Metformin attenuates bleomycin-induced scleroderma by regulating the balance of Treg/Teff cells and reducing spleen germinal center formation

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ABSTRACT

Scleroderma is an inflammatory autoimmune disease characterized by extensive tissue fibrosis. The imbalance of effector T (Teff) and regulatory T (Treg) cells and the production of autoantibodies contribute to the pathogenesis of this disease. Metformin (MET) has anti-inflammatory and anti-fibrotic effects, but its effect on the *in vivo* pathogenesis of scleroderma remains unknown. Therefore, we investigated the potential therapeutic effects of MET treatment of mice with bleomycin (BLM)-induced scleroderma. Scleroderma was induced in female C57BL mice by daily subcutaneous injections of BLM for 28 days. After each 2 h BLM injection, mice received MET (200, 100 or 50 mg/kg) or saline (control) by intraperitoneal injection. At the end of the fourth week, spleen mononuclear cells were collected for flow cytometry analysis. Skin samples were harvested for immunohistochemistry and quantification of other biological parameters. Our results showed that BLM increased dermal thickness, collagen deposition, and hydroxyproline level, and MET markedly mitigated these effects. MET also restored the Treg/Teff cell balance. Accordingly, the level of IL-17A and RORγt (related to Th17 cells) decreased, but Foxp3 (related to Treg function) increased in a dose-dependent manner. In addition, MET treatment inhibited spleen germinal center formation. These results indicate that the immunomodulatory and anti-fibrosis effects of MET on BLM-induced scleroderma are mediated by the upregulation of Treg cell differentiation, inhibition of Teff cell differentiation, and suppression of spleen germinal center formation. These results suggest that MET may be a potential therapeutic for scleroderma.

1. Introduction

Scleroderma is an autoimmune disease characterized by fibrosis of the skin and other organs, vascular impairment, and abnormal immune response (Wang et al., 2016; Kajii et al., 2011). Although the details of

its pathogenesis remain unclear, pro-inflammatory cytokines, autoimmunity, and high levels of autoantibodies contribute to microvascular damage, inflammation, and fibrosis (Yang et al., 2014). However, the exact roles of lymphocytes and autoantibodies in the pathogenesis of scleroderma have not been clarified (Lei et al., 2016a,

2016b). Therefore, an increased understanding of the roles of lymphocytes and autoantibodies may further elucidate the pathogenesis of scleroderma.

Interleukin-17A (IL-17A) is produced by Th17 cells, and retinoic-acid-receptor- related orphan receptors gamma t (RORγt) affects the function of these cells, whereas transcript factor forkhead box protein 3 (Foxp3) plays a key role in the function of CD4$^+$CD25$^+$Foxp3$^+$Treg cells (Zhu et al., 2017). Previous studies of an animal model of systemic sclerosis (SSc) showed that secretion of IL-17A contributes to immune inflammation and the development of scleroderma (Lei et al., 2012, 2015; Okamoto et al., 2012; Lei et al., 2016a, 2016b). In contrast, Treg cells have an anti-inflammatory role, and their function is downregulated in autoimmune diseases (Lee et al., 2009; Jhun et al., 2015; Park et al., 2016; Son et al., 2014). Therefore, a therapeutic approach that simultaneously increases Treg cells and reduces Th17 cells may be a promising scleroderma treatment. In addition, Th1 and Th2 cells have significant correlations with SSc by secreting soluble profibrotic factors that promote fibroblast matrix deposition (Lee et al., 2017). Follicular helper T (Tfh) cells assist in the function of B cells, and are indispensable for the formation of germinal centers (GCs). The dysregulation of Tfh cells contributes to the development of autoreactive B cells, production of autoantibodies, and aggravation of autoimmune diseases (Lee et al., 2014; Zhang et al., 2013; Mi et al., 2016). Thus, the imbalance of Th17/Treg cells and the production of pathogenic autoantibodies appear to be at least partly responsible for skin fibrosis in scleroderma. Drugs such as glucocorticoids and immunosuppressive agents are currently used to treat scleroderma (Kajii et al., 2011), but their long-term use can lead to a range of side effects. Therefore, it is urgent and necessary to find more safe and effective treatments for scleroderma.

Metformin (MET), which was originally introduced as a biguanide antidiabetic medication, has anti-inflammatory effects due to its activation of AMP-activated protein kinase (AMPK), a major sensor that modulates lipid and glucose metabolism (Lee et al., 2014; Zhou et al., 2001; Sato et al., 2016). Recent studies have reported that MET ameliorates the development of lung fibrosis and inflammatory bowel disease (Seon-Yeong et al., 2015). At the molecular level, MET-activated AMPK suppresses mammalian target of rapamycin (mTOR), which regulates effector T cell differentiation *in vitro* and *in vivo* (Lei et al., 2012). mTOR is a serine/threonine protein kinase that functions in the initiation of inflammatory cascades and regulation of Th17/Treg balance (Bo et al., 2016). Due to its anti-fibrotic effects on immune T cells, MET may have potential for the treatment of scleroderma.

We used a murine model of BLM-induced scleroderma to examine the effect of MET on the pathogenesis of scleroderma. More specifically, we investigated the effect of MET on skin fibrosis, the Th17/Treg balance, the formation of spleen GCs during the pathogenesis of scleroderma.

