

# Metformin Inducing the Change of Functional and Exhausted Phenotypic Tumor-Infiltrated Lymphocytes and the Correlation with JNK Signal Pathway in Triple-Negative Breast Cancer

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**Background:** Triple-negative breast cancer (TNBC) is an aggressive subtype of breast cancer. Metformin has been shown to have the potential to inhibit the proliferation of malignant cells. This study aimed to investigate the regulatory effect of metformin on phenotypic tumor-infiltrated lymphocytes (TILs) and mechanisms in TNBC.

**Methods:** Microarray analysis was performed on 4T1 cells post metformin treatment. BALB/c mice were inoculated with 4T1 cells with knockdown/overexpression of C-Jun N-terminal kinase (JNK), and administered with metformin. Phenotypic TILs in the tumor microenvironment (TME) were visualized by immunofluorescence staining.

**Results:** Metformin inhibited 4T1 cell proliferation and increased expression of JNK by 21% in vitro. In vivo, Metformin increased cell counts of CD4<sup>+</sup> and CD8<sup>+</sup>TILs by 100% and 85%, respectively, and the increase of TILs was associated with JNK pathway. Cell counts of CD4<sup>+</sup>/PD-1<sup>+</sup> and CD8<sup>+</sup>/PD-1<sup>+</sup>TILs were reduced by 64% and 58%, respectively, post metformin treatment, but the reduction of exhausted TILs was not associated with JNK pathway. Metformin induced a 11% and 20% reduction of IL-6 and TNF- $\alpha$  level in the TNBC model.

**Conclusion:** Our study demonstrated that metformin increased the functional phenotype of TILs and associated with JNK pathway, and suppressed the exhausted phenotype of TILs independently to JNK pathway in TNBC microenvironment. Further studies are needed to explore the basic mechanism of action of the drug. Metformin has potentially enhanced efficacy when used in combination with immunotherapy against TNBC.

**Keywords:** triple-negative breast cancer, metformin, phenotypic tumor-infiltrated lymphocytes, C-Jun N-terminal kinase

## Introduction

Breast cancer (BC) has become the most common female malignancy worldwide, accounting for 30% of new malignant cases and 15% of deaths in women.<sup>1</sup> Chinese BC patients have particular clinicopathological characteristics, ie low expression of hormone receptor and high expression of human epidermal growth factor receptor 2 (HER2).<sup>2</sup> Triple-negative breast cancer (TNBC), featured of negative expression of estrogen receptors (ER), progesterone receptors (PR) and HER2, is a more aggressive BC subtype and has the worst prognosis.<sup>3</sup> TNBC had higher expression of programmed death protein-1 (PD-1) and programmed death protein ligand-1 (PD-L1) than other subtypes, indicating the suppressed TME of TNBC.<sup>4</sup>

The TME of BC was reported to affect patients' prognosis.<sup>5</sup> BC recurrence and metastasis were related with phenotypes of tumor-infiltrated lymphocytes (TILs), especially CD4<sup>+</sup> and CD8<sup>+</sup>T lymphocytes. Functional TILs improved the prognosis of TNBC; however, exhausted TILs had a negative association with survival.<sup>6</sup> High levels of TILs increased disease-free survival and overall survival in the TNBC.<sup>7,8</sup> The complexity of TME is regulated by multiple signaling pathways, cytokines, cellular oxidative metabolism and other factors.<sup>9</sup> The Ras/MAPK signal pathway was reported to regulate the immunosuppression.<sup>10</sup> C-Jun N-terminal kinase (JNK) is an important component of MAPK signaling pathway and regulates the activation and proliferation of T cells.<sup>11</sup>

Metformin not only has good efficacy and safety in the prevention and treatment of diabetes,<sup>12</sup> but also may become a candidate drug for cancer prevention and treatment.<sup>13</sup> Metformin could regulate immune cells and immune-related molecules, affect TME, and then exert anti-tumor immune response.<sup>14,15</sup> This effect depends on a variety of pathways, including the classical AMP-activated protein kinase (AMPK) signaling pathway and regulation of the insulin-like growth factor family.<sup>16</sup> Metformin could regulate the immunosuppression of TME in TNBC, suppress BC cell proliferation and growth, and reduce overall BC risk and mortality.<sup>17,18</sup> Additionally, metformin increased the number and activity of TILs in TME<sup>14</sup> and significantly decreased the expression of PD-L1. Current studies had shown that metformin can promote AMPK phosphorylation, thereby increasing the number of CD8<sup>+</sup>TILs and improving the immune status of TME.<sup>16</sup> However, whether metformin could regulate functional and debilitating TILs through MAPK signaling pathway remains unclear.

This study aimed to investigate regulatory effect of metformin and JNK signal pathway on the functional and exhausted TILs in TME of TNBC models.

## Methods

### Ethics Approval

Animal experiments were approved by the Medical Ethics Committee of Beijing Shijitan Hospital, Capital Medical University and were carried out in accordance with the Animal Welfare Guidelines of the Medical Ethics Committee of Beijing Shijitan Hospital, Capital Medical University, in line with the guidelines for the care and use of laboratory animals by the Chinese Institute of Health, and in compliance with all regulatory guidelines.

### Cell Viability Assays

Cell viability was determined by MTT assay. 4T1 cells (Nanjing Kebai Biotechnology Co., LTD) were seeded into 96-well plates (5000 cells/well), cultured for 24h and discarded the supernatant. A gradient concentration (0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0mmol/L) of metformin (Shanghai Squibb Pharmaceutical Co., LTD) was added on the following day. Cells were incubated with 5% CO<sub>2</sub> at 37°C for 24h. 20μL (5g/L) MTT was added into each well and cultured for 4h. The supernatant was extracted and 100μL DMSO was added into each well for 15min, and then the absorbance was measured at 570nm. The experiment was repeated three times.

### MAPK Pathway Analysis

The effect of metformin on MAPK signaling pathway in 4T1 cells was determined by protein microarray (Raybiotech, Inc., Raybio C1). 4T1 cells were lysed post metformin treatment (10.0mmol/L). The membrane chip was incubated with diluted protein lysates followed by incubation with 1mL biotin labeled antibody. The membrane was then incubated with HRP-streptavidin. ImageQuant LAS4000 chemiluminescence imaging analysis system (General Electric Company) was used for scanning. The experiment was repeated twice. The scanned data were normalized by EXCEL formula, including subtracting background and averaging. The normalized data were analyzed using R language. The selection criteria of potential signal protein were set as upregulation more than 20.0% and down-regulation more than 16.7%.

