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Low-dose metformin reprograms the tumor immune microenvironment in human esophageal cancer: Results of a phase II clinical trial

Shuhong Wang[1,2†], Yusheng Lin[1,2,3†], Xiao Xiong[1], Lu Wang[1], Yi Guo[4], Yuping Chen[5], Shaobin Chen[5], Geng Wang[5], Peng Lin[1], Hongcai Chen[6], Sai-Ching Jim Yeung[7,8*], Edwin Bremer[3], Hao Zhang[1*]

Authors affiliations

[1]Department of General Surgery, The First Affiliated Hospital of Jinan University, and Institute of Precision Cancer Medicine and Pathology, Jinan University Medical College, Guangzhou, Guangdong, China.

[2]Shantou University Medical College, Shantou, Guangdong, China.

[3]Department of Hematology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands.

[4]Endoscopy Center, Affiliated Cancer Hospital of Shantou University Medical College, Shantou, Guangdong, China.

[5]Department of Thoracic Surgery, Affiliated Cancer Hospital of Shantou University Medical College, Shantou, Guangdong, China.

[6]Department of Immunotherapy and Gastrointestinal Oncology, Affiliated Cancer Hospital of Shantou University Medical College, Shantou, Guangdong, China.

[7]Department of Emergency Medicine, University of Texas MD Anderson Cancer Center, Houston, Texas, USA.

[8]Department of Endocrine Neoplasia and Hormonal Disorders, University of Texas MD Anderson Cancer Center, Houston, Texas, USA.

[*]**Correspondence to:** Professor Hao Zhang, Institute of Precision Cancer Medicine and Pathology, Jinan University Medical College, 601 Huangpu Avenue West, Guangzhou, Guangdong 510632, China, Tel./Fax: +86 20 85224770, E-mail address: haozhang@jnu.edu.cn; Professor Sai-Ching Jim Yeung, Department of Emergency Medicine, University of Texas MD Anderson Cancer Center, Houston, Texas, USA, E-mail address: syeung@mdanderson.org.

[†]Shuhong Wang and Yusheng Lin share co-first authorship.

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Translational Relevance

The tumor immune microenvironment (TIME) has an important impact on response to immune checkpoint inhibitor therapy for cancer. Therefore, therapeutic strategies that can reshape TIME towards a more active state may improve the treatment outcome. Here, we described the first phase II clinical trial of low-dose metformin (250 mg/day) in human esophageal squamous cell carcinoma (ESCC) that demonstrated the impact of metformin on B cells, T cells and macrophages in TIME. Our mouse model data corroborated and extended the findings that the impact on the TIME is sustained with long-term low-dose metformin treatment. The current discovery highlights that low-dose metformin reprograms the TIME to an activated state and may be a safe and efficacious pre-treatment/combination option to boost the effectiveness of immunotherapy (*e.g.*, CD47 blocking agents) in future clinical trials. Low-dose metformin may be a suitable immune response modifier in ESCC patients that can be easily integrated in routine care.

Abstract

Purpose: The tumor immune microenvironment (TIME) has an important impact on response to cancer immunotherapy using immune checkpoint inhibitors. Specifically, an "infiltrated-excluded"/"cold" TIME is predictive of poor response. The antidiabetic agent metformin may influence anti-cancer immunity in esophageal squamous cell carcinoma (ESCC).

Experimental Design: We analyzed matched pre- and post-treatment ESCC specimens in a phase II clinical trial of low-dose metformin treatment (250 mg/day) to evaluate direct anti-ESCC activity and TIME-reprogramming. Follow-up correlative studies using a carcinogen-induced ESCC mouse model were performed with short-term (1 week) or long-term (12 weeks) low-dose metformin (50 mg/kg/day) treatment.

Results: In the clinical trial, low-dose metformin did not affect proliferation or apoptosis in ESCC tumors as assayed by Ki67 and cleaved caspase-3 immunostaining. However, metformin reprogrammed the TIME towards "infiltrated-inflamed" and increased the numbers of infiltrated $CD8^+$ cytotoxic T-lymphocyte and $CD20^+$ B-lymphocyte. Further, an increase in tumor-suppressive ($CD11c^+$) and a decrease in tumor-promoting ($CD163^+$) macrophages were observed. Metformin augmented macrophage-mediated phagocytosis of ESCC cells *in vitro*. In ESCC mouse model, short-term metformin treatment reprogrammed the TIME in a similar fashion to humans, whereas long-term treatment further shifted the TIME towards an active state (*e.g.*, reduction in $CD4^+$ $FoxP3^+$ Tregs) and inhibited ESCC growth. In both humans and mice, metformin triggered AMPK activation and STAT3 inactivation, and altered the production of effector cytokines (*i.e.* TNF-α, IFN-γ, IL-10) in the immune cells.

Conclusions: Low-dose metformin reprograms the TIME to an activated status and may be a

suitable immune response modifier for further investigation in ESCC patients.

INTRODUCTION

Complex microenvironments have evolved during tumorigenesis to facilitate cancer growth (1). The tumor microenvironment contains many types of immune cells: dendritic cells (DC), natural killer (NK) cells, macrophages, granulocytes, and mast cells from the innate immune system; and T and B lymphocytes from the adaptive immune system (2-4). This tumor immune infiltrate contributes to define a pathologically active and specialized cancer niche, the Tumor Immune Microenvironment (TIME).

The TIME in solid tumors can be characterized as three states: active (A-TIME), equilibrated (E-TIME), and suppressive (S-TIME) (5-8). A hallmark of A-TIME is strong infiltration with $CD8^+$ effector T cells and tumor-suppressive macrophages. The S-TIME is characterized by increased $CD4^+$ helper and regulatory $FoxP3^+$ T cells and tumor-promoting macrophages (1,9). A major hallmark of the S-TIME is T-cell exhaustion induced by activation of inhibitory immune checkpoints, such as PD-1/PD-L1 (6). The E-TIME is characterized by equal infiltration of immune effector cells and immunosuppressive cells (10-12). Patients with A-TIME were found to have more favorable clinical outcomes compared to patients with S-TIME (10). Patients with E-TIME also had longer survival than those with S-TIME (10-12). Therefore, therapeutic strategies that can reshape the TIME towards a more reactive phenotype would be expected to improve the outcome of cancer immunotherapy.

Metformin, a widely used drug for type II diabetes, is being investigated in many clinical trials for human cancers, with doses ranging from 250 mg/day to 2000 mg/day (13-17). Previously, we reported that metformin also inhibited the growth and progression of esophageal squamous cell carcinoma (ESCC) in preclinical models (18,19). A recent retrospective study

revealed that metformin benefited ESCC patients with type II diabetes (20). In recent studies, there is evidence that metformin impacts on the TIME, an immunomodulatory effect that is yet to be completely defined (21-23). Recent evidence also suggests that the antitumor activity of metformin can, at least partly, be attributed to immune activation (24). Thus, metformin appears to have immunomodulatory effects that may be harnessed for cancer immunotherapy.

Here, we performed the first randomized phase II clinical trial in ESCC patients with a low dose (250 mg/day) and short-term treatment with metformin to determine: 1) the direct impact of metformin on ESCC cells, 2) the impact of metformin on the TIME status. The human data were further corroborated and extended using an orthotopic ESCC mouse model and *in vitro* immune functional experiments.

MATERIALS AND METHODS

Patient cohort

We recruited 128 patients in a phase II trial (Registration number: ChiCTR-ICR-15005940) from the Cancer Hospital of Shantou University Medical College (CHSUMC) between September 2014 and September 2016. Written informed consents were obtained from all participants in accordance with the principles established by the Helsinki Declaration. The clinical protocol was approved by the Institution Ethics Committee and Institutional Review Board of CHSUMC (2014060938). The inclusion criteria were: 1) 18-75 years of age, 2) clinical diagnosis of ESCC, 3) scheduled to undergo surgical resection without neoadjuvant chemotherapy. The exclusion criteria were: 1) renal insufficiency, 2) pregnant or lactating females, 3) history of other malignancies, 4) unstable angina, uncontrolled ischemic cardiomyopathy or congestive heart failure with symptoms (for example: New York Heart Association functional class III or IV), 5) diabetics receiving insulin, metformin or sulfonylurea medications, 6) history of lactic acidosis, 7) chronic liver disease or cirrhosis, 8) allergic to or intolerant of metformin, 9) inability to provide written informed consent.

Clinical study design

In this double-blind study, enrolled patients underwent endoscopic biopsy of esophageal tumors, and participants with a pathological diagnosis of ESCC were randomized to receive metformin 250 mg orally per day or placebo tablets of identical appearance in the same regimen for a convenient duration of 7 to 14 days before surgery. Surgical samples of cancer from study subjects were freshly collected for evaluation. Fasting blood glucose was checked before and

after drug treatment. The primary objective of this trial was to investigate the impact of metformin on cell proliferation and apoptosis markers, Ki67 and cleaved Caspase 3, in ESCC tissues. The secondary objective was to evaluate the impact of metformin on immune components in ESCC tissues. The study subjects were staged according to the Union for International Cancer Control (UICC) Tumor-Node-Metastasis (TNM) staging system (7th edition) (25).