2. Material and methods

2.1. Animals

Fifty female C57BL mice, which were 6 weeks old and weighed 20–25 g, were used for experiments. The mice were randomly separated into 5 groups (10 per group) and maintained in an animal facility that was climate-controlled, had a 12-h light/dark cycle, and provided free access to food and water. Mice were acclimatized for at least 7 days before use in experiments. All animal experimental protocols were reviewed and approved by the Laboratory Animal Ethics Committee of Shanxi Medical University.

Mice in the control group received 100 μg/day of subcutaneous normal saline (N.S.), which was injected into the shaved skin of the upper back. To induce dermal fibrosis, the remaining 4 groups received 100 μg of BLM (Nippon Kayaku, Tokyo, Japan) in the same region. After each 2 h BLM injection, the 4 groups of BLM-treated mice were also

given intraperitoneal MET (200, 100 or 50 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) or N.S. for 4 weeks. At the end of the fourth week, the spleens and skin tissues of all mice were collected for analysis. Spleen mononuclear cells were isolated for flow cytometry (FCM) analysis. The shaved dorsal skin at the injection site were collected for immunohistochemistry (IHC) and isolation of RNAs for analysis by the real-time quantitative polymerase chain reaction (RT-qPCR), and the remaining parts for the hydroxyproline (HYP) assay.

2.2. Histological and IHC examination

Skin tissues were fixed in a 10% formalin solution, embedded in paraffin, and cut into 4-μm-thick sections. These sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome to observe inflammation and collagen deposition (Koca et al., 2016; Lei et al., 2016a, 2016b). Dermal thickness, from the dermal-epidermal interface to the intradermal fat, was measured using Image-Pro-Plus software. Five randomly selected independent microscopic fields were counted for each sample (Yang et al., 2014). Data were analyzed using a computer-assisted image analysis system. IHC was performed using antibodies against α-smooth muscle actin (α-SMA; Abcam), IL-17A (Abcam), and Foxp3 (Abcam). All sections were independently examined by two investigators who were blinded to the group allocations.

2.3. Measurement of HYP concentration

HYP is a major component of collagen, and the total HYP content is an indicator of collagen deposition in skin tissues (Lei et al., 2016a, 2016b). The alkaline hydrolysis colorimetry assay was used to measure the concentration of HYP in skin tissues, as previously described (Wang et al., 2016). Skin samples (stored at −80 °C) were hydrolyzed with sodium hydroxide at 100 °C for 10 min. After neutralization with 6 N hydrochloric acid, the hydrolysates were diluted with distilled water. The concentration of HYP was determined using a hydroxyproline assay kit with measurement of absorption at 550 nm (Nanjing Jiancheng Bioengineering Institute), according to the manufacturer's instructions. Values were are as μg/mg skin.

2.4. RNA isolation and RT-qPCR

Total RNA was extracted from skin samples using the E.Z.N.A.° Total RNA Kit I (Omega Bio-tek, Inc.), and reverse-transcribed into cDNA according to the manufacturer's protocol (Prime Script RT Reagent Kit, Takara). The target genes were identified using SYBR Premix Ex Taq II (Takara) on a 7500 Fast Real-Time PCR sequence detection system (Bio-Rad, Hercules, CA, USA). The real-time-PCR conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 31 s. Expression was normalized to the housekeeping gene GAPDH, and the levels of α-SMA, collagen, Foxp3, RoRγt, and IL-17A transcripts were determined using the $2^{-\Delta\Delta Ct}$ method (Okamoto et al., 2012).

2.5. FCM analysis

Spleen mononuclear cells were freshly isolated by Ficoll-Hypaque density gradient centrifugation, washed twice in PBS, and then incubated with CD4-PerCP, CD25-APC (Tonbo Biosciences), CXCR5-PE, Bcl-APC, and GL-7-Alexa Fluor antibodies for 30 min in darkness at room temperature. All antibodies except CD25 were purchased from BD Bioscience. Intracellular Foxp3 (PE, eBioscience) staining was performed according to the manufacturer's instructions (He et al., 2016; Son et al., 2014). For detection of intracellular cytokine production, cells were stimulated with 20 ng/ml of PMA and 1000 ng/ml of ionomycin in the presence of Golgi-Stop (BD Biosciences) for 5 h. They were then incubated with anti-CD4 antibody and stained with anti-IL-17-APC (Miltenyi Biotec), IFN-γ- FITC (EXBIO Praha), and IL-4-PE (BD

Biosciences) after fixation and permeabilization (eBioscience). The stained cell images were acquired using the FACSCanto II flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA), and analyzed with FlowJo V.7.6.1 software (Treestar Inc, Ashland, Oregon, USA; He et al., 2016).

2.6. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 22 for Windows (Chicago, IL, USA). For data with normal distributions and homogenous variances, the statistical significance of differences was calculated using one-way analysis of variance (ANOVA). For data that did not have normal distributions and homogeneous variances, the statistical significance of the differences was calculated using the nonparametric two-tailed Kruskal-Wallis test. P values less than 0.05 were considered statistically significant. Data are presented as means ± standard deviations (SDs).