**Table 1** The Sequences of shRNA for JNK and PCR Primers

Genes		Sequence (5'-3')
<b>shRNA JNK</b>		
Control		5'-TTCTCCGAACGTGTCACGT-3'
shRNA 1		5'-GATCCGCGCGCTACCGAGAA-3'
shRNA 2		5'-GATCCGCGAGCCTAAATACGCT-3'
shRNA 3		5'-GATCCGTGAT TCAGATGGAGTT-3'
<b>qRT-PCR</b>		
JNK	Forward	5'-AGCAGAAGCAAACGTGACAAC-3'
	Reverse	5'-GCTGCACACACTATTCCCTTGAG-3'
Actin	Forward	5'-CATGTACGTTGCTATCCAGGC-3'
	Reverse	5'-CTCCTTAATGTCACGCACGAT-3'

## Knockdown/Overexpression of JNK in 4T1 Cells

Lentiviral-mediated JNK overexpression vector and shRNA vectors were obtained from GeneChem (Shanghai, China). Target sequences were shown in (Table 1). Cells were seeded at 70–80% confluency in medium and transfected with lentivirus and polybrene A reagent according to the manufacturer's instructions. The empty vector served as a negative control. After 12h, the medium was removed and replaced with fresh culture medium. Three days later, green fluorescent protein gene expression was observed under a fluorescence microscope, and the experiment was repeated twice; then, the cells were collected for subsequent culture.

## Animal Model

4T1 cells with wildtype JNK (Wildtype 4T1), 4T1 cells with JNK knock-down (4T1 JNK KD), 4T1 cells with overexpressed JNK (4T1 OVE JNK) were inoculated subcutaneously in the abdomen of 6-week female BALB/c mice (Spaefer Experimental Animal Co. Ltd.). Mice were divided into vehicle control group and metformin group, and metformin was administered (500mg/Kg) via oral gavage once a day for 4 weeks. All mice were anesthetized and the tumor tissues were aseptically harvested at the end of the experiment. After weight measurement, part of the tumor specimens was excised for IL-6 and TNF- $\alpha$  detection. Remaining tumor tissues were fixed in formalin and 4 $\mu$ m-thick formalin fixed paraffin-embedded sections were processed for TILs detection.

## Phenotypic TILs Detection

Phenotypic TILs markers were stained and visualized by immunofluorescence (IF) staining. Antibodies used for the staining included CD4 (Ab183685, Abcam), CD8 (Ab22378, Abcam), and PD-1 (Ab214421, Abcam) monoclonal antibodies. Each entire tumor section was evaluated for TILs by using an objective lens (Olympus IX51,  $\times$ 200), and 5 independent areas were selected randomly for cell counting. Each photograph was counted three times by the same investigator without awareness of hypothesis and early results. The average TILs counts were obtained for further analysis.

## Detection of IL-6, and TNF- $\alpha$

IL-6 and TNF- $\alpha$  levels were examined by ELISA Kit (Elabscience Biotechnology Co., Ltd). After the tumor tissue was harvested, part of the tumor tissue was cut into pieces. The cut tissue and the corresponding volume of PBS were added to the glass homogenizer and ground fully on ice. The homogenate was centrifuged at 5000 $\times$ g for 10 minutes. 100 $\mu$ L supernatant was added into ELISA kits for 20–25min at room temperature. The absorbance of IL-6 and TNF- $\alpha$  was measured with a microplate reader (SpectraMax iD5) at a wavelength of 450 nm to calculate the sample concentration.

## Statistical Analysis

GraphPad Prism (GraphPad Software Inc., San Diego, California, USA) was used for data analyses. All data was expressed as the median and interquartile range (IQR). Kruskal–Wallis test was used to estimate the difference between

groups. Pairwise comparisons were conducted by Wilcoxon tests with Bonferroni adjustments. All tests were two-sided and  $p < 0.05$  was considered statistically significant.

## Results

### Metformin Inhibited the Viability of 4T1 Cells

Metformin had no suppressive effect on 4T1 cell viability at low concentration (0.1–5.0 mmol/L), while the inhibitive effect was observed at higher concentration (10.0 mmol/L and 20.0 mmol/L), with a dose-response relationship (Figure 1).

### Metformin Affected the MAPK Pathway in 4T1 Cells

Among MAPK signal pathway, JNK expression level was 2462.0 (IQR = 265.02) in 4T1 cells with control vehicle treatment and increased to 2991.7 (IQR = 264.86) with metformin treatment, by a 21.4% increase ( $p < 0.05$ , Figure 2A–C).

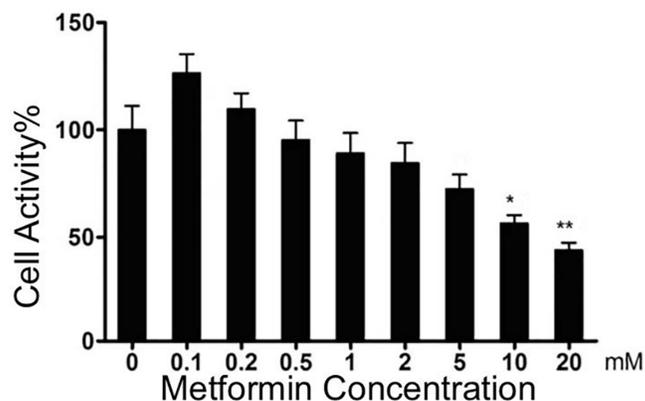
### No Change of the Weight of Xenograft Tumors in Mice

Metformin did not affect the weight of xenograft tumors in mice between groups ( $p > 0.05$ , Figure 3).

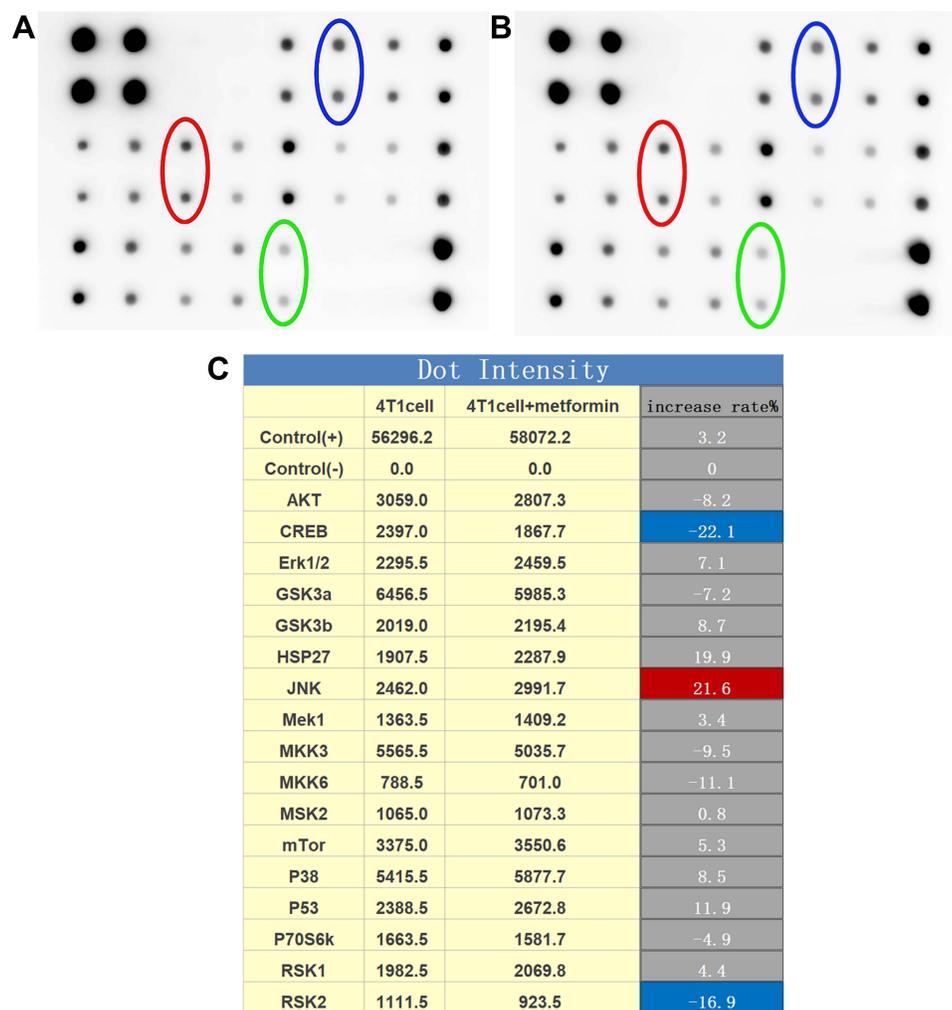
### Metformin and JNK Pathway Affected the Phenotypic of TILs