TIME status was analyzed and categorized with some modifications according to previous reports (1,9,10). In brief, the median of the percentage of marker-positive cells was used as a cut-off value to dichotomously classify each immune cell type into a rich or poor status. Markers used were CD8, CD4 and FoxP3 for T cell populations, CD20 for B cells (and CD19 for the mouse studies), and CD68, CD11c and CD163 for macrophages (with F4/80 and CD206 for mouse studies) (1,9,10). The strong infiltration of the lesion with $CD8^+$ effector T cells (1,9,21,26), tumor-suppressive macrophages ($CD68^+$, $CD11c^+$) (1,9,27), and B cells ($CD20^+$) (28-30) were reported to be the hallmark of inflamed status, whereas the immunosuppressed status was characterized by rich $CD4^+$ helper T cells (1,9,31), regulatory $FoxP3^+$ T cells (1,9,32) and tumor-promoting macrophages ($CD163^+$) (1,9). Here, the TIME was considered as activated (A-TIME) if a majority (≥4) of 7 markers contributing to an inflamed status (CD8-rich, CD4-poor, FoxP3-poor, CD20-rich, CD68-rich, CD11c-rich, CD163-poor) were detected simultaneously. The TIME was considered as suppressive (S-TIME) if a majority (≥4) of 7 markers contributed to an immunosuppressed status (CD8-poor, CD4-rich, FoxP3-rich, CD20-poor, CD68-poor, CD11c-poor, CD163-rich). In all other cases, the TIME was considered as in equilibrium (E-TIME). The TIME status pre- and post-treatment were analyzed as

aforementioned, and treatment-induced changes were classified as: 1) no change, 2) positive (anti-cancer) change and 3) negative (pro-cancer) change.

Animal experiments

All protocols and procedures for animal experiments were reviewed and approved by Ethics Committee of Shantou University Medical College (SUMC) and the Chancellor's Animal Research Committee at SUMC (SUMC2014-148). Six-week-old female C57BL/6 mice (Vital River Lab Animal Technology Co Ltd., Beijing, PR China) were given the carcinogen 4-Nitroquinoline N-oxide (4-NQO, Cat. N8141; Sigma) in drinking water (100 μg/mL) for 16 weeks to induce ESCC. The mice were randomized into four groups (10 /group). To investigate the short-term effects of low-dose metformin on ESCC, metformin (50 mg/kg) or PBS (same volume) was injected intraperitoneally for 1 week and then the mice were euthanized. To investigate the long-term effects, another two groups were treated with metformin or PBS the same way except for 12 weeks.

Statistical analysis

SPSS 20.0 statistical software package (Armonk, NY, IBM Corp., USA) and R Version 3.5.3 (The R Project for Statistical Computing, http://www.r-project.org) were used. Values before and after treatment for the same study subjects were compared by the paired *t* test. Comparisons between independent groups were performed with the independent *t* test or Wilcoxon test, where appropriate, for numeric values. Ratios and proportions were analyzed with χ^2 test or Fisher exact test where appropriate. Bonferroni correction was applied for multiple testing where appropriate. Correlation between immune makers was examined by Pearson's correlation test. A multivariate regression analysis was conducted to determine the relationship between immune markers expression and clinicopathological variables.

Details for biochemical assays are included in supplementary materials and methods.

RESULTS

Low-dose metformin treatment does not affect blood glucose balance nor directly affects human ESCC cells

In the clinical trial, subjects with pathologically confirmed ESCC were randomized into two arms with 46 receiving 250 mg metformin per day and 42 receiving placebos (Supplementary Figure 1A). Subjects in both arms took the study medication for a convenient duration of 7 to 14 days until tumor resection (Supplementary Figure 1B). Eventually, 38 evaluable patients from each group were analyzed. The mean treatment duration for the metformin and placebo groups were 10.05 days and 10.34 days respectively. The demographic and clinicopathological characteristics were summarized (Supplementary Table S1). Neither treatment with metformin nor placebo changed fasting blood glucose level (Supplementary Figure 2A). Further, metformin or placebo treatment did not change cell proliferation in ESCC specimens as determined by Ki67 staining of cancer in surgical specimens (Supplementary Figure 2B). Similarly, no impact on the amount of cleaved Caspase-3 (a marker of apoptosis) was detected after treatment with metformin or placebo (Supplementary Figure 2C). Thus, low-dose metformin neither had an impact on blood glucose homeostasis nor a direct effect on proliferation and apoptosis of ESCC cells.

Low-dose metformin increased CD8 T cell and CD20 B cell infiltration in human ESCC

Next, we determined whether low-dose metformin had any impact on the TIME by determining the composition of immune cell infiltrates before and after treatment. To profile the impact of metformin on adaptive immunity, CD8, CD4, FoxP3 and CD20 were immunostained in pre- and

post-treatment tumor samples, with no significant difference in any of these markers between placebo and metformin-treated group in the pre-treatment samples (Figure 1A, B). However, the infiltration of $CD8^+$ T cells was significantly increased in the metformin group, but not placebo group, in post-treatment samples (P<0.001, Figure 1A). Correspondingly, in post-treatment conditions, a significantly higher percentage of $CD8^+$ T cells was detected in the metformin group compared to the placebo group (P<0.001, Figure 1A). For the infiltration of $CD4^+$ and $FoxP3^+$ T cells, no significant change was detected in pre- versus post-treatment specimens in either study arm, although a significant decrease in $CD4^+$ T cells was detected post-treatment in the metformin group compared to placebo group (Figure 1A). Finally, metformin treatment, but not placebo, significantly increased $CD20^+$ B cell infiltration (P=0.002, Figure 1B), although no significant change was detected post-treatment between metformin and placebo group (Figure 1B). These IHC results were confirmed using multi-color fluorescence microscopy (Figure 1C). $CD8^+$ T cells and $CD20^+$ B cells were increased in metformin-treated patients (P=0.035, P<0.001, respectively; Figure 1C), but not $CD4^+$ T cells. Although PD-L1 is a crucial checkpoint protein associated with T cell exhaustion and S-TIME (33,34), no change in PD-L1 expression was detected either with IHC or IF in either study arm (Figure 1D, Supplementary Figure 3). Collectively, these data showed that short-term low-dose treatment with metformin altered the composition of adaptive immune cells infiltrated in ESCC. Importantly, in multivariate regression analyses metformin treatment remained an independent factor for change in both CD8 and CD20 staining (P<0.001 and P=0.001, respectively, Supplementary Figure 4).

Low-dose metformin altered macrophage composition in human ESCC

In addition to adaptive immune cells, innate cells such as macrophages are critical players in the TIME. Tumor-promoting macrophages are associated with S-TIME and tumor-suppressive macrophages with A-TIME. In line with the results on adaptive immunity, none of the myeloid markers tested (CD68, CD11c, CD163) were significantly differently expressed between the placebo and metformin group in the pre-treatment samples (Figure 1E). Further, the total infiltration of macrophages (defined by $CD68^+$ cells) was not changed in either metformin or placebo group in pre- versus post-treatment tumor samples (Figure 1E). However, in the metformin group, but not placebo group, the post-treatment samples had a significant increase in $CD11c^+$ tumor-suppressive macrophages ($P<0.001$, Figure 1E). Correspondingly, a significantly higher percentage of $CD11c^+$ tumor-suppressive macrophages was detected in post-treatment samples of metformin-treated patients compared to placebo-treated patients ($P<0.001$, Figure 1E). Reversely, metformin treatment decreased the percentage of $CD163^+$ tumor-promoting macrophages in post-treatment samples ($P<0.001$, Figure 1E), whereas again no significant difference was detected in pre- versus post-treatment samples upon placebo treatment (Figure 1E). Correspondingly, a significantly lower percentage of $CD163^+$ tumor-promoting macrophages was detected in post-treatment samples of metformin-treated patients compared to placebo-treated patients ($P<0.001$, Figure 1E). Metformin remained an independent factor for change in CD11c and CD163 in multivariate regression analysis ($P=0.001$, $P<0.001$, respectively, Supplementary Figure 4).

Low-dose metformin shifts the TIME towards an anticancer state

In order to define the cumulative impact of the changes in individual markers, the Tumor Immune Micro Environment (TIME) status was calculated for pre- and post-treatment samples and defined as suppressive (S-TIME), equilibrated (E-TIME) or activated (A-TIME). Treatment with metformin clearly altered the TIME composition, with 17 patients (45%) undergoing a favorable change in TIME after treatment compared to only 3 patients (8%) in placebo control (Figure 2A; Supplementary Table S2). Indeed, metformin-treated tumors were statistically significantly more likely to undergo a positive TIME change than placebo with an odds ratio (OR) of 9.44 (95%CI: 2.47–36.12, P=0.001) of positive TIME change *vs.* no or negative TIME change in metformin *vs.* placebo.

