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EDITORIAL

Harnessing the soil: reshaping the tumor microenvironment towards an antitumor immune state by low-dose metformin

Metformin, a biguanide derivate, has been in use as an anti-diabetic agent for over 60 years and has been widely accepted as first-line therapy for the management of type II diabetes. Interestingly, the treatment of type II diabetes with metformin is associated with reduced risk and improved prognosis for many types of cancers [1]. This observation has led to an explosion of interest in the potential application of metformin for cancer chemopreventive and direct antitumor effects, but also more recently for potential immunomodulatory activity.

Cancer immunotherapy has a major impact on the treatment outcomes of various solid tumors, with long-term remission and even cure in certain patients. Nevertheless, the heterogeneity of the tumor microenvironment, such as those found in esophageal cancer, can influence the response to immunotherapy [2]. The use of drugs such as metformin may help to alter the tumor immune microenvironment (TIME) and could improve the efficiency of immunotherapy. The antitumor immune activity of metformin has only become a focus in the past 2-3 years in preclinical studies, including enhancing the quality and quantity of T cells [3], switching the phenotype of tumor-promoting macrophages to tumor-suppressive [3], altering cytokine expression and downregulating programmed death-ligand 1 (PD-L1) [4, 5].

We recently reported on a phase II clinical trial of low-dose metformin treatment in patients with esophageal squamous cell carcinoma (ESCC) that provided clear evidence of the immunomodulatory properties of metformin in the TIME of cancer patients [6]. In this double-blinded study, patients underwent endoscopic biopsy of esophageal tumors. Upon pathological diagnosis with ESCC, patients were randomized to receive tablets of 250 mg metformin or placebo of identical appearance for 7-14 days before surgery (Figure 1). Interestingly, although no impact on cell proliferation or apoptosis levels was detected in ESCC tissues, metformin treatment markedly altered the immune repertoire in ESCC tissues. In pre-treatment biopsies, there was no significant difference between placebo- and metformin-treated groups in any of the markers assayed for innate or adaptive immunity. However, in post-treatment biopsies of metformin-treated patients, $CD8^+$ T cell infiltration was significantly increased, whereas $CD4^+$ T cell infiltration was significantly decreased. For innate immune cells, metformin treatment significantly increased the percentage of $CD11c^+$ tumor-suppressive macrophages and decreased the percentage of $CD163^+$ tumor-promoting macrophages. In multivariate regression analysis, metformin treatment

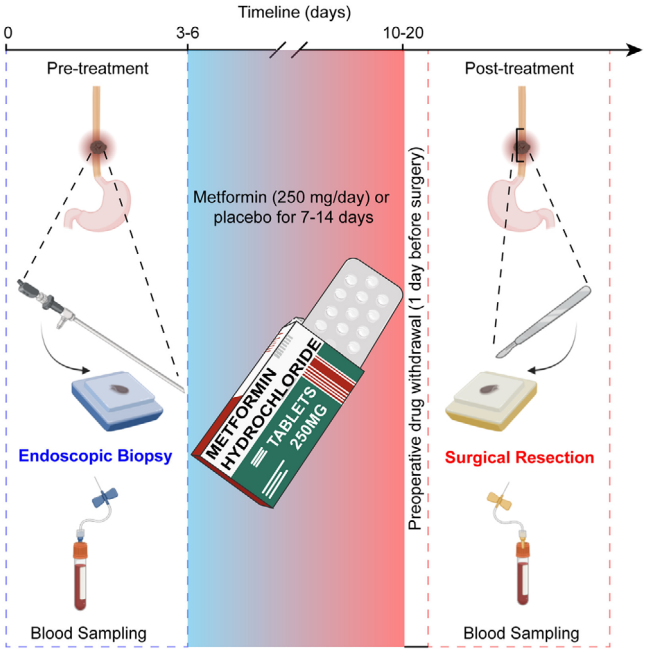

FIGURE 1 Patient treatment and sample collection scheme for the clinical trial of low-dose metformin treatment in patients with esophageal squamous cell carcinoma

Abbreviations: 4-NQO, 4-Nitroquinoline 1-oxide; AMPK, adenosine monophosphate-activated protein kinase; ESCC, esophageal squamous cell carcinoma; FoxP3, forkhead box P3; IFN-γ, interferon-gamma; IL, interleukin; LKB1, liver kinase B1; PD-L1, programmed death-ligand 1; SIRP, signal regulatory protein; STAT3, signal transducer and activator of transcription 3; TIME, tumor immune microenvironment; TNF, tumor necrosis factor

remained an independent factor for changes in $CD8^+$ T cells, $CD11c^+$ myeloid cells, $CD163^+$ myeloid cells, and $CD20^+$ B cells. Notably, although PD-L1 is a crucial checkpoint protein associated with T cell exhaustion and non-inflamed TIME [7], PD-L1 expression did not change in either study arm.