3. Results

3.1. MET ameliorated the effect of BLM on skin thickness and collagen deposition

We induced skin fibrosis in C57BL mice by daily subcutaneous administration of BLM or N.S. (healthy control, HC) for 28 days. We then collected skin at the injection site and prepared tissue sections for staining by H&E and Masson's trichrome. We found that the mice with scleroderma (BLM + saline) had clear disruptions of structural integrity, apparent inflammatory infiltrates, and fibrosis than HC groups (Fig. 1A). Notably, mice that received MET treatments in addition to BLM had thinner skin and less collagen deposition, and this effect was dose-dependent. Furthermore, quantitative analysis indicated that dermal thickness was significantly greater in BLM + saline mice than in HC mice (171.28 ± 27.18 *vs.* 88.28 ± 12.45 μm; $P < 0.01$; Fig. 1B). Although MET reduced dermal thickness, mice that received high-dose MET (200 mg/kg) had significantly thinner skin than BLM + saline mice (171.28 ± 27.18 *vs.* 131.11 ± 25.25 μm, $P < 0.05$; Fig. 1B).

In addition, BLM + saline mice had significantly increased collagen deposition relative to HC mice (182.2 ± 27.62 *vs.* 78.27 ± 39.85, $P < 0.001$; Fig. 1C). However, MET significantly reduced the collagen deposition at all tested doses, with the greatest effect at 200 mg/kg. The effects of 100 mg/kg and 200 mg/kg of MET were not significantly different.

3.2. MET reduced skin levels of α-SMA and collagen mRNA, but not HYP

We next examined the expression of three indicators of fibrosis (α-SMA, collagen, and hydroxyproline) in the skin of the different mice. The IHC results showed there were more α-SMA positive cells in the BLM + saline group than in the HC group, and that MET reduced the number of α-SMA-positive cells in a dose-dependent manner (Fig. 2A and B). The difference between the 100 mg/kg and 200 mg/kg MET groups was statistically significant ($P < 0.05$).

To further verify whether MET can modulate mRNA levels of different fibrosis index during BLM-induced mice, we then detected the *α-SMA and* collagen levels by RT-qPCR in the skin of different mice (Fig. 2C and D). These results indicate greater levels of *α-SMA* ($P < 0.001$) and collagen ($P < 0.001$) in the BLM + saline group than in the HCs. However, MET significantly downregulated *α-SMA* and collagen accumulation in a dose-dependent manner. In paralleling, we characterised the contents of HYP in the skin from the model and treatment groups of mice, and found that mice in the BLM + saline group had a greater skin level of HYP than the HCs (5.00 ± 0.37 *vs.* 3.95 ± 0.83 μg/mg, $P < 0.05$). MET slightly reduced HYP levels, but this effect was not statistically significant (Fig. 2C). These results show that BLM + saline treatment increased the levels of collagen and α-

SMA, and that MET reversed these effects.

3.3. MET restored the Th17/Treg balance and inhibited cytokine production

Previous studies have suggested that Th17 and Treg cells have opposing effects on autoimmune responses (Fenoglio et al., 2011; Tatiana S. and Janette, 2012; Fenoglio et al., 2012). We examined this effect in these mice by performing FCM analysis of spleen mononuclear cells (Fig. 3A, B and C). The results show that BLM + saline mice had increased proportions of Th17 cells ($P < 0.001$) whereas decreased proportions of Treg cells ($P < 0.05$). MET treatment alleviated the effect of BLM in a dose-dependent manner.

We also determined the expression of specific cytokines and transcription factors related to Th17 and Treg cells (*IL-17A*, *RoRγt*, and *Foxp3*) using RT-qPCR. We found that the BLM + saline group had greater expression of Th17-related cytokines and transcription factors, and reduced expression of Foxp3 ($P < 0.01$; Fig. 3D and E). Inversely, MET treatment partially reduced the effect of BLM in a dose-dependent manner.

In addition, we measured the infiltration of IL-17^{+} and Foxp3^{+} cells in skin biopsy specimens by IHC (Fig. 3F, G and H). We found that the BLM + saline mice had significantly increased infiltration of IL-17^{+} cells while decreased infiltration of Foxp3^{+} cells than HCs. As noted above, MET treatment partially reversed the effect of BLM in a dose-dependent manner. These findings suggest that the therapeutic effects of MET in this murine model of scleroderma may be related to its restoration of the balance of Th17/Treg cells.

3.4. MET decreased the proportions of Th1 and Th2 cells among spleen mononuclear cells

To study the differentiation of naive CD4^{+}T cells to Th1 and Th2 cells, we stained spleen mononuclear cells with anti-CD4, IFN-γ, and IL-4 antibodies and then performed FCM analysis (Fig. 4). The results show that the percentages of Th1 cells ($P < 0.001$) and Th2 cells ($P < 0.001$) were greater in the BLM + saline group than in the controls. Administration of MET partially reversed the effect of BLM in a dose-dependent manner.