In mice inoculated with Wildtype 4T1, metformin increased the cell counts of CD4<sup>+</sup> and CD8<sup>+</sup>TILs in TME that cell counts of CD4<sup>+</sup>TILs were 41/HPF in control vehicle group and increased to 82/HPF in metformin group; CD8<sup>+</sup>TILs were 47/HPF in control vehicle group and increased to 87/HPF in metformin group ( $p < 0.05$ , Figures 4A, 4B, 5A, 5B, 6A and 6B). The mice inoculated with 4T1 JNK KD, had a reduced level of CD4<sup>+</sup>TILs (30/HPF) and CD8<sup>+</sup>TILs (30/HPF) than those inoculated with Wildtype 4T1 (Figures 4C, 5C, 6A and 6B); but metformin administration reversed CD4<sup>+</sup>TILs and CD8<sup>+</sup>TILs to 62/HPF and 63/HPF, respectively, in mice with inoculation of 4T1 JNK KD ( $p < 0.05$ , Figures 4E, 5E, 6A and 6B). The mice inoculated with 4T1 OVE JNK had the CD4<sup>+</sup> TILs and CD8<sup>+</sup> TILs increased to 66/HPF and 65/HPF, respectively, compared with mice inoculated with Wildtype 4T1 ( $p < 0.05$ , Figures 4D, 5D, 6A and 6B), and metformin administration increased the cell counts of CD4<sup>+</sup>TILs and CD8<sup>+</sup>TILs to a higher extent (90/HPF and 110/HPF) in mice with inoculation of 4T1 OVE JNK ( $p < 0.05$ , Figures 4F, 5F, 6A and 6B). But the CD8<sup>+</sup>/CD4<sup>+</sup>TILs ratio had no significant difference between groups ( $p > 0.05$ , Figure 6C).

The mice inoculated with Wildtype 4T1, had the cell counts of CD4<sup>+</sup>/PD-1<sup>+</sup>TILs and CD8<sup>+</sup>/PD-1<sup>+</sup>TILs being 28/HPF and 26/HPF in control vehicle group and decreased to 10/HPF and 11/HPF in metformin group, respectively ( $p < 0.05$ , Figures 7A, 7B, 8A, 8B, 9A and 9B). Compared with mice inoculated with Wildtype 4T1 cells, the mice inoculated with 4T1 JNK KD, had similar cell counts of CD4<sup>+</sup>/PD-1<sup>+</sup> (22/HPF) and CD8<sup>+</sup>/PD-1<sup>+</sup>TILs (21/HPF) in



**Figure 1** The effects of metformin on 4T1 cell viability compared with control group. \* $p < 0.05$ , \*\* $p < 0.01$ .



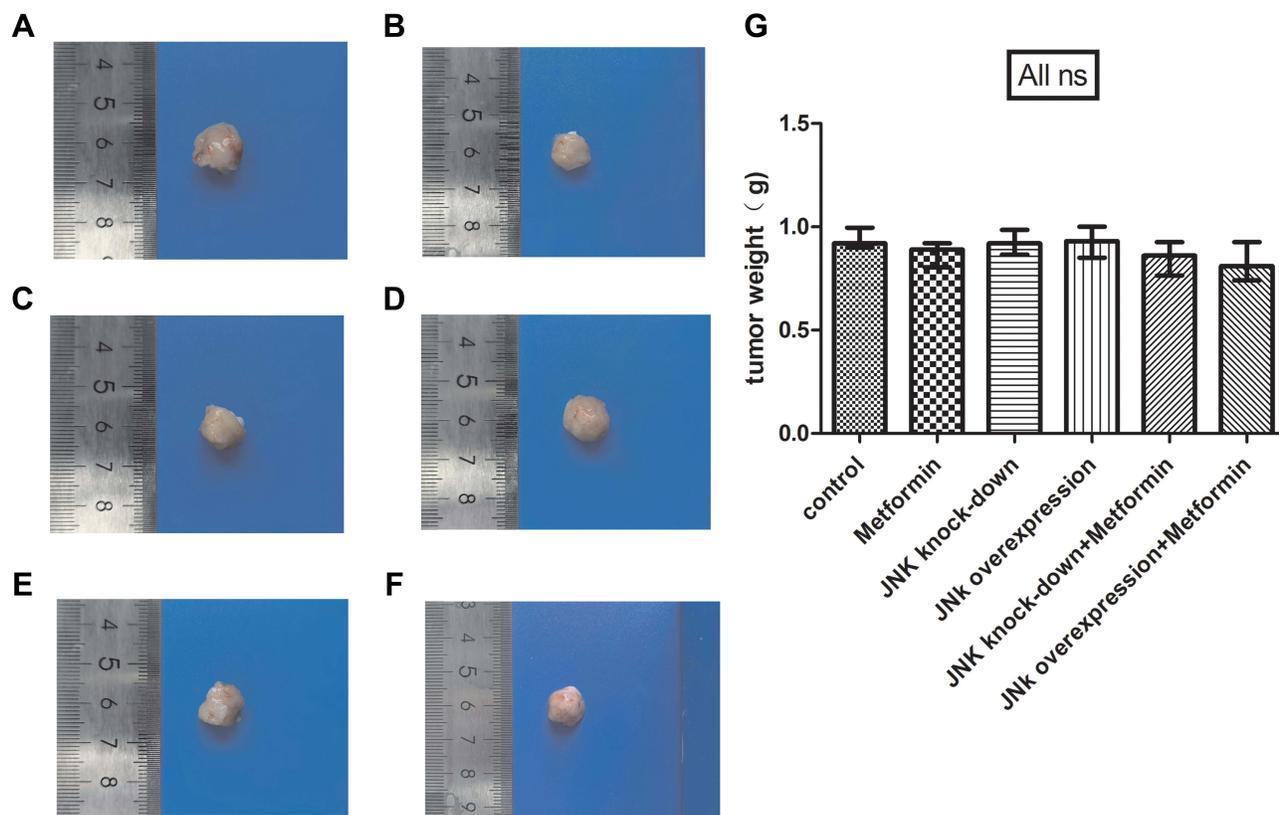
**Figure 2** The expression of MAPK pathway in 4T1 cells. (A) The original blot of protein expression in 4T1 cells (JNK: red circle; RSK2: green circle; CREB: blue circle). (B) The original blot of protein expression in 4T1 cells with metformin treatment (JNK: red circle; RSK2: green circle; CREB: blue circle). (C) Quantitative results of the original blot.