These clinical observations were closely recapitulated in an ESCC mouse model induced by carcinogen 4-nitroquinoline 1-oxide (4-NQO), where low-dose and short-term metformin treatment yielded almost identical changes in TIME as detected in ESCC patients. Upon extended treatment with metformin, the antitumoral TIME changes of the mice became more pronounced. Specifically, the percentage of $CD11c^+$ tumor-suppressive macrophages was further increased, whereas $CD206^+$ tumor-promoting macrophages were strongly suppressed. In line with this, treatment of ESCC cells with low-dose metformin *in vitro* significantly potentiated phagocytic uptake of ESCC cells via macrophages. Further, the percentage of forkhead box P3 $(FoxP3)^+$ regulatory T cells was reduced. In contrast to short-term treatment, the expression of PD-L1 was significantly decreased following long-term metformin treatment compared to placebo treatment. Moreover, long-term metformin treatment decreased ESCC cell proliferation as well as the number of tumors per mouse. Thus, the mouse model mirrored the alterations of metformin-induced TIME reprogramming in ESCC patients and provided a window to capture the impact of extended treatment with low-dose metformin in ESCC.

One of the main mechanisms of action for the antitumor activity of metformin is thought to be the regulation of liver kinase B1 (LKB1)/adenosine monophosphate-activated protein kinase (AMPK) pathways. In addition, dephosphorylation of signal transducer and activator of transcription 3 (STAT3) has been recently identified to be triggered by metformin [8]. Both AMPK and STAT3 are known to participate in the modulation of the metabolic reprogramming and immunoregulation in immune cells (*i.e.*, $CD8^+$ T cells and $CD11c^+$ tumor-suppressive macrophages) [9, 10]. Indeed, clear activation of AMPK and inactivation of STAT3 were detected in intra-tumoral immune cells, associating with significant increases in tumor necrosis factor-α(TNF-α)$^+$CD8$^+$ T cells, interferon-γ(IFN-γ)$^+$CD8$^+$ T cells, TNF-α^+CD11c$^+$ macrophages, and IFN-γ^+CD11c$^+$ macrophages. At the same time, a significant decrease in interleukin (IL)-10$^+$CD11c$^+$ macrophages was detected. Thus, these data provide evidence that low-dose metformin mediates metabolic-immune signaling pathways, most likely via AMPK-STAT3 signaling, leading to an increase in pro-inflammatory cytokine expression in key immune effector cell types in the tumor microenvironment.

From our data, it is clear that metformin alters the TIME in patients and in a closely-matched mouse model, possibly via metabolic reprogramming. However, the potential implementation of metformin as a bona fide immuno-metabolic adjuvant in clinics still warrants further evaluation. Its effect may also be influenced by particular body conditions including high blood glucose, obesity, and other metabolic disturbances which may change the metabolism locally or systemically [4]. In addition, it has been reported that the metabolic program is different between resting immune cells and proliferative immune cells, as well as between normal cells and cancer cells [11, 12]. Further, the TIME contains a host of diverse cell types with distinct and flexible metabolic states. Thus, the exact outcome of metformin treatment may partly depend on the composition of the TIME before treatment. Future efforts on the immuno-metabolic mechanism contributing to the antitumor actions of metformin are warranted, and the strategy targeting metabolic reprogramming may have the potential to be optimized to selectively target cancer cells or the TIME.

Of note, metformin increased the number of tumor-suppressive macrophages and decreased that of tumor-promoting macrophages. In addition, the phagocytic activity of metformin-treated macrophages toward ESCC cells was enhanced, indicating that metformin plays an important role in (re)activating macrophage-mediated immunity. Our findings, thus, suggest that the potential combination of metformin with innate immune-targeting strategies, such as combination with CD47-blocking or CD24-blocking antibodies that remove CD47/signal regulatory protein-alpha- or CD24/Siglec-10-mediated "don't eat me" signaling, might be worth further (pre)clinical investigation [13, 14].

Low-dose metformin also clearly increased the number of B lymphocytes. Currently, increasing evidence supports a pivotal role of B lymphocytes in tumor immunology but the function of tumor-infiltrating B cells remains controversial [15, 16], suggesting that the specific subpopulation and function of tumor-infiltrating B cells need to be carefully assessed. Therefore, the potential impact of metformin-induced functional alterations on B lymphocytes will need to be delineated in further studies.

Classification of the TIME into subtypes has been attempted previously to give a readily interpretable and broad-stroke overview of the tumor microenvironment status. Such subtype classification of the TIME is a potentially useful tool for evaluating the immunological status and predicting immunotherapy response and prognosis [17]. Using histochemistry and immunofluorescence, we similarly developed a generalized model for the microenvironment in our study, with a suppressive (S-TIME), equilibrated (E-TIME), or activated (A-TIME) status, based on

the relative presence of immune cell subtypes. Metformin treatment clearly and positively impacted the TIME status, with significant shifts of TIME in ESCC patients from S-TIME or E-TIME towards A-TIME status. It will be interesting to implement additional technologies to get a further detailed understanding and optimize the TIME definitions for ESCC. Hereto, alternative approaches, such as flow cytometry or single-cell sequencing can be exploited, with the landscape of immune cells in ESCC microenvironment in a mouse model and patients recently being reported [2, 18]. Therefore, the conclusions of metformin treatment for the TIME remodeling in ESCC may be further refined and fine-tuned to provide better predictive value.