3.5. MET suppressed differentiation of Tfh cells and GC formation

Follicular helper T (Tfh) cells are specialized providers of help to B cells, and are important for the formation of GCs. Once GCs are formed, Tfh cells maintain them and regulate GC B cell differentiation into PCs and memory B cells. Tfh cells express high levels of Bcl-6 and CXCR5 (Lee et al., 2012). GL-7 is a GC marker on GC B cells, thymocytes, and some neuronal cell types (Cervenak et al., 2001). Our FCM analysis (Fig. 5) indicated that BLM + saline mice had greater levels of Tfh cells ($P < 0.001$) and GC B cells ($P < 0.001$) than HCs, but MET treatment reversed this effect. However, there were no significant differences in the different MET groups.

4. Discussion

Scleroderma is a chronic autoimmune disease characterized by extensive tissue fibrosis and damage of multiple organs. Immunological disorders, especially Th17/Treg imbalance and the production of autoantibodies, play major roles in its pathogenesis, suggesting that targeting of these disorders may provide a novel treatment. We examined the therapeutic potential of MET due to its anti-fibrosis and immune regulatory effects.

Our study adds important new data to the existing body of knowledge on the pathogenesis of scleroderma. In particular, we demonstrated for the first time that the immunomodulating effect of MET on scleroderma is mediated by its alteration of T and B lymphocytes. More specifically, our data show that MET increased the proportion of Treg
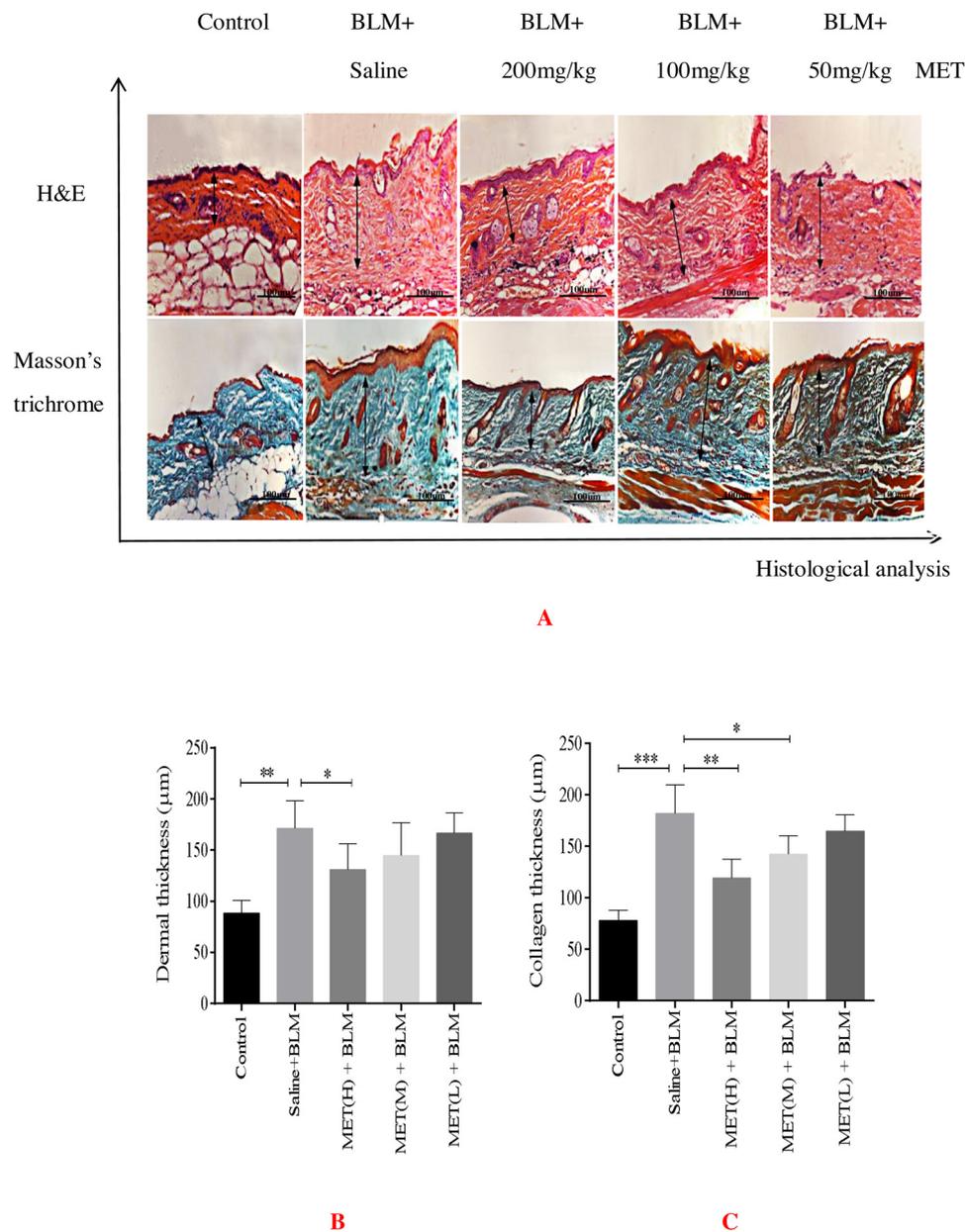
Fig. 1. MET ameliorated tissue damage. (A) Representative photomicrographs of skin tissues stained with hematoxylin and eosin (H&E) and Masson's trichrome (×200; scale bar: 100 μm). (B and C) Quantitative analysis of dermal thickness and collagen in skin tissues. Black arrows indicate skin thickness (H&E) and cutaneous fibrosis (Masson's trichrome). *P < 0.05, **P < 0.01 and ***P < 0.001 for the indicated groups.

cells and decreased the proportion of Teff (Th17, Th1, Th2 and Tfh) cells, and restored GC formation. Therefore, MET may be a potential therapeutic for scleroderma.