control vehicle group; but the mice with 4T1 JNK KD inoculation and metformin administration had cell counts of CD4<sup>+</sup>/PD-1<sup>+</sup> and CD8<sup>+</sup>/PD-1<sup>+</sup>TILs reduced to 12/HPF, with reference to those with 4T1 JNK KD inoculation ( $p<0.05$ , Figures 7C, 7E, 8C, 8E, 9A and 9B). The mice inoculated with 4T1 OVE JNK had similar cell counts of CD4<sup>+</sup>/PD-1<sup>+</sup>TILs (22/HPF) and CD8<sup>+</sup>/PD-1<sup>+</sup>TILs (20/HPF) to those with Wildtype 4T1 inoculation; however, the mice receiving 4T1 OVE JNK inoculation and metformin administration had the CD4<sup>+</sup>/PD-1<sup>+</sup>TILs and CD8<sup>+</sup>/PD-1<sup>+</sup>TILs reduced to 10/HPF and 12/HPF, respectively, lower than those with 4T1 OVE JNK inoculation ( $p<0.05$ , Figures 7D, 7F, 8D, 8F, 9A and 9B).

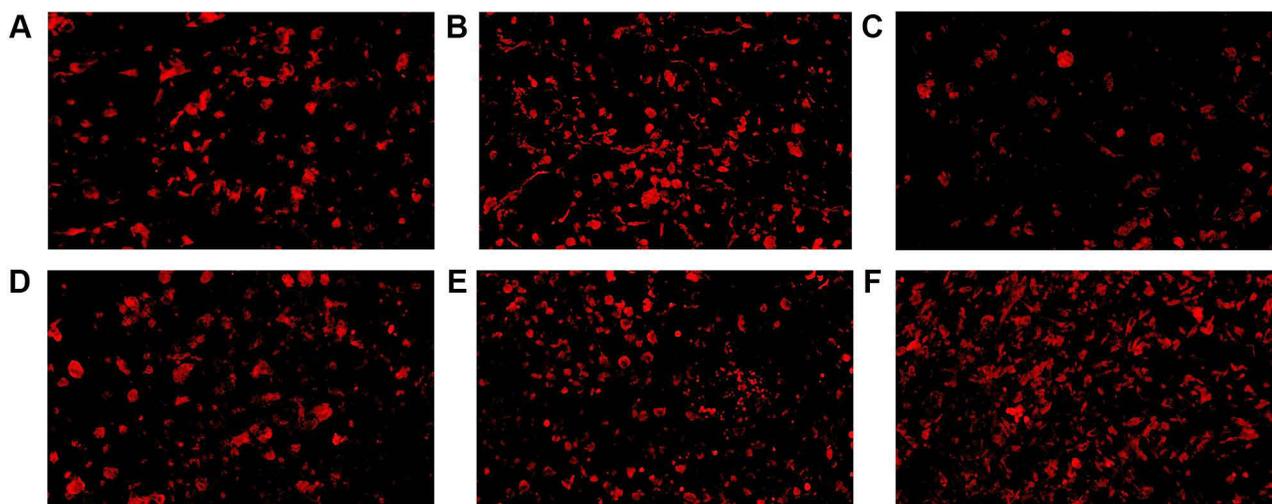
### Metformin Reduced the IL-6 and TNF- $\alpha$ Production

Mice inoculated with Wildtype 4T1 and administered metformin, had reduced IL-6 (452.2pg/mL) than those inoculated with Wildtype 4T1 (509.6pg/mL,  $p<0.05$ , Figure 10A). Compared with mice with Wildtype 4T1 inoculation, JNK KD and OVE JNK inoculation did not have significant effect on IL-6 production (Figure 10A). However, metformin administration reduced the IL-6 to 462.2pg/mL in mice inoculated with 4T1 JNK KD and 456.3pg/mL in mice inoculated with 4T1 OVE JNK, respectively (Figure 10A).

Similarly, metformin administration reduced TNF- $\alpha$  level to 432.3pg/mL from 539.6pg/mL in mice inoculated with Wildtype 4T1 ( $p<0.05$ , Figure 10B). Inoculation with 4T1 JNK KD and 4T1 OVE JNK had no effect on TNF- $\alpha$