When assessing matched pre- and post-treatment TIME status, only minimal positive changes and many negative changes in TIME status were detected in placebo-treated patients (Figure 2B). In contrast, in metformin-treated patients a high number of positive TIME changes were detected (Figure 2B). Specifically, in placebo-treated patients a positive change from S-TIME or E-TIME was detected in only a few patients (Figure 2C, 2 samples, 20% and 1 sample, 6%, respectively), whereas in metformin treated patients a large proportion of patients with pre-treatment S-TIME or E-TIME underwent a positive TIME changes (Figure 2C, 10 samples, 77% and 7 samples, 47%, respectively). Of note, in pre-treatment samples with an E-TIME composition a negative change from E-TIME to S-TIME was detected with a similar frequency for placebo and metformin treatment (Figure 2B, 5 samples each; 29% *vs.* 33%). However, a negative change from A-TIME to S-TIME was only detected in the placebo group (Figure 2B, 3 samples; 27%). Taken together, low-dose metformin had a strong positive effect on TIME status,

with a predominant shift towards a more antitumoral composition.

Specific correlations among immune cells were investigated in the two study arms. Particularly in the metformin arm, increased CD8 expression was accompanied by decreased CD163 in majority of the post-treatment samples of metformin treated patients (22 out of 38 patients, 57.89%) compared to pre-treatment with a strong negative correlation (Figure 2D, P=0.001). Further, increased CD8 expression was accompanied by increased CD20 in majority of the post-treatment samples of metformin treated patients (20 out of 38 patients, 52.63%) compared to pre-treatment with a positive correlation (Figure 2D, P=0.038).

Treatment of metformin in carcinogen-induced ESCC mouse model mirrors metformin-induced TIME changes in ESCC patients

Next, we confirmed these clinical findings in an ESCC mouse model in which the carcinogen 4-NQO was provided in the drinking water for 16 weeks (Figure 3A). Treatment with a low dose of metformin (50 mg/kg/day) for 1 week (week 16 to week 17) did not significantly impact on ESCC proliferation or the number of tumors per mouse compared to SHAM-treated mice (Supplementary Figure 5). However, tumors from mice in the metformin-treated group had significantly more A-TIME status than the SHAM-treated group (60% *vs.* 10%, P=0.041; Figure 3B). Specifically, $CD8^+$ T cells and $CD19^+$ B cells were increased in the metformin group compared to the control group (P=0.005 and P=0.011, respectively, Figure 3C-E), with no significant change in $CD4^+$ T cells, $FoxP3^+$ Treg cells, or expression of PD-L1. Further, metformin treatment did not significantly increase the total number of $F4/80^+$ macrophage populations (Figure 3F), but $CD11c^+$ tumor-suppressive macrophages increased (P=0.005,

Figure 3F) along with a concomitant decreased level of $CD206^+$ tumor-promoted macrophages (P=0.007, Figure 3F). Collectively, these data indicate that low-dose and short-term metformin treatment in this ESCC mouse model closely recapitulated the effect of metformin in human ESCC patients.

When low-dose treatment was extended to 12 weeks, ESCC cell proliferation as well as the number of tumors per metformin-treated mouse was reduced compared to SHAM-treated mice (P=0.005, Figure 4A; P=0.030, Figure 4B). Moreover, the number of mice with A-TIME status was increased in metformin-treated mice compared to SHAM-treated mice (70% *vs.* 10%, P=0.013; Figure 4C).

Similar to the results from the short-term metformin experiment, long-term treatment also significantly increased the percentage of $CD8^+$ T cells and $CD19^+$ B cells (P=0.002, Figure 4D; P=0.002, Figure 4E). In addition, this long-term metformin treatment did reduce the percentage of $CD4^+$ T cells and particularly of $FoxP3^+$ T cells (P=0.033 and P=0.035, respectively, Figure 4D). Moreover, expression of PD-L1 was significantly decreased after long-term metformin treatment compared to SHAM-treatment (P<0.001, Figure 4F). Regarding macrophage content, the long-term metformin treatment also increased $CD11c^+$ tumor-suppressive macrophages and severely suppressed $CD206^+$ tumor-promoting macrophages, yielding a net decrease in total number of $F4/80^+$ macrophages (P=0.005, P<0.001 and P=0.034, respectively; Figure 4G). Thus, long-term metformin treatment changed TIME towards E- and A-TIME and suppressed tumor growth in the mouse model.

Metformin augments macrophage phagocytic activity

One of the more prominent TIME changes in all analyses was the increase in tumor-suppressive macrophages and simultaneous decrease in tumor-promoted macrophages, suggesting metformin may augment anti-tumor innate immunity. To substantiate this finding in *in vitro* phagocytosis assays, ESCC cell line KYSE140 was pretreated with different concentrations of metformin (1 mM, 3 mM and 5 mM) for 48h, with no induction of apoptosis at 1 mM and a slight trend towards apoptosis at 3 mM in KYSE140 (Supplementary Figure 6A). Interestingly, in mixed culture phagocytosis assay, a significant increase in phagocytic uptake of tumor cells was observed when macrophages pre-treated with metformin for 48h with or without KYSE140 cells pre-treated with metformin for 48h (Supplementary Figure 6B, C). Similar results were observed in another ESCC cell line (Supplementary Figure 7). These findings indicate that low-dose short-term metformin exposure specifically activated macrophage phagocytic activity.

Low-dose metformin triggered metabolic signaling and altered the immune cell cytokine expression profile

To identify metformin-mediated activity and modulation of the TIME, we evaluated phosphorylation of AMPK as well-established sensor of metformin-mediated activity (32,35,36) and phosphorylation of STAT3, which has recently been identified as regulated by metformin (19,37,38). In ESCC patients, metformin treatment significantly increased the percentage of $CD8^+$ T cells positive for phospho-AMPK (p-AMPK), with an increase from ~16% to 49% in pre-treatment versus metformin treatment (P<0.001; Supplementary Figure 8A). Similarly, the percentage of $CD11c^+$ tumor-suppressive macrophages positive for p-AMPK increased from ~14% to ~55% (P<0.001; Supplementary Figure 8B). In contrast, no change in p-AMPK was detected in placebo-treated groups for either CD8 or CD11c-positive cells (Supplementary Figure 8). Conversely and in line with recent publications, treatment with metformin significantly

decreased the percentage of $CD8^+$ T cells and $CD11c^+$ macrophages positive for phospho-STAT3 (p-STAT3), from 63% to 10% and 61% to 9%, respectively (P<0.001; Supplementary Figure 8). Again, in placebo-treated patients no change was detected in p-STAT3 in pre- or post-treatment samples (Supplementary Figure 8).

In close agreement with these findings, the low-dose and short-term metformin treatment in the ESCC mouse model significantly increased the percentage of p-AMPK positive $CD8^+$ T cells and $CD11c^+$ macrophages compared to SHAM-treated mice (P<0.001; Supplementary Figure 9), while decreasing the percentage of p-STAT3 positive cells (P<0.001; Supplementary Figure 9). These effects on phosphorylation of AMPK and dephosphorylation of STAT3 became even more pronounced in the low-dose and long-term metformin treated ESCC mice (P<0.001; Supplementary Figure 10). Thus, low-dose metformin metabolically activated AMPK and inactivated STAT3 in both cytotoxic T cells and tumor-suppressive macrophages.

As modulation of AMPK and STAT3 is known to alter the cytokine secretion profile of immune cells (26,31,39), we determined effector cytokine expression in $CD8^+$ T cells and $CD11c^+$ macrophages. In placebo treated patients no significant changes were detected in either CD8 or CD11c positive cells for any of the cytokines analyzed (Figure 5). However, metformin treatment significantly increased the proportion of $CD8^+$ T cells expressing TNF-α from ~12% to ~46% (P<0.001; Figure 5A), whereas no effect was detected on IFN-γ (Figure 5A). A similar increase in terms of TNF-α positive $CD11c^+$ macrophages was detected, from ~14% to ~47% (P<0.001; Figure 5B). Further, metformin treatment significantly decreased the percentage of IL-10 positive $CD11c^+$ tumor-suppressive macrophages form ~46% to ~7% (P<0.001; Figure 5B). Again, these results were faithfully replicated in mice treated with low-dose and short-term metformin, with a significant increase in $CD8^+$ and $CD11c^+$ cells expressing TNF-α (P<0.001; Supplementary Figure 11), a significant decrease in IL-10 positive $CD11c^+$ tumor-suppressive macrophages (P<0.001; Supplementary Figure 11B), and no change in IFN-γ expression

(Supplementary Figure 11A). In mice treated with low-dose metformin for long-term, the changes in cytokine were analogous yet more pronounced (P<0.001; Supplementary Figure 12). Furthermore, these more pronounced changes in TNF-α and IL-10 were accompanied by a significant increase in $CD8^+$ T cells expressing IFN-γ, from ~17% to 62% (P<0.001; Supplementary Figure 12A).

