of PD-L1, and this phosphorylation induces abnormal glycosylation of PD-L1. Eventually, abnormal PD-L1 is degraded by means of the endoplasmic reticulum-associated protein degradation (ERAD) pathway, which recovers CD8+ CTL activity. Furthermore, metformin-treated breast cancer patients’ data and animal work strongly support that a combination of metformin with a non-PD-1/PD-L1 targeting ICB can be effective in treating patients with PD-L1-expressing tumors. Similar to these results, Verdure et al. mentioned that metformin improves cytotoxicity of CD8+ T-cells by reducing the membrane-bound PD-L1 level increased by IFN-γ treatment (68). Consistently, Han et al. showed that poly ADP ribose polymerase inhibitor (PARPi)-resistant cells up-regulate PD-L1 expression, and metformin treatment reverses the PD-L1 level, which sensitizes PARPi-resistant cells to CTL (69). These studies all suggest that metformin can increase T-cell immunity by reducing PD-L1 level in the TME.

The effect of metformin in metabolic reprogramming of T-cells

In adaptive immunity, effector CD8+ CTLs educated by phagocytic APCs in the drain lymph nodes can specifically recognize and kill tumor cells (70). However, effector CTLs undergo metabolic exhaustion in TME, which causes their malfunction and apoptosis (71). The cancer cell relies on ineffective aerobic glycolysis as the main source of ATP, not oxidative phosphorylation, and fills the need by actively absorbing glucose (72). An unusual metabolic property called the ‘Warburg effect’ has an advantage for cancer cells in survival under hypoxia. Interestingly, the metabolism of effector CTL is converted to aerobic glycolysis after binding with a cancer antigen in the tumor (73). This metabolic similarity encourages glucose-uptake competition between cancer cells and CTL, leading to metabolic exhaustion of effector CD8+ CTL (74). In this regard, the metabolic reprogramming of exhausted T-cells can be a therapeutic target to increase anti-tumor immunity.

Since AMPK and mTOR, downstream factors of metformin,
are the main regulators in energy sensing and mitochondrial function, metformin may play on the metabolic reprogramming of exhausted T-cells (Fig. 1D and Table 1) (75). Indeed, metformin treatment has been demonstrated to increase the number of CD8+ CTL and prevent their exhaustion in the tumor (10, 11). AMPK is reported to maintain consistent activity of CTL by activating oxidative metabolism under glucose depletion (76). Rao et al. showed that genetic ablation of AMPKα1 in T-cells cause apoptosis of CD8+ CTL during in vitro activation and in vivo tumor development, and promote tumor growth in an immune-competent mouse model (77). Consistently, when tuberous sclerosis complex 2 (TSC2), an mTORC1 negative regulator, is genetically ablated, the mice showed the generation of highly glycolytic and potent effector CTL (78).

Most CD8+ T-cells that have completed their mission are removed by means of apoptosis, and some of them differentiate into CD8+ memory T-cells, which respond faster and more effectively to the same enemy, preventing recurrence (79). This transition to CD8+ memory T-cells also requires metabolic reprogramming (80). Interestingly, Elikawa et al. showed that metformin not only increases the multi-functionality of CD8+ CTL but also improves long-term memory immunity (10). In this study, metformin treatment increased the effector memory T-cell/central memory T-cell (TEM/TCM) ratio. Indeed, the AMPK-mTOR pathway is known to regulate memory CD8+ T-cell but also improves long-term memory immunity (10).

In vivo activation of CD8+ memory T-cells central memory T-cell (TEM/TCM) ratio. Indeed, the AMPK-mTOR pathway is known to regulate memory CD8+ T-cell but also improves long-term memory immunity (10). Consistent with this result, mTOR inhibition with rapamycin (87), and (3) activated T-cells cause apoptosis of CD8+ CTL during in vitro activation and in vivo tumor development, and promote tumor growth in an immune-competent mouse model (77). Consistently, when tuberous sclerosis complex 2 (TSC2), an mTORC1 negative regulator, is genetically ablated, the mice showed the generation of highly glycolytic and potent effector CTL (78).