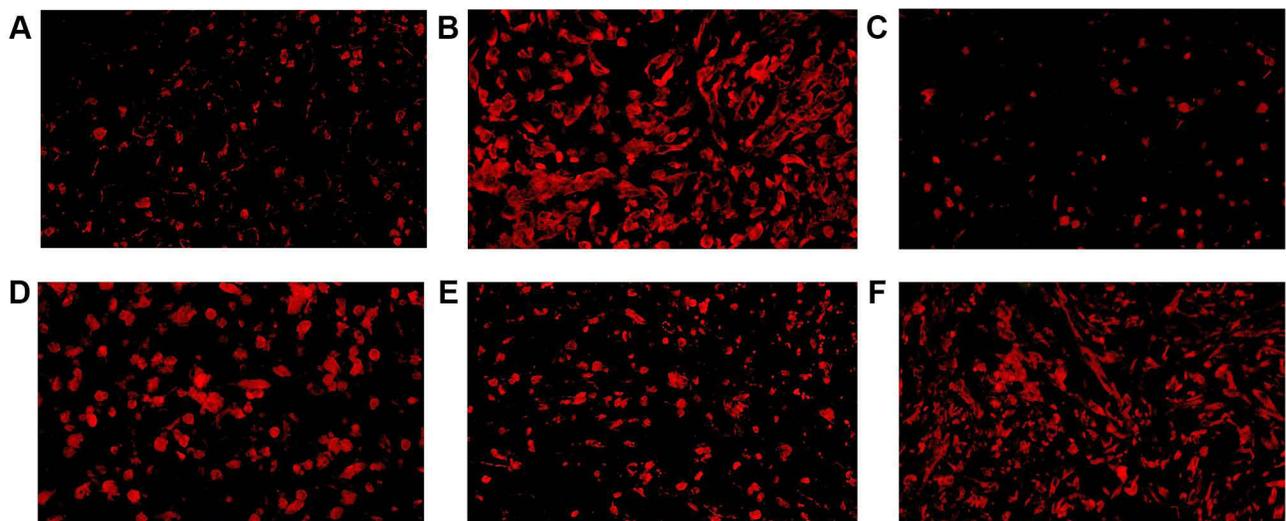


**Figure 3** The tumor weight of mice model. (A) Tumor specimens of 4T1 cells. (B) Tumor specimens of 4T1 cells and metformin administration. (C) Tumor specimens of 4T1 cells with JNK knock-down. (D) Tumor specimens of 4T1 cells with JNK overexpression. (E) Tumor specimens of 4T1 cells with JNK knock-down and metformin administration. (F) Tumor specimens of 4T1 cells with JNK overexpression and metformin administration. (G) statistical result of tumor weight. ns  $p > 0.05$ .

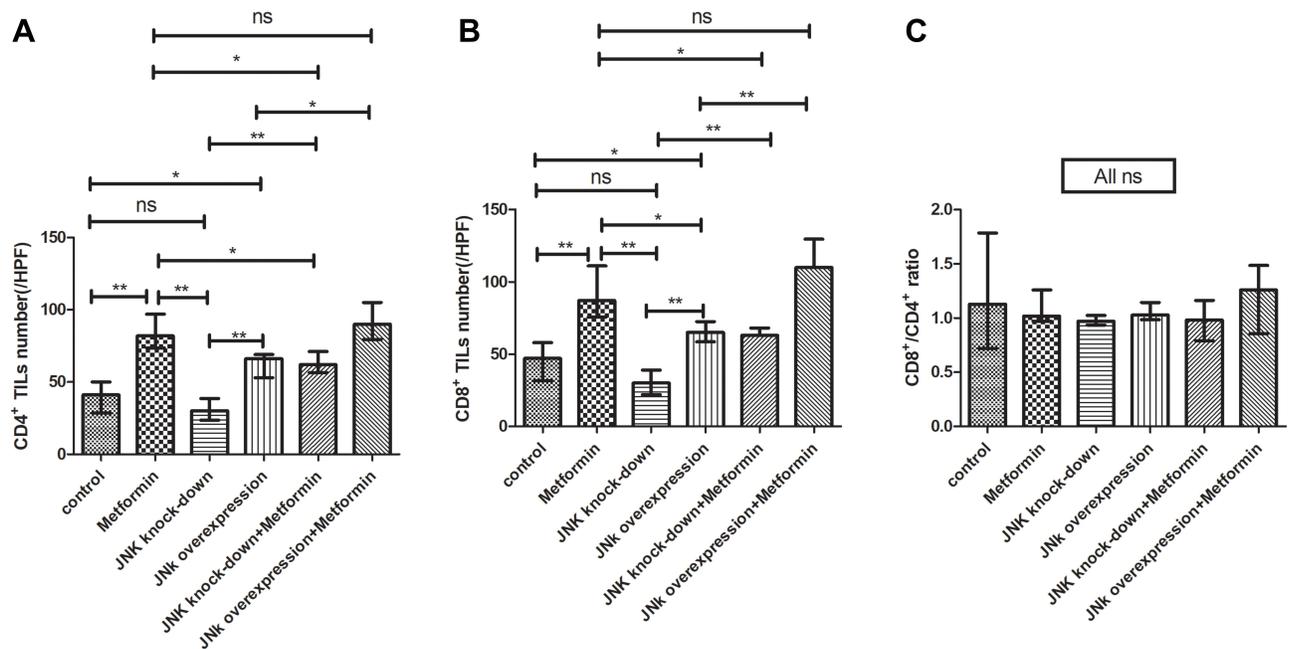


**Figure 4** CD4<sup>+</sup> TILs in tumor microenvironment of mice inoculated with breast cancer cells. (A) Inoculation of 4T1 cells. (B) Inoculation of 4T1 cells and metformin administration. (C) Inoculation of 4T1 cells with JNK knock-down. (D) Inoculation of 4T1 cells with JNK overexpression. (E) Inoculation of 4T1 cells with JNK knock-down and metformin administration. (F) Inoculation of 4T1 cells with JNK overexpression and metformin administration.

secretion, referring to the mice with Wildtype 4T1 inoculation ( $p > 0.05$ , Figure 10B). However, metformin administration reduced TNF- $\alpha$  level to 436.7pg/mL in mice with 4T1 JNK KD inoculation and 443.1pg/mL in mice with 4T1 OVE JNK inoculation ( $p < 0.05$ , Figure 10B).



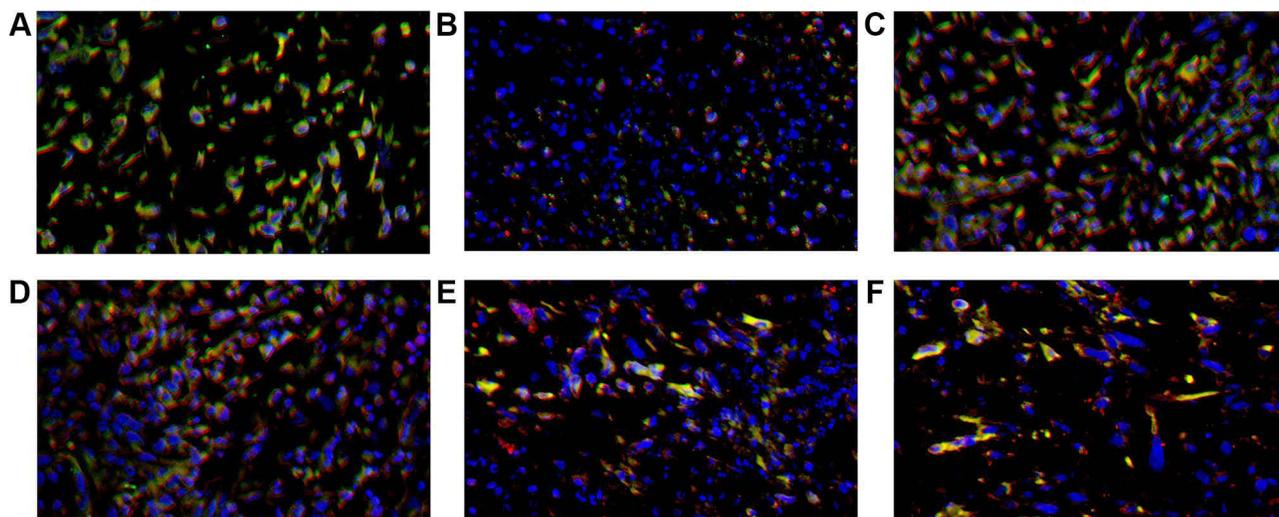
**Figure 5** CD8<sup>+</sup> TILs in tumor microenvironment of mice inoculated with breast cancer cells. (A) Inoculation of 4T1 cells. (B) Inoculation of 4T1 cells and metformin administration. (C) Inoculation of 4T1 cells with JNK knock-down. (D) Inoculation of 4T1 cells with JNK overexpression. (E) Inoculation of 4T1 cells with JNK knock-down and metformin administration. (F) Inoculation of 4T1 cells with JNK overexpression and metformin administration.