Taken together, these data indicate that metformin treatment increased AMPK signaling, leading to an increase in pro-inflammatory cytokine expression in key immune effector cell types in the tumor. These results were closely mirrored in the mouse model clearly indicating that short-term metformin treatment triggered a pro-inflammatory and anti-tumoral reshaping of the TIME. Long-term treatment with metformin further augmented this antitumoral TIME shift.

DISCUSSION

In the current study, short-term administration of low-dose metformin reprogrammed the TIME in ESCC patients from unfavorable S-TIME (immune suppressive) to E-TIME (equilibrated) or A-TIME (activated). The changes in ESCC patients were almost identical to those observed in the 4-NQO induced ESCC mouse model. Long-term metformin treatment in this mouse model further resulted in a more robust TIME alteration and inhibition of tumor progression. To the best of our knowledge, this is the first reported phase II clinical trial investigating the usage of low-dose metformin in human ESCC and the first report detailing the impact of low-dose metformin on the TIME in ESCC.

The pro- or anti-tumoral state of the immune infiltrate in cancer is held to be of crucial importance for the efficacy of cancer (immune) therapy (1-3). For instance, S-TIME not only provides a driving force for tumor progression, but also facilitates drug resistance (40), whereas A-TIME associates with better prognosis. Our results indicate that metformin is able to trigger such TIME shifts at a low-dose and in a short treatment schedule, which would make this metformin protocol fully compatible with integration into standard-of-care treatment in ESCC patients.

Although initial focus was on direct anti-cancer activity of metformin, metformin-mediated immunomodulatory effects have started to be recognized as well. In this respect, high-dose metformin treatment (1000 mg twice daily) for a mean duration of 13.6 days was recently reported to increase $CD8^+$ T cells and $FoxP3^+$ T cells in head and neck squamous cell carcinoma (HNSCC) patients (13). Such a high dose of metformin is in line with the majority of clinical studies in cancer patients, with dosing typically ranging from 500–2000 mg per day (14,15,41).

Further, metformin improves antitumor T cell immunity in ovarian cancer patients (21). Correspondingly, immune-mediated anticancer effects were uncovered using immunocompetent mouse models (TRAMP mice with prostatic cancer and BALB/c mice bearing RLmale1 leukemia cells) (23,26). Further, metformin improves antitumor T cell immunity in preclinical models of renal cell carcinoma, melanoma, fibrosarcoma, leukemia, or hepatocellular carcinoma (26,32,42), either by boosting $CD8^+$ T cell immunity or attenuating the ability of naive $CD4^+$ cells to differentiate into $Foxp3^+$ regulatory T cells. Notably, although isolated reports on immune-modulation in humans and various mouse models exist, the overall impact of metformin on TIME is unclear nor is there a clear correlation established between preclinical and clinical effects of metformin. Our immunocompetent mouse model clearly demonstrates a large spectrum of immune-mediated antitumor effects of metformin even at a low dosage of metformin. These effects on immunity precede an impact on ESCC cells, suggesting TIME remodeling is perhaps the primary mode-of-action of this low dose of metformin. More importantly, there is a remarkable uniformity in TIME compositional changes observed in ESCC patients and the mouse model we employed. Thus, this mouse model may well serve as a relevant tool to predict response for potential metformin-based cancer immunotherapy combinations.

One of the prominent effects of metformin was an increase in antitumoral tumor-suppressive macrophages and a decrease in protumoral macrophages. Interestingly, *in vitro* metformin-treated macrophages proved to have higher phagocytic activity toward ESCC cells, together indicating that metformin may (re)activate macrophage-mediated immunity. These data are in line with previous studies in which metformin activated macrophage-mediated immune responses in hepatocellular carcinoma and glioma (42,43). These findings suggest that

metformin treatment reprograms macrophages into an antitumor mode and set the stage for potential combination innate immune-targeting strategies such as combination with CD47-blocking or CD24-blocking antibodies that remove CD47/SIRP-alpha- or CD24/Siglec-10-mediated ‘don’t eat me’ signaling (44). The mechanism for this metformin-mediated shift in innate immune balance remains to be determined, but at least part appears to be due to direct impact on macrophage biology.

Although there is no clear molecular mechanism through which metformin modulates tumor-associated immune cells, it has been widely appreciated that AMPK activation correlates with metformin treatment (35). Metformin-mediated activation of AMPK may induce the downregulation of NF-kB and STAT signaling (26,45), although AMPK-independent regulation of such inflammatory signaling by metformin has been reported in some settings (19,37,38). Our results derived from both humans and mice clarified that low-dose metformin treatment activated AMPK and inactivated STAT3 in tumor suppressive macrophages and cytotoxic T cells. Compared with short-term treatment, long-term treatment in the mouse model induced a more robust and sustained response of these signaling pathways. Given that both AMPK and STAT3 are central regulators of cellular metabolism and inflammation, our data favor the notion that metformin mediates the metabolic-inflammatory signaling pathways. Short-term metformin treatment also triggered an increase in TNF-α and a decrease in IL-10 expression in tumor-suppressive macrophages, and increased expression of TNF-α in cytotoxic T cells. Long-term treatment with metformin in addition increased IFN-γ expression in cytotoxic T cells. These cytokines are clearly associated with antitumoral activity of immune cells. These data provide insights with regard to metformin-dependent functional differentiation of immune cells.

Of note, we also identified a higher proportion of B cells in ESCC patients and the mouse model after metformin treatment. Tumor-infiltrating B cells have been reported in mouse cancer models (46) and human solid tumors (30), but the role of tumor-infiltrating B cells remains unclear. Future studies are being set-up to examine the specific composition and function of tumor-infiltrating B cells up-regulated by metformin, *e.g.* by assessing the impact of CD20-mediated B cell depletion in the ESCC mouse model.

Our studies demonstrate that low-dose metformin treatment clearly shifts the TIME in ESCC patients from S-TIME toward a more activated E-TIME and A-TIME. To gain insight into the clinical relevance of this finding, follow-up prospective studies that correlate TIME alterations with relapse rate, progression free survival and overall survival are warranted. Moreover, development and potential expansion of tumor-reactive T cell clones due to metformin treatment should be evaluated, *e.g.* by prospective monitoring of tumor-reactive T cell clones. In this respect, ESCC patient blood is known to be contain NY-ESO-1 and PRAME-reactive T cells.

Several limitations exist for of our study. First, the clinical trial was designed as a randomized controlled trial with relatively small number of participants to examine pharmacodynamic response biomarkers. Although our mouse model data showed that a longer treatment duration resulted in a more robust and lasting response, the pharmacodynamic effects of long-term low-dose metformin treatment in ESCC patients need to be recapitulated. Second, the TIME was highly heterogeneous among patients since TIME can be influenced by many factors. In addition to the main composition of TIME (T cells, B cells and macrophages) investigated in our study, other immune cells such as dendritic cells (DC), natural killer (NK)

cells, granulocytes, and mast cells were not included in our analyses. Whether metformin affected polarization of macrophage and which B cell subsets were altered by metformin remain significant questions for further investigation.

In conclusion, with this phase II clinical trial and correlative mouse model studies we demonstrate that low-dose metformin treatment shifts the TIME in ESCC from a pro-tumoral state towards a more anti-tumoral state (Figure 6). These results provide the basis for designing future clinical trial for evaluating the potential impact of metformin on long-term outcome as well as set the stage for clinical trials with cancer immunotherapeutics such as CD47 or CD24 blocking agents, in which metformin treatment provides a safe immunomodulatory strategy.

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Contributors: Clinical trial and research studies design: HZ and SJY; provision of study materials or patients and implementation of clinical trials: YL, YG, YC, SC, GW, HC; patient specimen staining and analysis: SW, YL and XX; mouse model constructions and mouse experiments: SW, YL and LW; data analysis and interpretation: HZ, SW, YL, XX, PL, SJY, and EB; project supervision: HZ; manuscript writing and reviewing: HZ, EB, SW, YL and SJY.

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FIGURE LEGENDS

Figure 1 Metformin treatment reshapes tumor immune microenvironment (TIME) in ESCC patients. **A-C,** Representative immunohistochemistry (IHC) images of CD8, CD4, and FoxP3 (A), or CD20 (B), or PD-L1 (C) expression in pre- and post-treatment ESCC tissues (left panel). Scale bars: 50 μm. Quantification of CD8-, CD4-, and FoxP3- (A), or CD20- (B), or PD-L1- (C) positive cells (right panel). **D,** Representative Multiplexed Immunofluorescence (mIF) images for CD20, CD4, and CD8 expression in pre- and post-treatment ESCC specimens from control group (top panel). Representative mIF images for CD20, CD4, and CD8 expression in pre- and post-treatment ESCC specimens from metformin group (middle panel). Scale bars: 50 μm. Quantitative determination of CD20-, CD4-, and CD8-positive cells (bottom panel). **E,** Representative IHC photomicrographs of CD68, CD11c, and CD163 staining in pre- and post-treatment ESCC tissues (left panel). Scale bars: 50 μm. Quantification of CD68-, CD11c-, and CD163-positive cells (right panel). Error bars indicate SEM. NS, non-significant, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, by the paired *t* test, $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ by one-way ANOVA followed by a Tukey-Kramer *post-hoc* test.