Recently, three different groups reported at similar times that distinct gut microbiota profiles are observed between ICB responders and non-responders, and that the therapeutic efficacy of ICB is greatly affected by the gut microbiota (86-88). The fecal microbial transplant (FMT) model showed that the efficacy of ICB is much higher in mice transplanted from ICB responders than in those from non-responders, and FMT from ICB responders highly improved T-cell immunity. In addition, microbes beneficial for ICB immunotherapy were identified as (1) Faecalibacterium prausnitzii (86), (2) Bifidobacterium longum (87), and (3) Akkermansia muciniphila (88).

Cuesta-Zuluaga et al. demonstrated the relevance of T2D, metformin, and gut microbiota (Fig. 1E and Table 1) (89). Interestingly, metformin shifted the gut microbiota profile of T2D patients, and in particular increased A. muciniphila and several beneficial microbes (85), suggesting that the balance between the immune system and the symbiotic microbiota is very important for maintaining our immunity. The following studies suggest that gut microbiota may be an important mediator of metformin which positively affects anti-tumor immunity.

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**Table 2.** Current clinical trials of metformin combined with ICB

| Title (NCT No.)                                      | Disease       | Drug           | Sponsor (Collaborator)              | Phases |
|------------------------------------------------------|---------------|----------------|-------------------------------------|--------|
| Nivolumab and Metformin Hydrochloride in Treating Patients With Stage III-IV Non-small Cell Lung Cancer That Cannot Be Removed by Surgery (NCT03048500) | NSCLC         | Metformin      | Northwestern University (BMS, NCI)  | Phase 2 |
| Nivolumab and Metformin in Patients With Treatment Refractory MSS Colorectal Cancer (NCT03800612) | Colorectal cancer | Metformin      | Emory University (BMS)              | Phase 2 |
| Combining Pembrolizumab and Metformin in Metastatic Head and Neck Cancer Patients (NCT04414540) | HNSCC         | Metformin      | UC Health (American Cancer Society) | Phase 2 |
| Anti-PD-1 mAb Plus Metabolic Modulator in Solid Tumor Malignancies (NCT04114336) | Melanoma      | Metformin      | University of Pittsburgh             | Phase 2 |
| A Trial of Pembrolizumab and Metformin Versus Pembrolizumab Alone in Advanced Melanoma (NCT03311308) | Melanoma      | Metformin      | University of Pittsburgh (Merck)    | Phase 1 |
**CONCLUDING REMARKS**

Most of the current cancer immunotherapy targets TIL and works by maintaining the activity of TIL in TME. In order to ensure their effectiveness, anti-tumor immune processes, including APCs infiltration, cancer antigen presentation, T-cell priming, and TIL activity maintenance, must be adequately achieved (92). Indeed, “hot” tumors that achieve these prerequisites respond well to cancer immunotherapy, but “cold” tumors that do not meet the prerequisites have a poor response to immunotherapy (93). If cold tumors can turn into hot ones, we could greatly improve the efficacy of current cancer immunotherapy, as well as expand the beneficiaries.

In this favorable conversion, the problem is that immunosuppressive TME in cold tumors inhibits multiple targets at different stages of anti-tumor immunity (94). Therefore, it would be difficult to expect an effective conversion with a single factor or path-adjusting approach. Although the choice of inhibitor cocktail can be considered to target multiple immunosuppressive factors, it would probably cause excessive side effects, such as accumulated toxicity. To support the conversion of cold tumors, the repositioning of previously well-tolerated drugs that have a multi-immunomodulatory effect would be a more effective and safer approach.

As summarized in this mini-review, metformin’s safety and tolerability have been proven by decades of clinical experience. In addition, this drug has multiple beneficial effects on anti-tumor immunity, such as by having anti-inflammatory and anti-hypoxic effects, increasing T-cell activity, and acting favorably on the gut microbiota (Fig. 1 and Table 2). Moreover, control-case studies showed that metformin reduces the incidence of various types of cancer, and the positive effect of metformin on anti-tumor immunity has been consistently observed by means of several animal models. These findings strongly suggest that metformin has a high potential to be an adjuvant to comprehensively overwhelm immunosuppressive TMEs by supporting the stable conversion of cold tumors.

In current clinical trials that reflect this possibility, metformin is being co-administered with ICB targeting PD-L1/PD-1 (nivolumab and pembrolizumab) in patients with advanced melanoma, colorectal cancer, hepatocellular carcinoma (HCC), head and neck squamous-cell carcinoma (HNSCC), NSCLC, and RCC (Table 2). In anticipation of the old friend’s re-evaluation, let’s pay attention to the upcoming results.
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