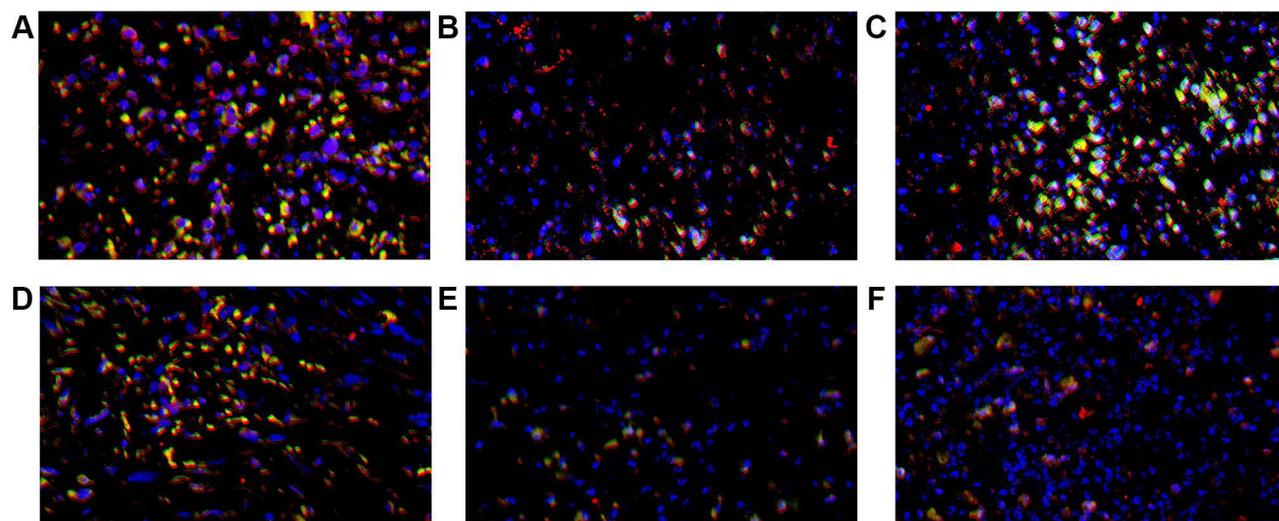
**Figure 6** Statistical result of CD4<sup>+</sup> and CD8<sup>+</sup> TILs in tumor microenvironment of mice inoculated with breast cancer cells. (A) CD4<sup>+</sup> TILs in tumor microenvironment. (B) CD4<sup>+</sup> TILs in tumor microenvironment. (C) CD8<sup>+</sup>/CD4<sup>+</sup> ratio. \**p* < 0.05, \*\**p* < 0.01, ns *p* > 0.05.

## Discussion

TNBC has an immunosuppressive TME among molecular subtypes of BC. Metformin and JNK pathway both had regulatory effect immune microenvironment of TNBC. The anti-tumor and immune regulatory effect from metformin was reported from previous studies.<sup>14,20,21</sup> Metformin could stimulate immune cells, particularly T cells, in TME, and we found that metformin increased the number of CD4<sup>+</sup> and CD8<sup>+</sup>TILs in TNBC, but the CD8/CD4 ratio did not change with metformin treatment or JNK knock-down/overexpression. The regulatory T cells (Tregs) are subtypes of CD4<sup>+</sup>T cells and suppress the proliferation and infiltration of CD8<sup>+</sup> T cells in the TME.<sup>22</sup> Therefore, the increase of CD4<sup>+</sup>T cells might not be positively correlated with the prognosis, while the increase of CD8/CD4 ratio could improve



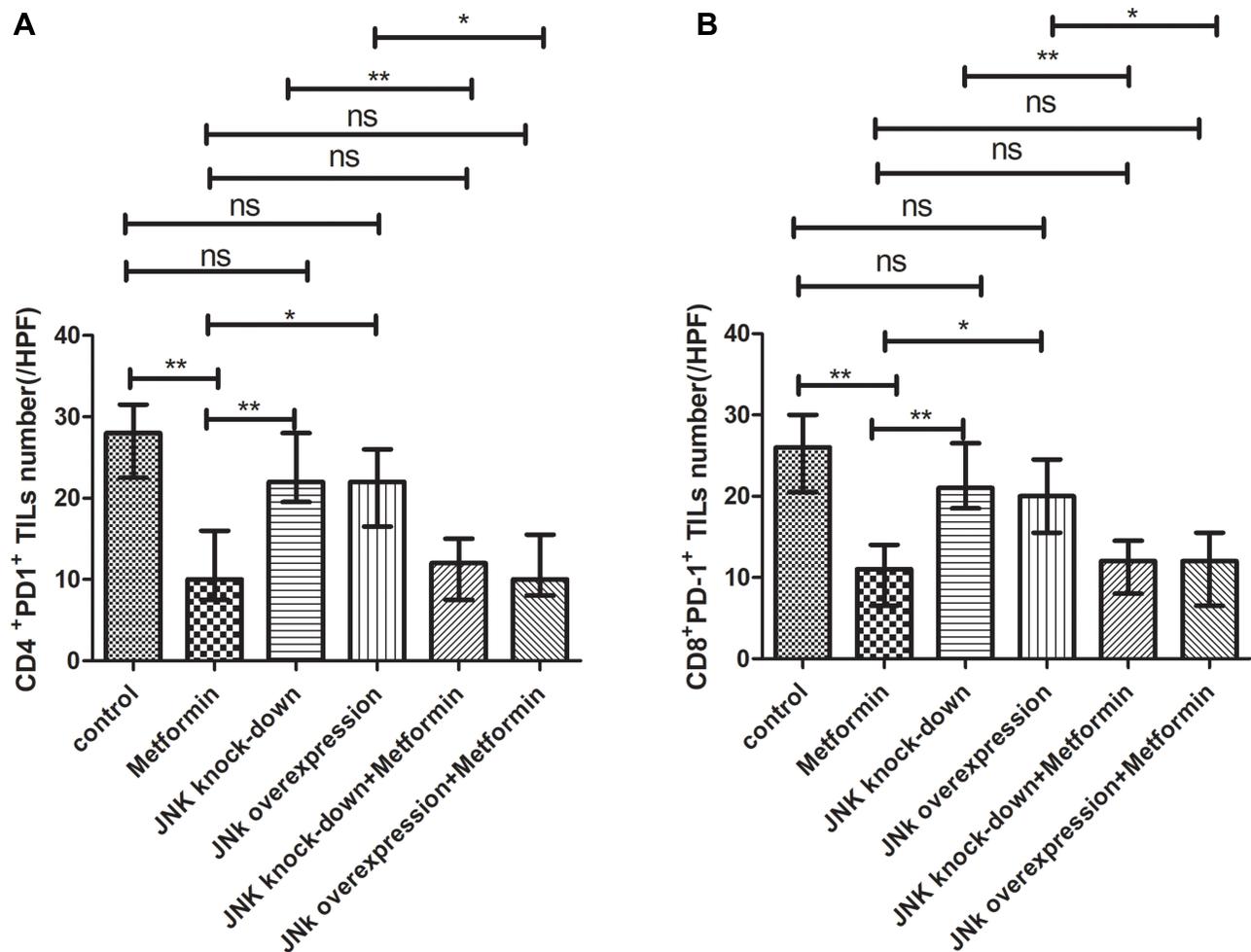
**Figure 7** CD4<sup>+</sup> TILs (red), PD-1<sup>+</sup> TILs (green) and DAPI (blue) expression in tumor microenvironment of mice inoculated with breast cancer cells. (A) Inoculation of 4T1 cells. (B) Inoculation of 4T1 cells and metformin administration. (C) Inoculation of 4T1 cells with JNK knock-down. (D) Inoculation of 4T1 cells with JNK overexpression. (E) Inoculation of 4T1 cells with JNK knock-down and metformin administration. (F) Inoculation of 4T1 cells with JNK overexpression and metformin administration.