Figure 2 TIME status analysis and correlogram of the immune markers in ESCC patients. **A,** Change in TIME status in control and metformin-treated patients defined as the percentage of the total population of patients. $^{**}P=0.001$ by χ^2 test. **B,** Sunburst plots of pre- (inner ring) and post-treatment (outer ring) TIME status in control and metformin-treated patients. As indicated, cyan is suppressive TIME (S-TIME), purple is equilibrated TIME (E-TIME) and maroon is activated TIME (A-TIME). **C,** Positive change in TIME status in control and metformin-treated patients

defined as the percentage of the population of E-TIME or S-TIME patients. **D,** Correlogram showing the Pearson's correlation between immune markers in ESCC patients. The color scale indicates the correlation coefficient, representing the strength and direction of correlation (red=negative correlation, blue=positive correlation and white=not related). The diameters of the color dots represent the *P* values which are the numbers on the dots (upper right in each panel). Corresponding scatter plots with regression lines were shown as well (lower left in each panel). Left panel: control group, right panel: metformin group.

Figure 3 Short-term treatment of metformin alters TIME in 4-NQO-induced orthotopic ESCC mice. **A,** Schematic model of longitudinal 4-NQO and metformin treatment: C57BL/6 mice were treated with 4-NQO for 16 weeks and then divided into short-term metformin-treated model (upper panel, *n*=10 per group, intraperitoneally injected with metformin or PBS daily for 1 week), and long-term metformin-treated model (lower panel, *n*=10 per group, intraperitoneally injected with metformin or PBS daily for 12 weeks). Tumors were harvested at the end of each experiment. **B,** Change in TIME status in control and metformin-treated mice defined as the percentage of the total population of mice. **C,** Representative mIF images of triple staining for CD8, CD4, and FoxP3 in tumor sections derived from mice that treated with short-term metformin and PBS (left panel). Scale bars: 50 μm. Quantitative determination of CD8-, CD4-, and FoxP3-positive cells (right panel). **D-E,** Representative immunofluorescence (IF) photomicrographs for CD19 (D) and PD-L1 (E) staining in tumors derived from mice that treated with short-term metformin and PBS (left panel). Scale bars: 50 μm. The percentage of CD19-positive (D) and PD-L1-positive cells (E) in tumor sections from metformin-treated mice

is plotted against that observed in controls (right panel). **F,** Representative mIF images of triple staining for F4/80, CD11c, and CD206 in tumor sections derived from mice that treated with short-term metformin and PBS (left panel). Scale bars: 50 μm. Quantitative determination of F4/80-, CD11c-, and CD206-positive cells (right panel). Error bars indicate SEM. NS, non-significant, $^{*}P<0.05$, $^{**}P<0.01$, by Student's *t* test.

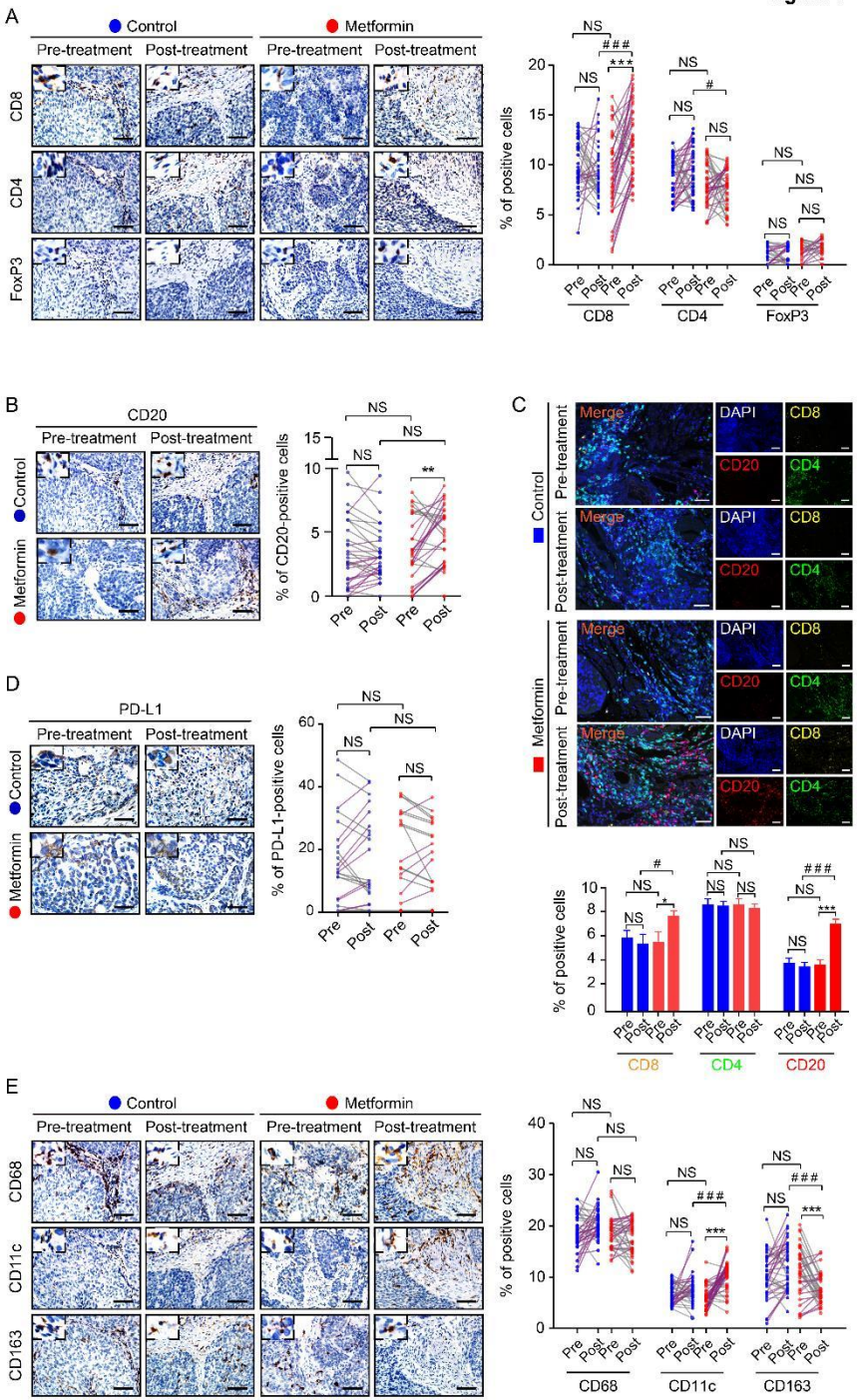
Figure 4 Long-term treatment of metformin inhibits tumor growth and alters tumor immune microenvironment in 4-NQO-induced orthotopic ESCC mice. **A,** Mice received long-term treatment of metformin developed less tumors per mouse in esophagus than did the control mice (left panel). Red arrows indicate esophageal tumors. Number of tumors in esophagus per mouse from metformin-treated mice was plotted against that observed in controls (right panel). **B,** IHC analyses indicated decreased Ki67 expression in the tumor tissues derived from mice that treated with long-term metformin compared with control tumors in PBS-treated mice (left panel). Scale bars: 50 μm. The percentage of Ki67-positive in tumor sections from metformin-treated mice is plotted against that observed in controls (right panel). **C,** Change in TIME status in control and metformin-treated mice defined as the percentage of the total population of mice. **D,** Representative mIF images of triple staining for CD8, CD4, and FoxP3 in tumors derived from mice that treated with long-term metformin and PBS (left panel). Scale bars: 50 μm. Quantification of CD8-, CD4-, and FoxP3-positive cells (right panel). **E-F,** Representative IF photomicrographs for CD19 (E) and PD-L1 (F) staining in tumor sections from mice that treated with long-term metformin and PBS (left panel). Scale bars: 50 μm. Quantification of CD19- (E) and PD-L1- (F) positive cells (right panel). **G,** Representative mIF images of triple staining for

F4/80, CD11c, and CD206 in tumors derived from mice that treated with long-term metformin and PBS (left panel). Scale bars: 50 μm. Quantification of F4/80-, CD11c-, and CD206-positive cells (right panel). Error bars indicate SEM. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, by Student's *t* test.