Our results confirmed that MET alleviates BLM-induced inflammation, fibrosis, and collagen deposition in skin tissues, consistent with recent reports of its effects on mTOR and in reducing inflammation and fibrosis in scleroderma (Zhu et al., 2017; Ursini et al., 2016). In addition, there are increased levels of phosphorylated mTOR in the fibroblasts of patients with scleroderma and mice with BLM-induced scleroderma (Zhu et al., 2017). The treatment of BLM-induced mice with rapamycin (an mTOR inhibitor) significantly reduced the inflammation and fibrosis in lesioned skin. These reports suggest that the pathogenesis of scleroderma is associated with mTOR activation (Zhu et al., 2017). Other studies have reported that the endothelial-to-mesenchymal transition (EndoMT) is an important pathological process that contributes to the activation of α-SMA$^+$ fibroblasts during tissue fibrosis (Jamshidian et al., 2013; Yamashita et al., 2016). Furthermore,

transforming growth factor (TGF) -β stimulates the production of extracellular matrix (ECM) and the deposition of collagen, which are essential processes in fibroblast activation and proliferation (Lei et al., 2016a, 2016b; Barnes et al., 2011). However, in our experiment, MET suppressed the BLM-induced upregulation of α-SMA mRNA and protein, and reduced collagen deposition in skin tissues. These data suggest that MET may exert anti-fibrotic effects by inhibiting BLM-induced EndoMT and TGF-β production, thereby promoting vessel stabilization.