**Figure 8** CD8<sup>+</sup> TILs (red), PD-1<sup>+</sup> TILs (green) and DAPI (blue) expression in tumor microenvironment of mice inoculated with breast cancer cells. (A) Inoculation of 4T1 cells. (B) Inoculation of 4T1 cells and metformin administration. (C) Inoculation of 4T1 cells with JNK knock-down. (D) Inoculation of 4T1 cells with JNK overexpression. (E) Inoculation of 4T1 cells with JNK knock-down and metformin administration. (F) Inoculation of 4T1 cells with JNK overexpression and metformin administration.

the prognosis.<sup>23</sup> However, the prognostic value of Tregs is varied with tumor types. Some studies<sup>24</sup> suggested FOXP3<sup>+</sup>Tregs are positively correlated with CD8<sup>+</sup>T cells in ER negative BC. Moreover, another clinical trial<sup>25</sup> showed that TNBC patients with higher infiltration of Tregs had better prognosis. Our study did not distinguish Tregs from CD4<sup>+</sup>TILs. Metformin was reported to prevent the differentiation of naive CD4<sup>+</sup> T cells into Tregs, inhibited the proliferation and function of Tregs, and reversed the suppressive TME. Although there was no significant difference in the weight of xenograft tumor specimens between metformin treatment groups, the non-significant change might be related to the short time or low dose exposure of metformin.

MAPK signaling pathway involved in the regulation of innate immunity,<sup>21</sup> and metformin increased the phosphorylation of JNK and decreased the phosphorylation of RSK2 and CREB. JNK pathway activation was reported to downregulate cancer cell proliferation<sup>27</sup> and upregulate T cell activation and differentiation.<sup>28</sup> CREB is often over-expressed in hematopoietic and solid tumors.<sup>29</sup> Activation of CREB was related to increased tumor growth, resistance to

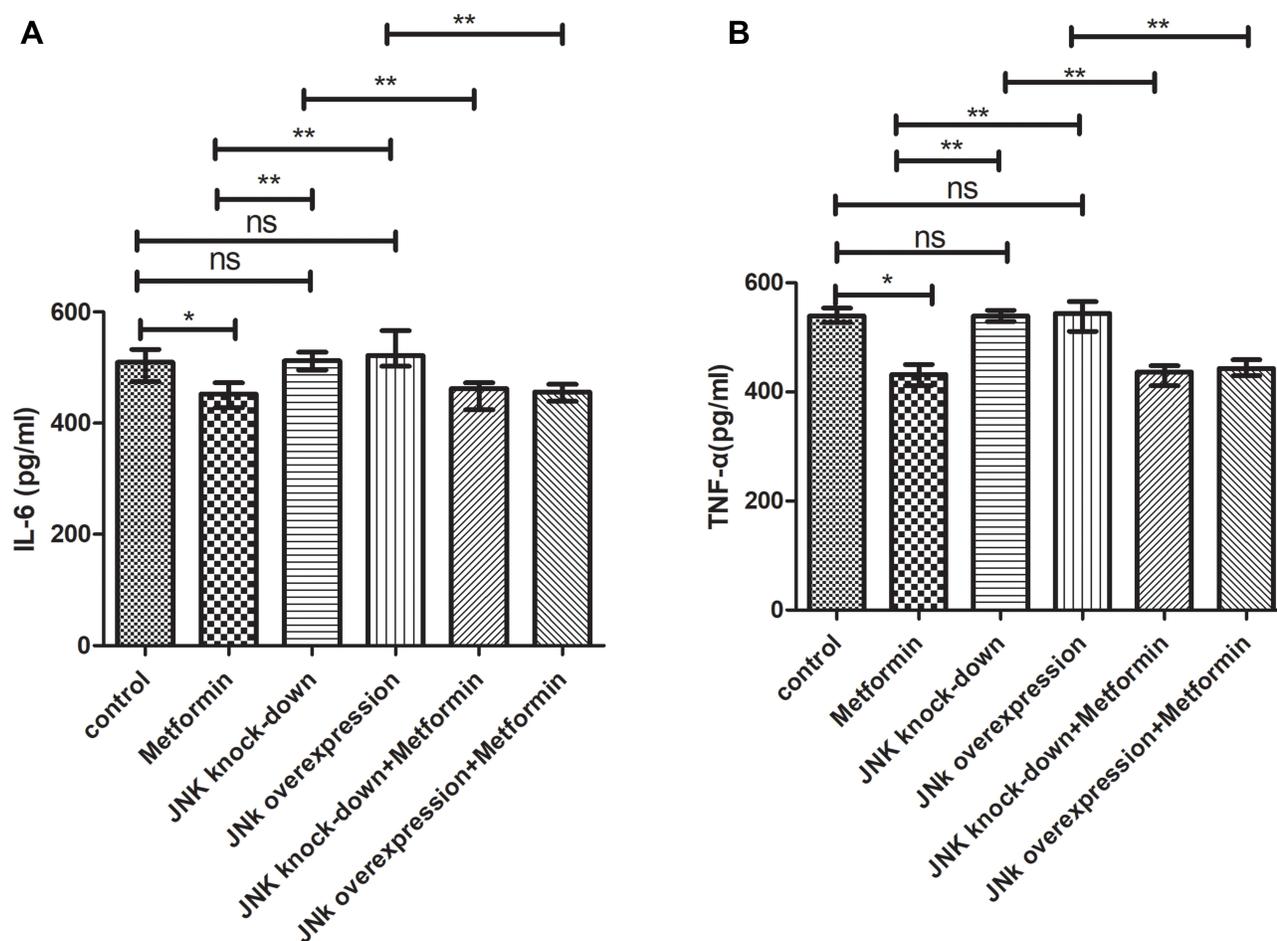


**Figure 9** Statistical result of CD4<sup>+</sup>PD-1<sup>+</sup> and CD8<sup>+</sup>PD-1<sup>+</sup>TILs in tumor microenvironment of mice inoculated with breast cancer cells. (A) CD4<sup>+</sup>PD-1<sup>+</sup>TILs in tumor microenvironment. (B) CD8<sup>+</sup>PD-1<sup>+</sup>TILs in tumor microenvironment. \* $p < 0.05$ , \*\* $p < 0.01$ , ns  $p > 0.05$ .