Figure 5 Short-term metformin increases TNF-α in $CD8^+$ T cells and $CD11c^+$ macrophages but decreases IL-10 in $CD11c^+$ macrophages in ESCC patients. **A,** Representative mIF images for TNF-α or IFN-γ expression in $CD8^+$ T cells in pre- and post-treatment ESCC specimens from control group (upper panel) and metformin group (middle panel). Quantitative determination of TNF-α- or IFN-γ-positive $CD8^+$ T cells (bottom panel). **B,** Representative mIF images for TNF-α or IL-10 expression in $CD11c^+$ macrophages in pre- and post-treatment ESCC specimens from control group (upper panel) and metformin group (middle panel). Quantitative determination of TNF-α- or IL-10-positive $CD11c^+$ macrophages (bottom panel). n=10 per group. Scale bars: 50 μm. Error bars indicate SEM. NS, non-significant, $^{***}P<0.001$, by the paired *t* test.

Figure 6 Metformin reprograms TIME in ESCC. Short-term metformin triggers anticancer immunity by reactivation of immune effectors, including $CD11c^+$ tumor-suppressive macrophages, $CD8^+$ T cells, $CD20^+$ B cells, and relief of immunosuppressive mechanisms, such as $CD163^+$ tumor-promoting macrophages, resulting in a shift from inhibited TIME to activated TIME. Long-term metformin further shifted the TIME towards an active state (*i.e.*, reduction in $CD163^+$ tumor-promoting macrophages, $CD4^+$ $FoxP3^+$ Tregs and PD-L1 expression; increase in $CD8^+$ T cells and $CD20^+$ B cells) and also inhibited ESCC tumor growth.

Figure 1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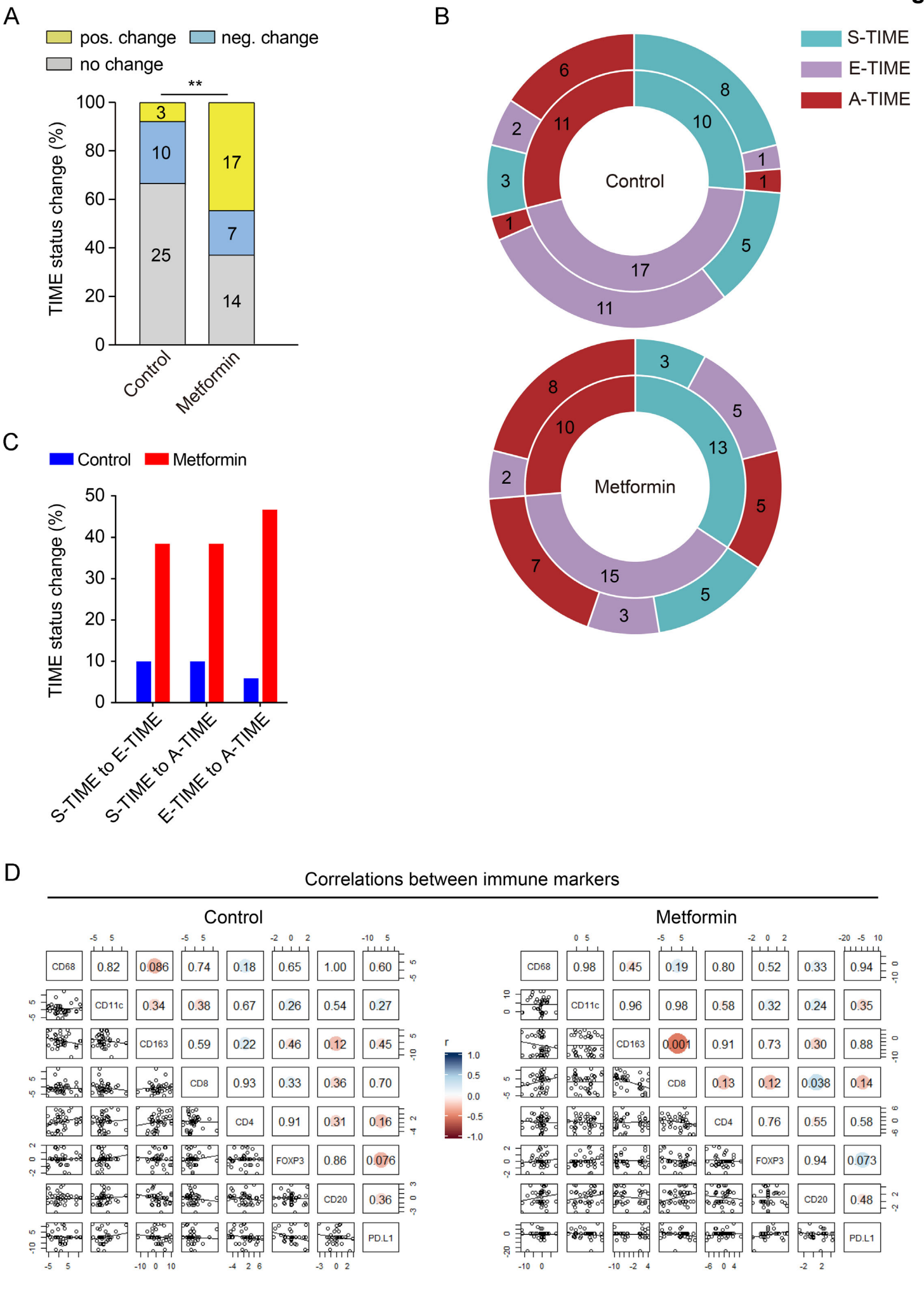
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6
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TIME status change (%)
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Metformin
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CD11c
CD163
CD8
CD4
FOXP3
CD20
PD.L1
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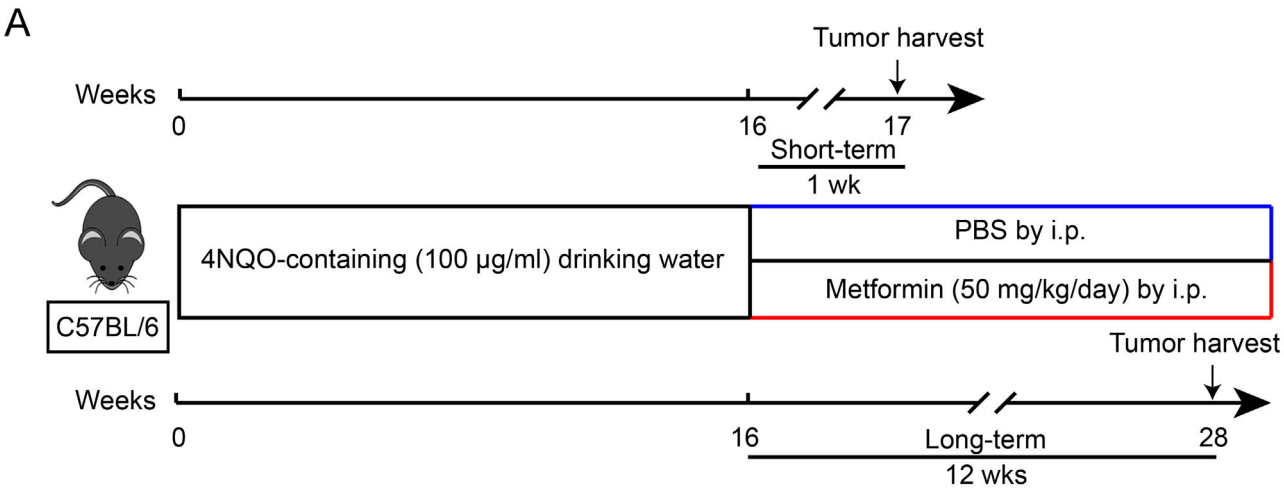
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Tumor harvest
Weeks
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Short-term
1 wk
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C57BL/6
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12 wks
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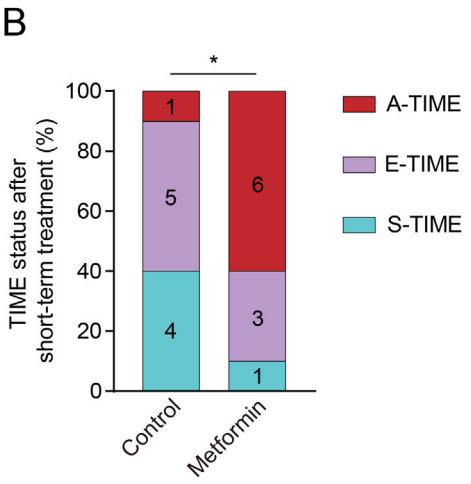
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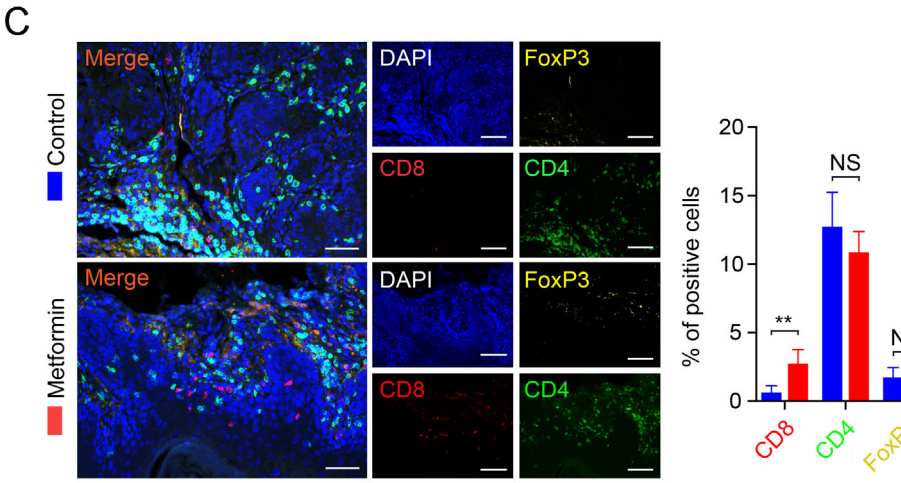
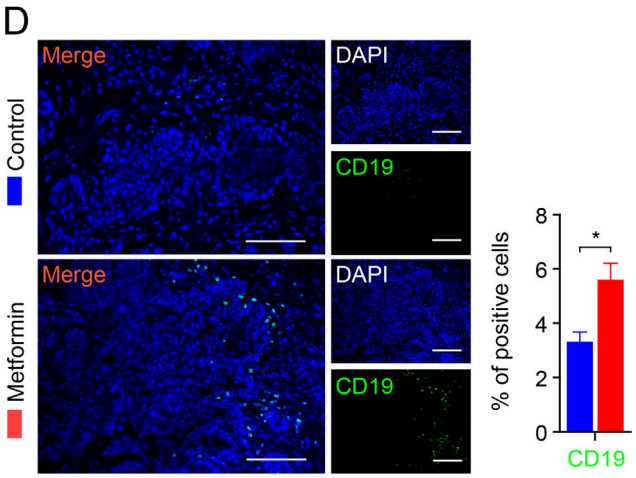
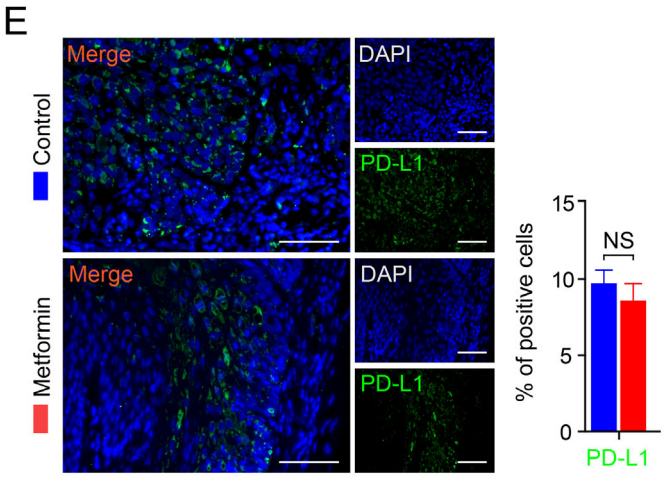
Control
Metformin