Th17 cells play a critical role in the induction of autoimmune tissue injury (Pernis, 2009). In contrast, Treg cells are essential for maintaining self-tolerance, and play key roles in regulating immune system homeostasis. Th17 and Treg cells can be interconverted, depending on the intracellular cytokine milieu (Lee et al., 2009). Our findings are in accordance with these results. Recent research also indicated that cellular metabolism is an important regulator of the Th17/Treg balance (Hanidziar and Koulmanda, 2010). Furthermore, Kang et al., 2013 reported that MET activated AMPK, and thereby inhibited the mTOR-
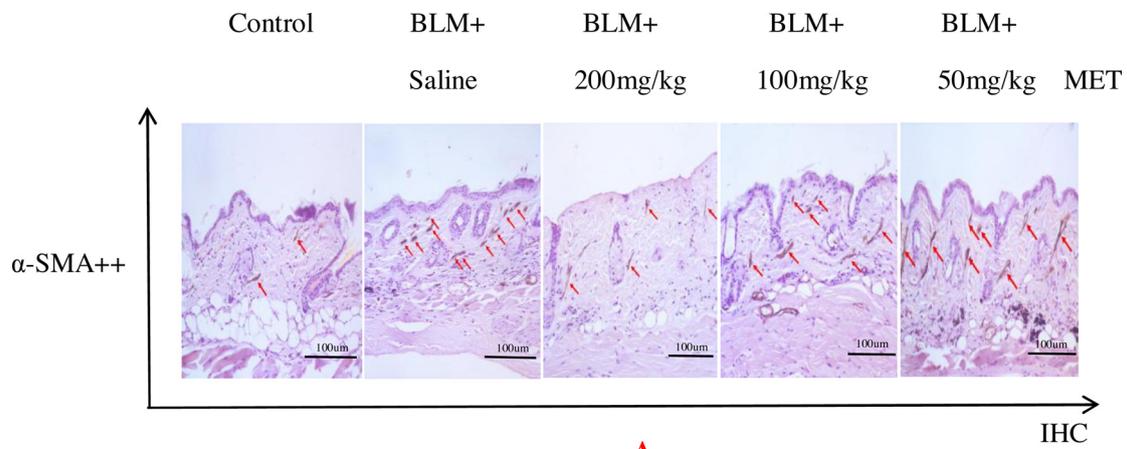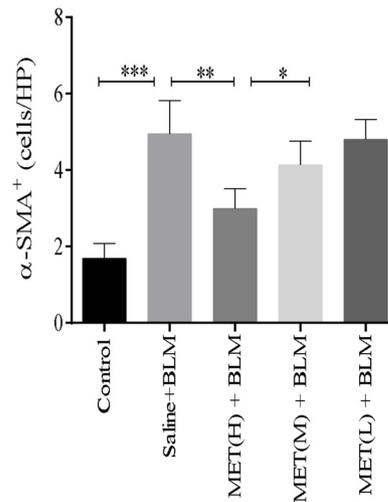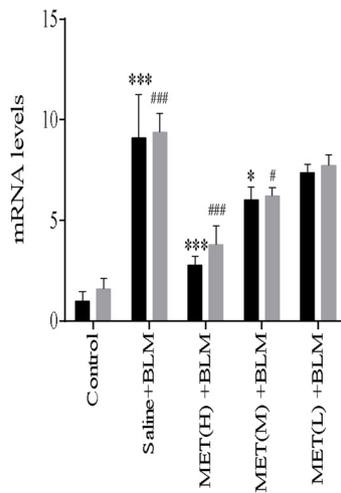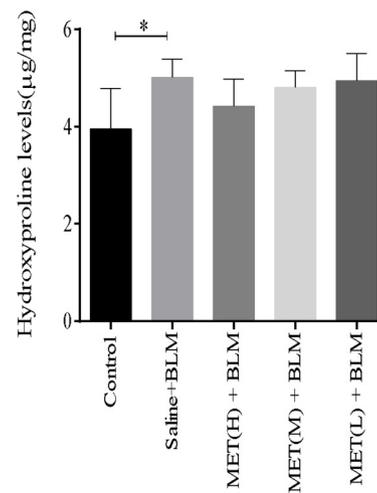
Fig. 2. MET reduced the skin fibrosis. (A) Expression of α-SMA$^+$ cells in skin sections *via* IHC staining (×200). (B) Quantitative analysis of α-SMA$^+$ cells. (C) Expression of *α-SMA* and collagen. (D) HYP concentration. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, for comparisons with the BLM + saline or BLM + MET groups (*α-SMA*). [#]$P < 0.05$, [##]$P < 0.01$, and [###]$P < 0.001$, for comparisons with the BLM + saline or BLM + MET groups (collagen).
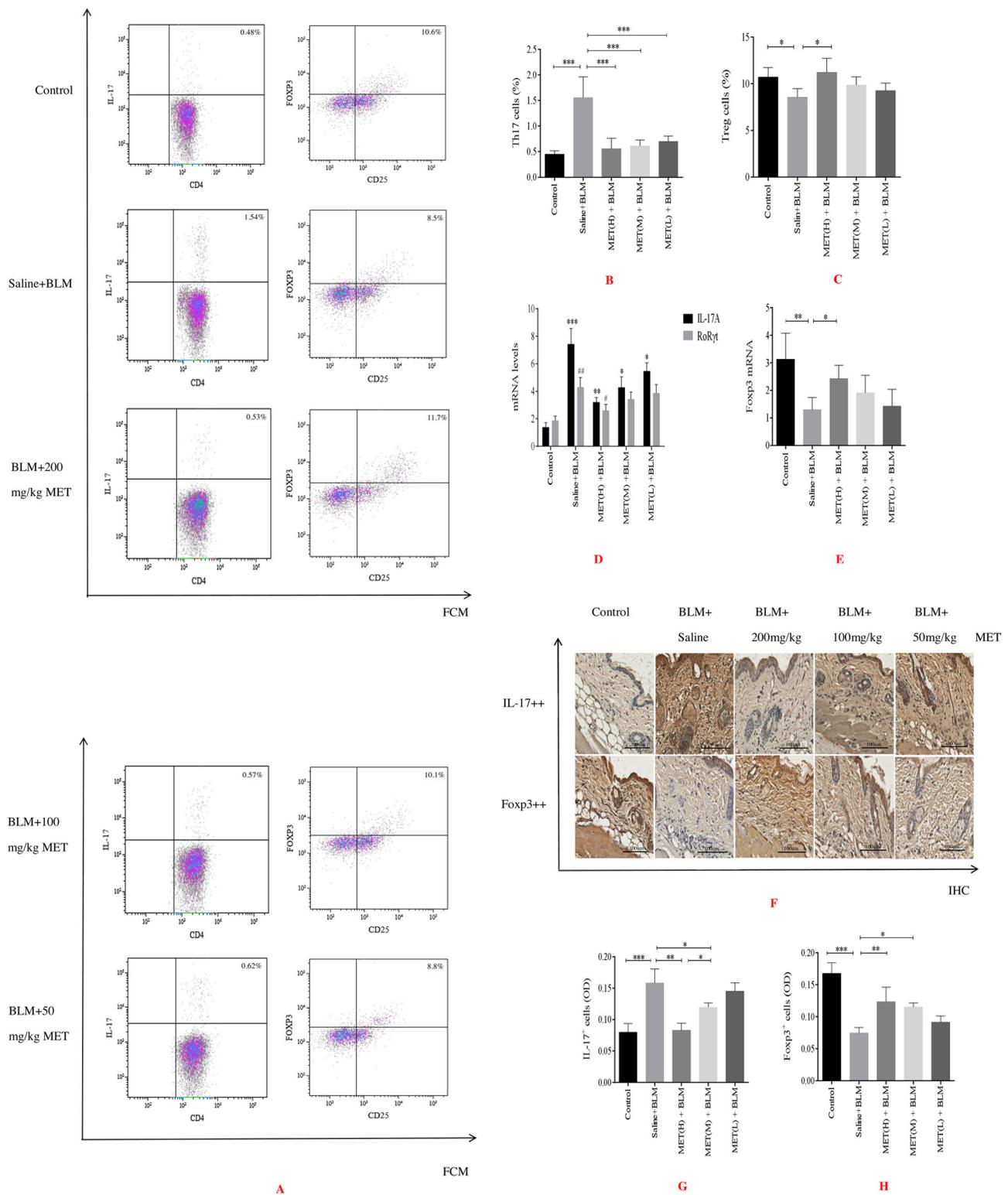
Fig. 3. MET reversed the molecular effects of BLM. (A) Representative flow cytometry analysis of spleen mononuclear cells, showing the proportions of Th17 and Treg cells in the different groups. (B and C) Quantitation of these results. (D and E) mRNA levels of *IL-17A*, *RORγt* and *Foxp3* in the skin of different mice. (F, G and H) IHC for IL-17 and Foxp3 (×200), and quantitation of these results. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, for comparisons with the BLM + saline or BLM + MET groups (IL-17A). #$P < 0.05$, ##$P < 0.01$ and ###$P < 0.001$, for comparison with the BLM + saline or BLM + MET groups (*RORγt*).