anti-proliferative signals, enhanced angiogenesis, increased metabolism, and decreased apoptosis.<sup>29</sup> Oncogenic EGFR, Ras and BRAF activated the ERK/MAPK/RSK pathways in many tumors,<sup>30</sup> and hyperactive RSK signaling was found to induce cell transformation and tumor growth.<sup>31</sup> The anti-proliferative effect of metformin was also related with the downregulation of CERB and RSK2.

4T1 cells are similar to human TNBC, with negative ER, PR and HER-2. Compared with other molecular subtypes of BC, TNBC had the highest content of FoxP3<sup>+</sup>Tregs, and activated PD-1/PD-L1 pathway.<sup>4,32,33</sup> Ras/MAPK pathway activation could induce TNBC immunosuppression, and inhibition of this pathway could reverse the immune response.<sup>34</sup> JNK is a component of the MAPK signaling pathway, suppressing malignant cell differentiation and inducing apoptosis.<sup>35</sup> T cell activation and proliferation.<sup>11</sup> Metformin activated JNK signal pathway, increased the functional CD4<sup>+</sup> and CD8<sup>+</sup>TILs infiltration and downregulated the exhausted CD4<sup>+</sup> and CD8<sup>+</sup>TILs infiltration in TNBC. Besides JNK pathway, metformin promoted phosphorylation of AMPK, increased the number of CD8<sup>+</sup>TILs and protected effector TILs from apoptosis and exhaustion characterized by decreased production of IL-2, TNF- $\alpha$ , and IFN- $\gamma$ .<sup>14</sup> Metformin transformed CD8<sup>+</sup>TILs from central memory status to effector memory status and decreased functional T cell exhaustion, by regulating AMPK-MTOR signaling pathway.

Although metformin reduced the number of CD4<sup>+</sup>/PD-1<sup>+</sup> and CD8<sup>+</sup>/PD-1<sup>+</sup>TILs, the JNK signaling pathway seemed to have no effect on the reduction. PD-1 is a checkpoint molecule in the immune response<sup>36</sup> and PD-1 expression on T cells indicated a state of function exhaustion.<sup>37</sup> PD-1 expression inhibited T cell proliferation and induced functional depletion.<sup>38</sup> Metformin increased the cell counts of functional TILs and decreased the cell counts of exhausted TILs.



**Figure 10** Secretion of inflammatory mediators by tumor tissue. (A) IL-6 secretion (B) TNF- $\alpha$  secretion. \* $p < 0.05$ , \*\* $p < 0.01$ , ns  $p > 0.05$ .

However, PD-1 expression on CD4<sup>+</sup> and CD8<sup>+</sup>T lymphocytes was increased under the stimulation of pro-inflammatory factors such as TNF- $\alpha$  and IL-6.<sup>39–41</sup> Metformin inhibited the synthesis of pro-inflammatory cytokines in the TME,<sup>42</sup> and we observed a reduction of the TNF- $\alpha$  and IL-6 production in our study. This reducing expression is linked with the inactivation of nuclear factor kappa B (NF- $\kappa$ B) and signal transducer and activator of transcription 3 (STAT3) signaling pathway.<sup>43</sup> Metformin reduced TNF- $\alpha$  and IL-6 expression by inhibiting NF- $\kappa$ B signaling pathway.<sup>44,45</sup> The reduction of PD-1 expression might be related with the downregulation of TNF- $\alpha$  and IL-6 in TNBC.

Although CD8<sup>+</sup> T cells could secrete perforin, IL-6, TNF- $\alpha$ , IFN- $\gamma$ , so as to achieve cytotoxic efficacy.<sup>46</sup> The reduce of IL-6 and TNF- $\alpha$  might be related to the activation of AMPK pathway, induced by metformin. Metformin suppressed the synthesis of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-6, IL-8<sup>42</sup> and VEGF, and the suppression was linked with the inactivation of NF- $\kappa$ B and STAT3, which were in an AMPK-dependent manner.<sup>47</sup> The activation AMPK pathway mediated the inactivation of NF- $\kappa$ B.

Metformin is a commonly used hypoglycemic drug and the dose used in our study was within a safe level. Thirty-eight adverse events were reported, suspected to be related with the combining treatment of metformin and immune checkpoint inhibitors. The study found that patients with lung cancer had an increased risk of inflammatory bowel disease and diarrhea during Nivolumab and metformin combination therapy.<sup>48</sup> Patients with hepatocellular carcinoma were prone to multiple organ dysfunction syndrome and lactic acidosis.<sup>48</sup> Patients with malignant melanoma had adverse events as interstitial lung lesions, decreased thyroglobulin, acute kidney injury, and pancreatic failure.<sup>48</sup> At present, there was little clinical evidence on metformin and the risk of immune-related adverse events.

Although we observed metformin affected the activation of JNK, RSK2 and CERB signal pathways, only JNK signal pathway was investigated by gene knockdown and overexpression. Metformin upregulated JNK pathway expression to affect functional TILs, but other MAPK pathways seemed to mediate the crosstalk effect between metformin and immune microenvironment in TNBC too, especially for exhausted TILs. It was the first limitation. The second limitation was that the immune regulatory effect of metformin in murine model needed to be validated in human studies.

## Conclusion

Metformin increased the cell counts of CD4<sup>+</sup> and CD8<sup>+</sup>TILs by increasing JNK phosphorylation in TNBC. Metformin also reduced the production of IL-6, TNF- $\alpha$  and the cell count of exhausted TILs in TNBC. Metformin had the potential to combined immunotherapy for TNBC.

## Data Sharing Statement

The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

## Acknowledgment

This study was financially supported by Beijing Municipal Committee of Science and Technology (Z19110006619041) and Beijing Hospitals Authority (XMLX202114) and Open project of Beijing Key Laboratory of Tumor Therapeutic Vaccine (2019-KF04) and Youth Fund of Beijing Shijitan Hospital (2020-q01). The supporting organizations had no role in study design, data collection, analysis and interpretation.

## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

## Disclosure

The authors declare no conflicts of interest in this work.

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