C
Merge
DAPI
FoxP3
CD8
CD4
Control
Metformin
% of positive cells
NS
CD8
CD4


D
Merge
DAPI
CD19
Control
Metformin
% of positive cells
CD19


E
Merge
DAPI
PD-L1
Control
Metformin
% of positive cells
NS
PD-L1

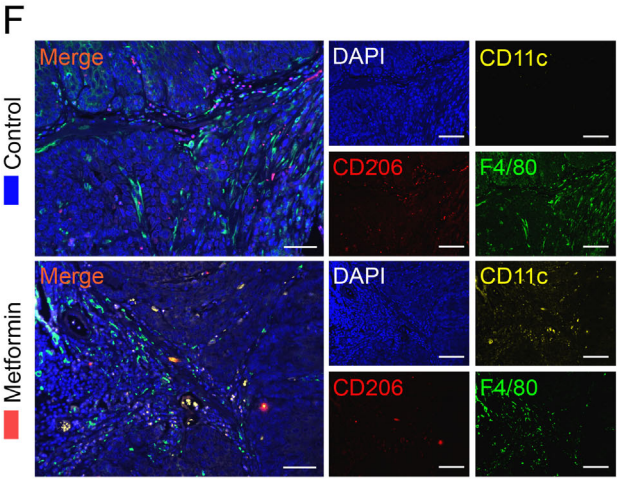

F
Merge
DAPI
CD11c
CD206
F4/80
Control
Metformin
% of positive cells
NS
F4/80
CD11c
CD206

Figure 4

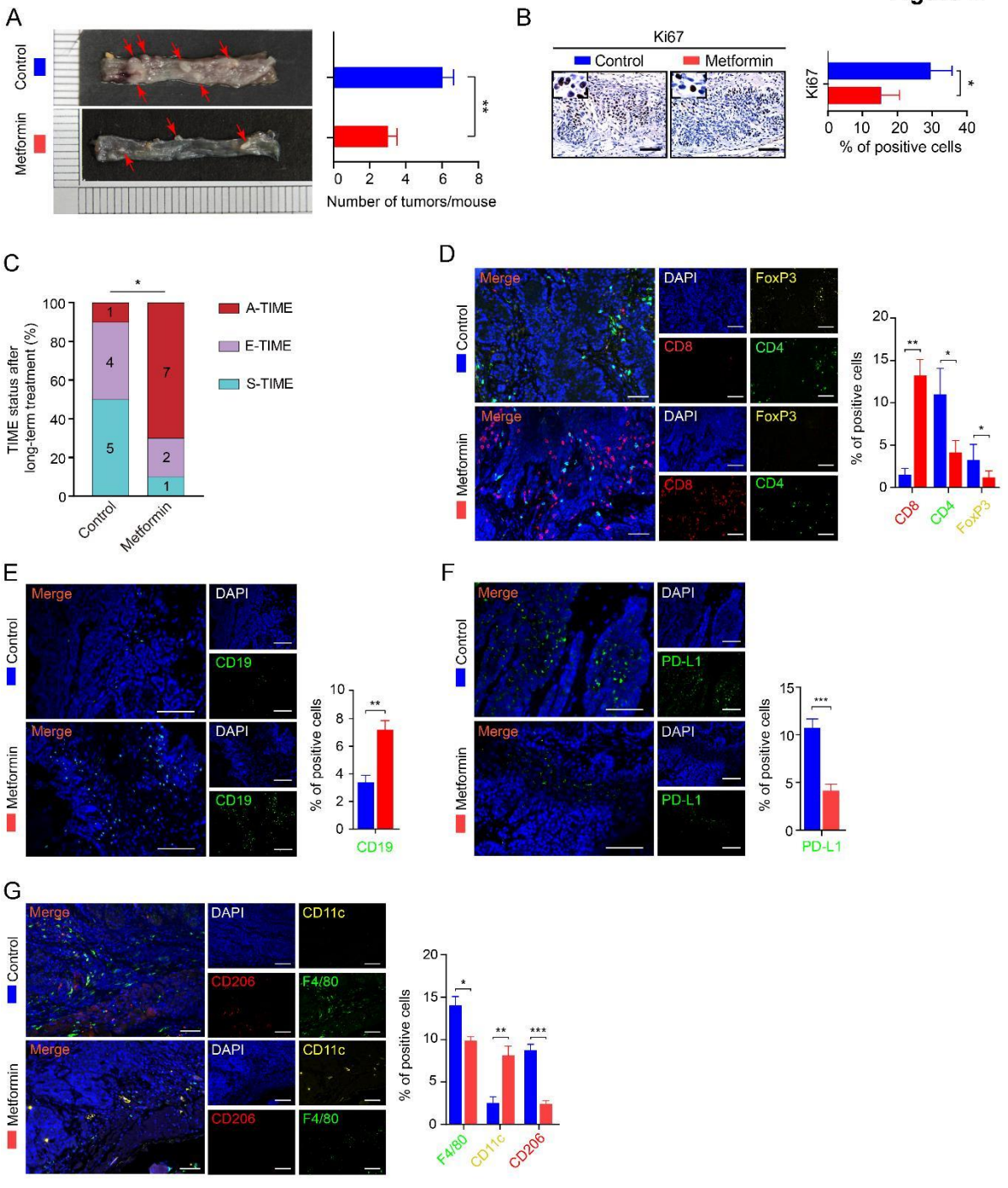

A
Control
Metformin
Number of tumors/mouse
B
Ki67
Control
Metformin
% of positive cells
C
TIME status after long-term treatment (%)
A-TIME
E-TIME
S-TIME
Control
Metformin
D
Merge
DAPI
FoxP3
CD8
CD4
% of positive cells
E
CD19
F
PD-L1
G
CD11c
CD206
F4/80

