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Invited Mini Review

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

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T-cell-based cancer immunotherapies, such as immune checkpoint blockers (ICBs) and chimeric antigen receptor (CAR)-T-cells, have significant anti-tumor effects against certain types of cancer, providing a new paradigm for cancer treatment. However, the activity of tumor infiltrating T-cells (TILs) can be effectively neutralized in the tumor microenvironment (TME) of most solid tumors, rich in various immunosuppressive factors and cells. Therefore, to improve the clinical outcomes of established T-cell-based immunotherapy, adjuvants that can comprehensively relieve multiple immunosuppressive mechanisms of TME are needed. In this regard, recent studies have revealed that metformin has several beneficial effects on anti-tumor immunity. In this mini-review, we understand the immunosuppressive properties of TME and how metformin comprehensively enhances anti-tumor immunity. Finally, we will discuss this old friend's potential as an adjuvant for cancer immunotherapy. [BMB Reports 2020; 53(10): 512-520]

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 cancer types, suggesting an anti-tumor effect of metformin beyond that against diabetes (2, 3). Indeed, several studies have reported an anti-proliferative effect of metformin against various types of cancer by means of different pathways (4). Metformin–activated AMPK inhibits the mammalian target of rapamycin (mTOR) pathway, which regulates protein synthesis, cell proliferation, 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 mitochondrion 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 (Axicabtageneciloleucel, and Tisagenlecleucel)] (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.

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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 (DC)s 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 (CTL) 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 (T regs), 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.

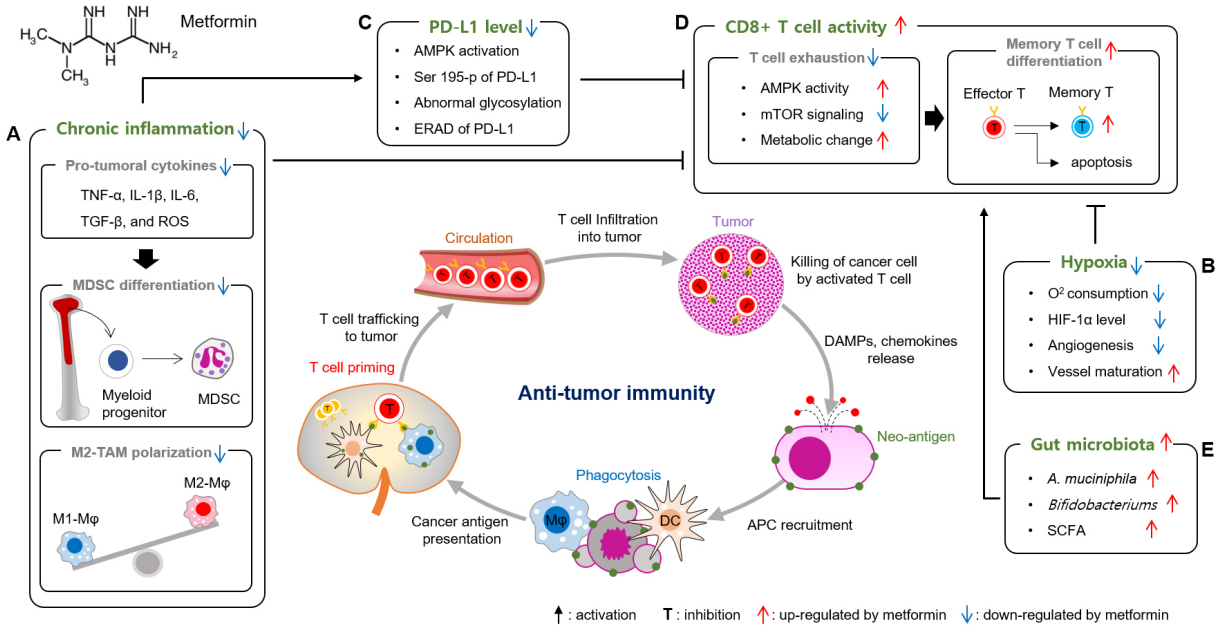

Fig. 1. Several beneficial effects of metformin on anti-tumor immunity. Metformin indirectly increases T-cell activity by negatively regulating (A) chronic inflammation, (B) hypoxia, and (C) PD-L1 levels that inhibit T-cell activity. (D) Metformin directly relieves T-cell exhaustion by means of metabolic reprogramming of TIL and promotes memory T-cell differentiation. (E) Metformin shifts the profile of gut microbiota more favorably to T-cell immunity (TAM) tumor-associated macrophages; (Mφ) macrophages; (MDSC) myeloid-derived suppressor cells; (T) T-cell; (DAMPs) Damage-associated molecular patterns; (APC) antigen presenting; (SCFA) short-chain fatty acid.