STAT3 pathway, contributing to a smaller proportion of Th17 cells. These findings suggest that MET may regulate the balance of Th17 and Treg cells *via* the AMPK-mTOR pathway. What's more, Teff and Treg require different energy sources to support their functions. Th17 cells are highly glycolytic, whereas Treg cells have high rates of lipid oxidation (Michalek et al., 2011). This is consistent with the hypothesis that T cell activation stimulates mTOR to upregulate glycolysis and downregulate lipid metabolism (Wieman et al., 2007). Activation of AMPK by MET and inhibition of mTOR by rapamycin leads to low glycolytic activity of Teff cells and high mitochondrial oxidative
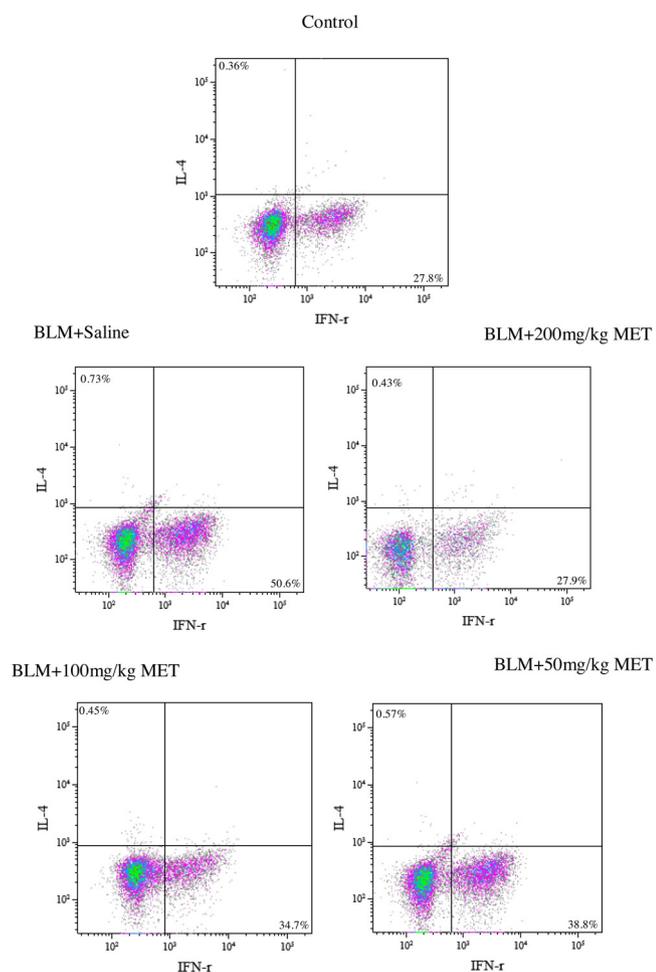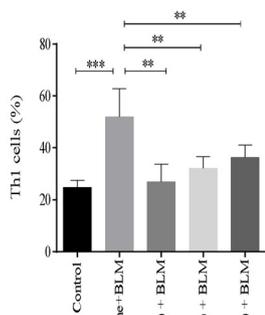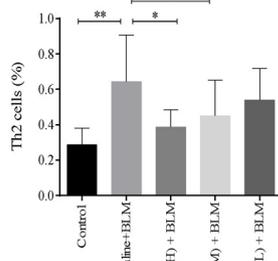
Fig. 4. MET restored the balance of Th1 and Th2 cells. (A) Representative fluorescence-activated cell sorting plots of Th1 and Th2 cells in the different groups. (B and C) Quantitation of these results. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$, for the indicated groups.