Figure 5

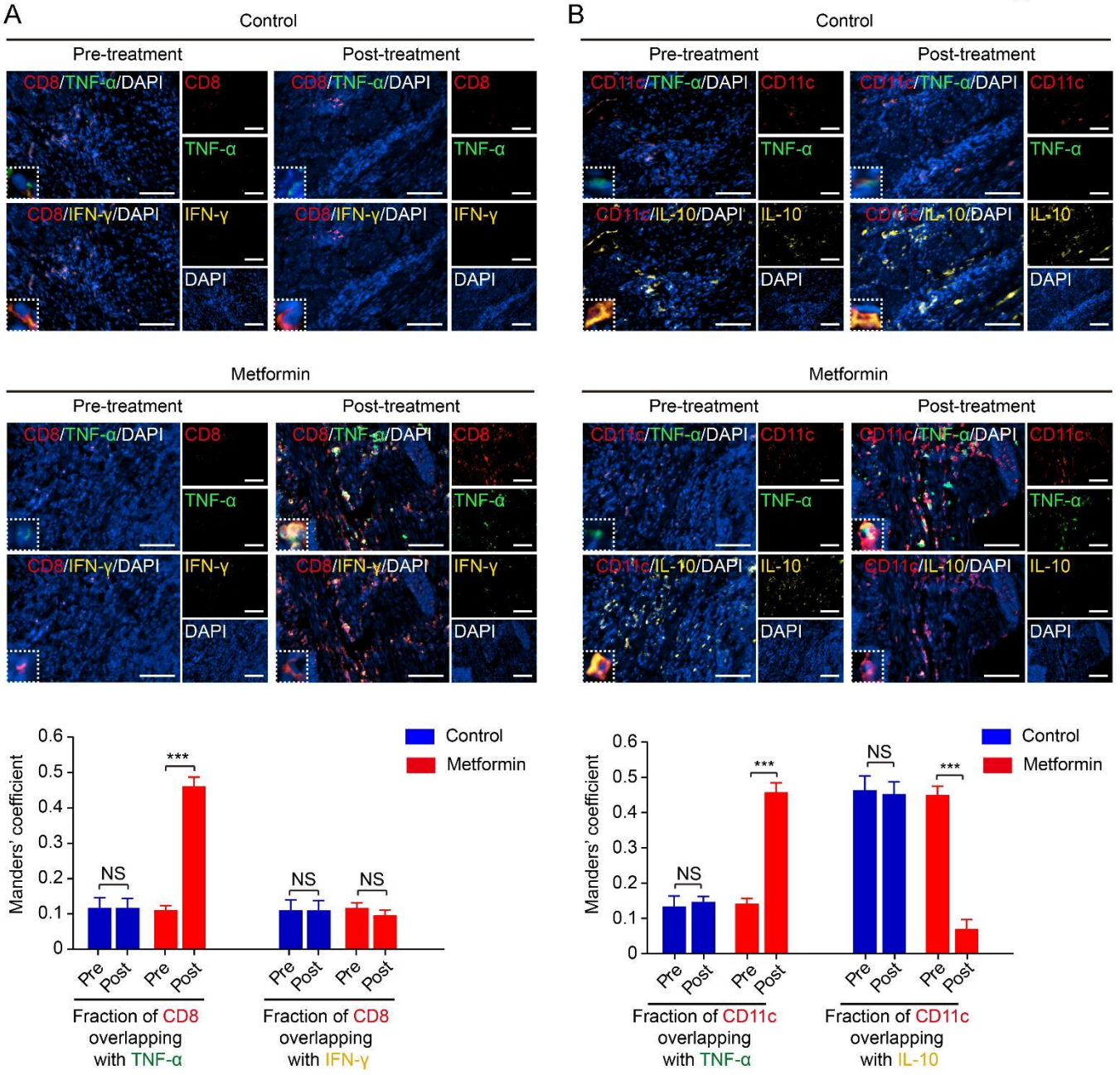
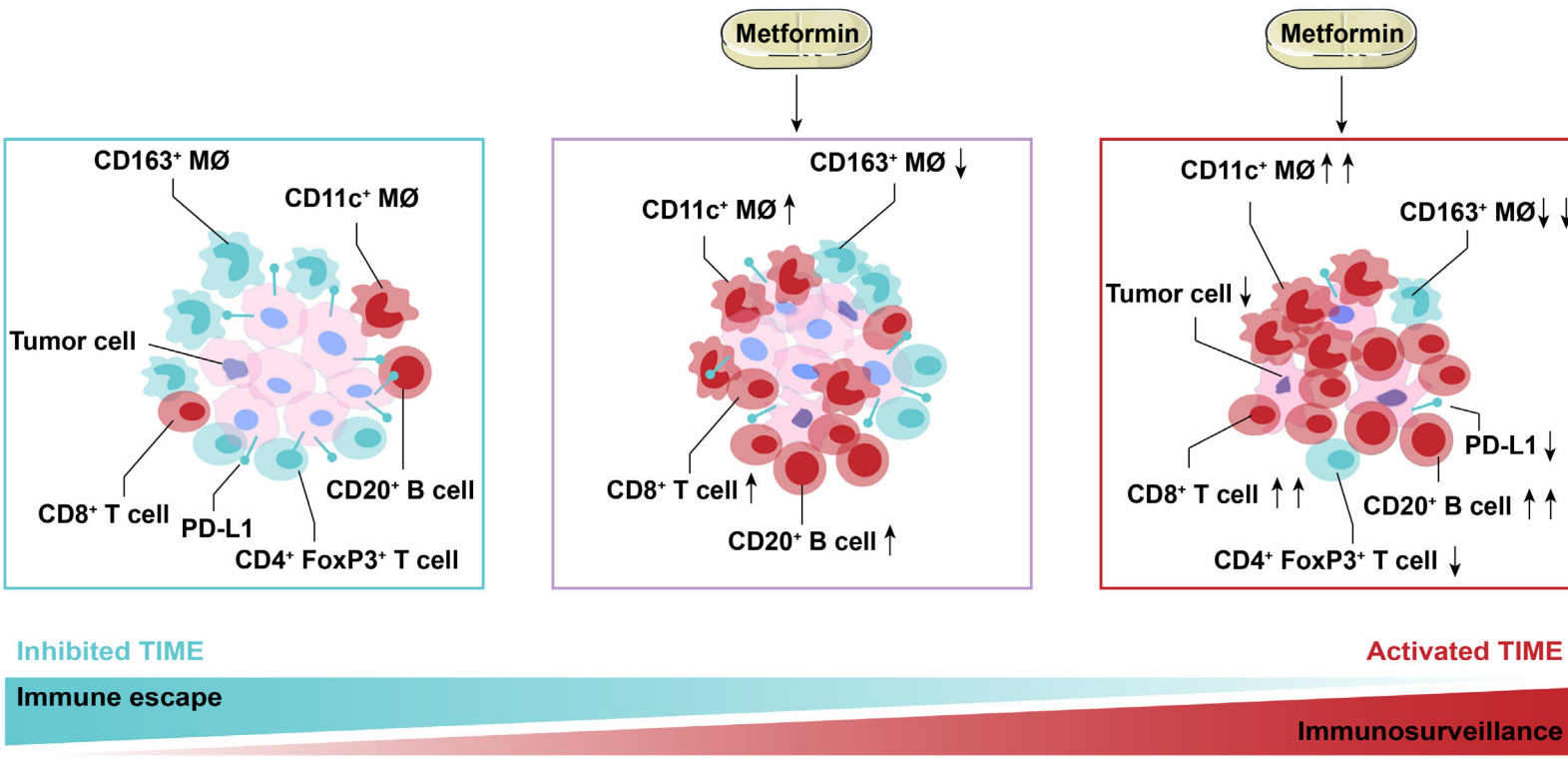

A
Control
Pre-treatment
Post-treatment
CD8/TNF-α/DAPI
CD8
TNF-α
CD8/IFN-γ/DAPI
IFN-γ
DAPI
Metformin
B
CD11c/TNF-α/DAPI
CD11c
CD11c/IL-10/DAPI
IL-10
Manders' coefficient
Control
Metformin
NS
***
Pre
Post
Fraction of CD8 overlapping with TNF-α
Fraction of CD8 overlapping with IFN-γ
Fraction of CD11c overlapping with TNF-α
Fraction of CD11c overlapping with IL-10

Figure 6


Before-treatment
Short-term treatment
Long-term treatment
Metformin
Metformin
CD163+ MØ
CD11c+ MØ
Tumor cell
CD8+ T cell
PD-L1
CD4+ FoxP3+ T cell
CD20+ B cell
CD163+ MØ
CD11c+ MØ
CD8+ T cell
CD20+ B cell
CD11c+ MØ
CD163+ MØ
Tumor cell
PD-L1
CD8+ T cell
CD20+ B cell
CD4+ FoxP3+ T cell
Inhibited TIME
Activated TIME
Immune escape
Immunosurveillance
Tumor mass