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). Consistently, 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 and pro-angiogenic factors (58). Consistently, metformin suppresses accumulation 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) (44, 45)
	ROS and IL-1β	Metformin decreases the level of inflammatory cytokines from LPS-activated macrophages by means of inhibition of mitochondrial complex I.	Inflammation	(46)
	TAM polarization	Metformin inhibit tumor growth by promoting M2-TAM polarization	Cancer	(47)
	MDSC chemotaxis	Metformin has anti-tumor effect by reducing MDSC accumulation in the TME via AMPK/DACH1/CXCL1 axis.	Cancer	(48)
Hypoxia	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)
	PD-L1	Metformin treatment reverses PD-L1 level, which sensitizes PARPi-resistant cells to CTL.	Cancer	(69)
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 underlining mechanism, metformin-activated AMPK directly phosphorylates at Serine 195 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*. also 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 differentiation. Cantrell *et al* showed that AMPKα1-deficient T-cells have a defect in generation of CD8 + memory T-cells (81). Consistent with this result, mTOR inhibition with rapamycin increased the population of CD8 + memory T-cells in a virus-infected mice and primate model (82). Overall, metformin probably increases anti-tumor efficacy of CTL and its *in vivo* persistence by means of metabolic reprogramming via the AMPK/mTOR pathway.

PERSPECTIVES AND FUTURE DIRECTIONS

Abnormal gut microbiota profiles have emerged in a variety of diseases related to immune disorder, such as inflammatory bowel disease, rheumatoid arthritis, myocardial metabolic disease, CNS autoimmune disease, and cancer (83, 84). Symptoms of such diseases have been improved by means of microbial modulation by dietary therapy or direct transplantation of 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.

Recently, three different groups reported at similar times that distinct gut microbiota profiles are observed between ICB responders and non-reponders, 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 IBC 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

Table 2. Current clinical trials of metformin combined with ICB

Title (NCT No.)	Schedule	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)	Start : Jun. 2017 End : Feb. 2021	NSCLC	Metformin Nivolumab	Northwestern University (BMS, NCI)	Phase 2
Nivolumab and Metformin in Patients With Treatment Refractory MSS Colorectal Cancer (NCT03800602)	Start: Jan. 2019 End : Jan. 2023	Colorectal cancer	Metformin Nivolumab	Emory University (BMS)	Phase 2
Combining Pembrolizumab and Metformin in Metastatic Head and Neck Cancer Patients (NCT04414540)	Start: Jul. 2020 End : Feb. 2024	HNSCC	Metformin Pembrolizumab	UC Health (American Cancer Society)	Phase 2
Anti-PD-1 mAb Plus Metabolic Modulator in Solid Tumor Malignancies (NCT04114136)	Start: May 2020 End : Nov. 2023	Melanoma RCC NSCLC HCC HNSCC	Metformin Pembrolizumab Nivolumab	University of Pittsburgh	Phase 2
A Trial of Pembrolizumab and MetforminVersus Pembrolizumab Alone in Advanced Melanoma (NCT03311308)	Start: Dec. 2017 End : Dec. 2028	Melanoma	Metformin Pembrolizumab	University of Pittsburgh (Merck)	Phase 1

Bifidobacterium species. As mentioned, *A. muciniphila* and B*ifidobacterium* are species identified as microbes beneficial for ICB immunotherapy (87, 88). Further, *A. muciniphila* and *Bifidobacterium* species are known to produce short-chain fatty acids (SCFA), like acetate, propionate, and butyrate, which have immune-modulatory and anti-inflammatory effects (90, 91). Such evidence strongly supports that metformin could affect anti-tumor immunity in a systematic manner via gut microbiota. However, it is largely undefined how metformin can shift a gut microbiota profile to be favorable to anti-tumor immunity and what is the link between metformin-increased germ species and anti-tumor immunity. To address these issues, future studies are needed, which are expected to greatly increase the usefulness of metformin and provide new insights and therapeutic targets for cancer immunity.

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 havinging 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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CONFLICTS OF INTEREST

The authors have no conflicting interest.

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