metabolism of Treg cells. The high glycolytic activity of Th17 cells is attributed to hypoxia-induced factor (HIF)-1, a downstream molecule in the mTOR pathway (Son et al., 2014). HIF-1 increases the expression of transcription factors specific for the activation of RORγt and suppression of Foxp3, and appears to contribute to altering the Th17/Treg balance (Yang et al., 2012). Therefore, the activation of AMPK and inhibition of mTOR is an effective method for the simultaneous regulation of Teff and Treg, which have important and opposing roles in
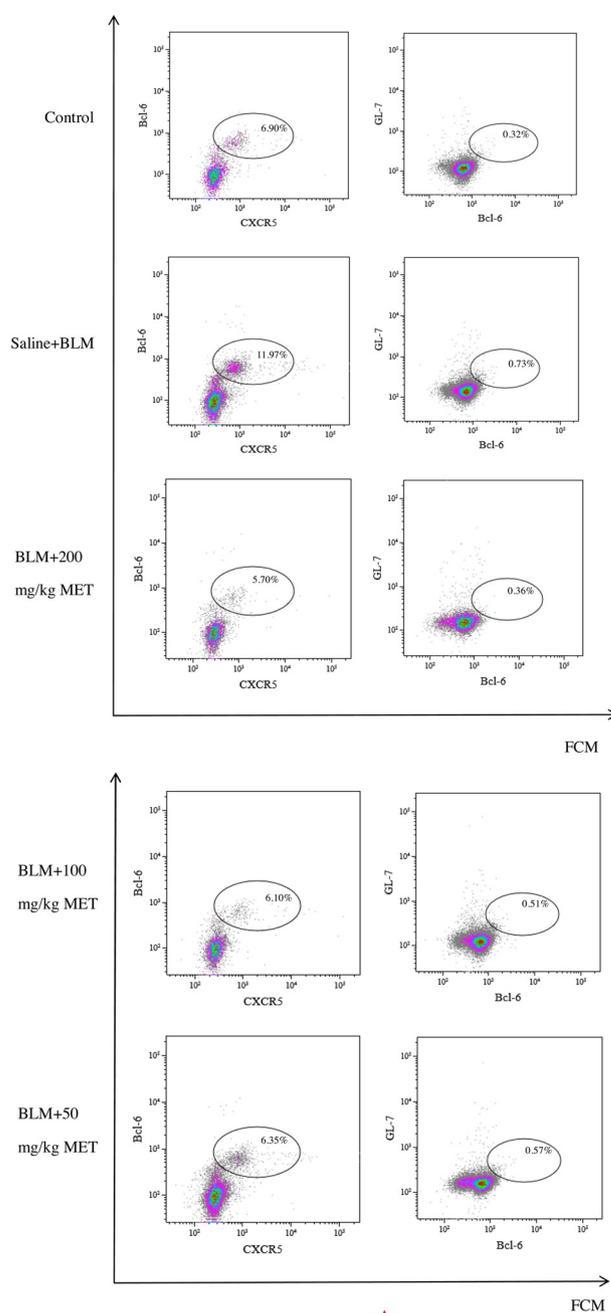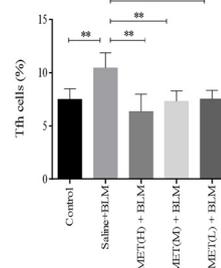
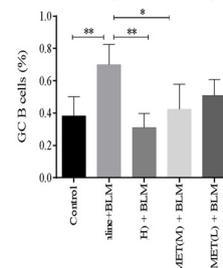**Fig. 5. MET reduced the levels of Tfh cells and GC B cells.** (A) Representative flow cytometry analysis of spleen mononuclear cells, showing the proportions of Tfh and GC B cells in the different groups. (B and C) Quantitation of these results. $*P < 0.05$ and $**P < 0.01$, for the indicated groups.

the metabolism and immune responses in scleroderma.

Tfh cells are essential for GC formation, and are regarded as specialized helpers of B cells. B cells can differentiate into plasma cells and memory B cells (Lee et al., 2017). Loss of self-tolerance and the production of autoantibodies by autoreactive B cells plays a central role in the activation of the immune system during scleroderma. This is supported by the presence of antinuclear antibodies in more than 90% of patients with scleroderma (Yoshizaki and Ayumi, 2018). Furthermore, our study showed that the proportions of Tfh cells and GC B cell formation were significantly greater in BLM + saline group, and the treatment with MET reversed these effects. Consistent with these findings, a recent study reported that autoantibodies may promote vasoconstriction and obliterative vasculopathy by direct contact with fibroblasts or dendritic cells and B cells (Raybuck et al., 2018). Collectively, these findings indicate that B cell abnormalities play major roles in the pathogenesis of scleroderma, and are thereby potential therapeutic targets. Further studies are needed to determine whether MET can also alter the production of autoantibodies in mice with BLM-induced scleroderma.

However, some limitations should be noted for our study. First, we investigated the effect of MET on BLM-induced inflammation and fibrosis without further evaluation of the underlying signaling pathway. Second, we did not examine a "post-BLM" experimental group. Finally, some of the experiments failed, so that data for some experiments were not available for all mice in different groups. Therefore, future studies are needed to validate the potential therapeutic and molecular mechanism of this therapy.

5. Conclusion

Our results indicate that mice with BLM-induced scleroderma had increased proportions of Teff cells (Th1, Th2, Th17, and Tfh cells), and decreased proportions of Treg cells. MET ameliorated the effects of BLM by reducing skin inflammation and fibrosis, restoring the balance of Treg and Teff cells, and reducing spleen GC formation. These results provide new insights into the immunological abnormalities present in scleroderma, and suggest that MET might be a potential candidate for the therapeutic modulation of scleroderma.

Authors' contributions

Jing Luo and Hui Guo contributed to the conception and design of the study. Shulan Zhang and Zhaojun Liang performed the immunohistochemistry and hydroxyproline assays, and analyzed the data. Xiangcong Zhao performed the flow cytometry analysis. Min Feng contributed to the interpretation and acquisition of data. Yanlin Wang drafted the manuscript. Chong Gao, Kaili Qin and Xiaofeng Li revised the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this manuscript.

Acknowledgments

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