Given the pivotal role of TIME status during immunotherapies, it would be of great worth to develop a "gold standard" method for defining the TIME status that may aid in defining the impact of drugs and combinatorial treatment strategies. With the development of quantitative multiplex immunohistochemistry, single-cell sequencing, and spatial transcriptomics, the classification and definition of TIME status could be further improved, and the relative impact of individual cell type changes can be better weighed. Ultimately, TIME classification may be implemented in clinical care for diagnosis and patient stratification.

Due to the asymptomatic feature of patients with early-stage ESCC, most ESCC cases present in late stages [19, 20], with ~48% of patients having stage III ESCC in our study. Thus, the potential impact of metformin in early developmental stages of ESCC (*i.e.*, its influence on inhibiting the formation of tumor metastasis) remains to be determined. It remains to be elucidated whether the antitumor immune response mediated by metformin is related to the treatment duration or dosage. Importantly, since the mouse model closely corresponds to the data obtained from patients, this mouse model may serve as a tool to more rapidly investigate different timing and treatment strategies in order to steer the design of next clinical trials with metformin in ESCC.

There are increasing clinical trials evaluating metformin for cancer prevention and treatment, with our study providing compelling evidence for the immunomodulatory role of metformin in the tumor microenvironment. For future clinical studies, one should bear in mind that the antitumor benefits of metformin include not only direct tumor-killing effects but also TIME activation with diverse mechanisms (Figure 2). A clinical study with a detailed physical examination, a meticulous assessment of tumor status, and a thoughtful plan for TIME evaluation would help to provide meaningful information and lead to successful outcomes. Before metformin can be properly implemented as an effective adjuvant for immune boosting in clinical practice, carefully designed prospective investigations are required to confirm the impact of metformin on the TIME.

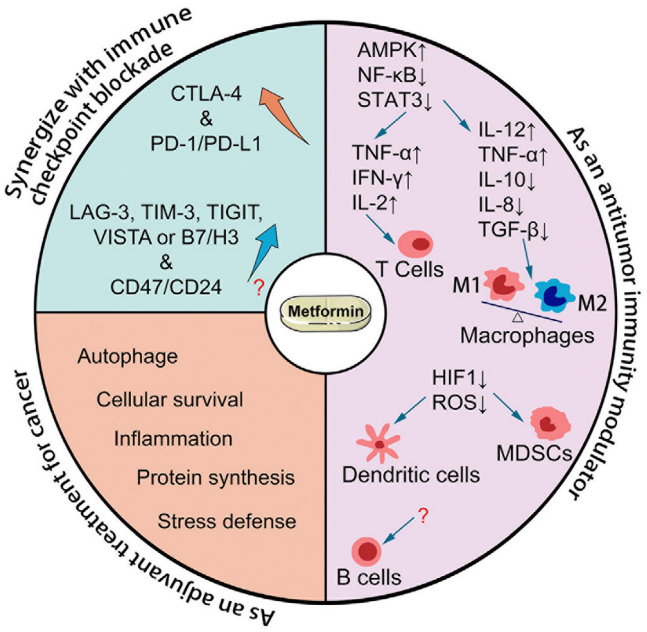

FIGURE 2 Model of metformin action in cancer cells and the tumor immune microenvironment. Abbreviations: CTLA-4, cytotoxic T-lymphocyte antigen 4; PD-1, programmed cell death 1; PD-L1, programmed death-ligand 1; LAG-3, lymphocyte activating 3; TIM-3, T cell immunoglobulin, and mucin domain-containing protein 3; TIGIT, T cell immunoreceptor with Ig and ITIM domains; VISTA, V-domain Ig suppressor of T cell activation; B7/H3, B7 homolog 3; AMPK, adenosine monophosphate-activated protein kinase; NF-κB, nuclear factor kappa B; STAT3, signal transducer and activator of transcription 3; TNF, tumor necrosis factor; IFN-γ, interferon-gamma; IL, interleukin; TGF-β, transforming growth factor-beta; HIF-1, hypoxia-inducible factor 1; ROS, reactive oxygen species; MDSCs, myeloid-derived suppressor cells

DECLARATIONS

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

CONSENT FOR PUBLICATION

All authors consent to publish the paper.

DATA AVAILABILITY STATEMENT

Not applicable.

AUTHORS' CONTRIBUTIONS

YL, SW, EB, and HZ wrote the manuscript. All authors read and approved the final manuscript.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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