

Regular Article

Metformin Suppresses LPS-Induced Inflammatory Responses in Macrophage and Ameliorates Allergic Contact Dermatitis in Mice *via* Autophagy

Mengjie Wang, Shulan Qu, Jun Ma, Xiaoyu Wang,* and Yifu Yang*

Experiment Center for Science and Technology, Shanghai University of Traditional Chinese Medicine; Shanghai 201203, China.

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Allergic contact dermatitis (ACD) is one of the most common skin diseases caused by hapten-modified proteins. Metformin, a drug commonly prescribed for type II diabetes, has been demonstrated to have various biological functions beyond its antidiabetic effects. However, its role in ACD remains unknown. In the present study, we found that metformin reduced the production of nitric oxide (NO) and the level of proinflammatory cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and IL-6 in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. These anti-inflammatory effects were also demonstrated on bone marrow-derived macrophages (BMDMs). Furthermore, metformin also enhanced autophagic flux, inhibited the phosphorylation of the serine/threonine protein kinase (AKT)/mammalian target of rapamycin (mTOR), mitogen-activated protein kinases (MAPKs) related protein levels and the level of miR-221 in LPS-stimulated RAW264.7 cells. Besides, metformin attenuated 2,4-dinitrofluorobenzene (DNFB)-induced ACD and inhibited proinflammatory cytokines in the ear. In addition, metformin ameliorated ACD partly through the inhibition of macrophage activation and the induction of autophagic flux. Taken together, our data indicated that metformin ameliorates ACD through enhanced autophagic flux to inhibit macrophage activation and provides a potential contribution to ACD treatment.

Key words metformin; allergic contact dermatitis; autophagy; macrophage; inflammation

INTRODUCTION

Allergic contact dermatitis (ACD), one of the most common skin diseases caused by hapten-modified proteins, has been defined as a cell-mediated type IV hypersensitivity reaction in which many cell types are involved.¹⁾ The strong skin sensitizer 2,4-dinitrofluorobenzene (DNFB) is known to provoke ACD mediated by CD8⁺ T cell and mast cell after repeated topical application, also brings about the change of the cutaneous cytokine circumstance from T-helper 1 (Th1) to Th2.^{2,3)} Macrophages also play important roles in the mouse dermis.^{4,5)} Suzuki *et al.* investigated macrophages recruitment in skin lesions of DNFB-induced ACD.⁶⁾ The production of inflammatory cytokines and chemokines in macrophages is involved in ACD reactions.⁷⁾ Corticosteroids are effective and powerful agents for ACD, but their doses should be restricted because of their adverse side effects.

Autophagy is a cellular degradation process in which cellular proteins and organelles are engulfed by double-membrane autophagosomes and degraded in lysosomes. The dysregulation of autophagy has been shown in the pathogenesis of various disease processes such as inflammation and tumor development. Transcriptome analyses data identified the up-regulation of autophagy-related genes was observed in skin of ACD patients, suggesting that autophagy is enhanced in ACD.⁸⁾ The autophagy-dependent anti-inflammatory is very important in suppressing activated T cells and ACD mice.⁹⁾ Recently, in a double-blind trial, some researchers found that a newly-synthesized peptide, which enhances the autophagy activity, can ameliorate ACD by restoring skin barrier and managing inflammation.¹⁰⁾ Therefore, regulation of autophagy

could be an attractive option in the treatment of ACD.

Metformin, an insulin-sensitizing biguanide, is used as an antidiabetic medication for the treatment of cardiovascular complications associated with type 2 diabetes. Recent reports have shown that metformin has various biological functions other than its anti-diabetic effects. It has been proposed that metformin exerts anti-cancer effects through the activation of AMP-activated protein kinase (AMPK). Furthermore, metformin may have anti-inflammatory and anti-oxidant effects.¹¹⁾ Metformin also impaired autophagic flux in rheumatoid arthritis and alleviated hepatosteatosis by restoring autophagy induction,^{12,13)} suggesting that metformin acts as an autophagy modulator. In the present study, we investigated the effect of metformin on macrophages and ACD, and its functional mechanism *in vitro* and *in vivo*.

MATERIALS AND METHODS

Reagents Metformin was purchased from Selleckchem (Houston, U.S.A.). Lipopolysaccharide (LPS) and antibodies against microtubule-associated protein light chain 3 (LC3) B (#L7543) were purchased from Sigma (St. Louis, U.S.A.). DNFB was purchased from Sinopharm (Beijing, China). Antibodies against extracellular signal-regulated kinase (ERK) (clone 137F5, #4695), p-ERK (clone 20G11, #4376), c-Jun N-terminal kinase (JNK) (#9252), p-JNK (clone 81E11, #4668), p38 mitogen-activated protein (MAP) kinase (p38) (clone D13E1, #8690), p-p38 (clone D3F9, #4511), the serine/threonine protein kinase (AKT) (#9272), p-AKT (clone C31E5E, #2965), mammalian target of rapamycin (mTOR) (clone 7C10, #2983), p-mTOR (clone D9C2, #5536), sequestosome 1

*To whom correspondence should be addressed. e-mail: 0000002733@shutcm.edu.cn; yangyifu@mail.shcnc.ac.cn

(SQSTM1) (clone D5E2, #8025), autophagy protein 5 (ATG5) (clone D5F5U, #12994) and α -tubulin (clone 11H10, #2125) were purchased from Cell Signaling Technology (Beverly, U.S.A.). Anti-F4/80 antibody was purchased from eBioscience (San Diego, CA, U.S.A.). mCherry-enhanced green fluorescent protein (eGFP)-LC3B adenovirus was purchased from TranSheepBio (Shanghai, China).

Animals and Induction of the ACD Model by DNFB

Male 6-week-old BALB/c mice were purchased from Shanghai Laboratory Animal Center of the Chinese Academy of Sciences and raised by the Experimental Animal Center of Shanghai University of Traditional Chinese Medicine (Shanghai, China). All animal experiments were approved by the Animal Care and Use Committee of the Shanghai University of Traditional Chinese Medicine. ACD was induced by repeated treatment with 0.5% DNFB in acetone–olive oil (4:1 (v/v)) solution to each hindfoot on day 0, and on the next day, 20 μ L of the DNFB solution was topically administered to each hindfoot. Five days after sensitization, mice were challenged by 0.2% DNFB (20 μ L) topically administered to the left ear. The right ear was treated with acetone/olive oil in the absence of DNFB. Metformin (100 mg/kg, 250 mg/kg) and the positive drug methotrexate (1 mg/kg) were orally administered once a day for 7 d. The mice were sacrificed at day 7, the ears were weighed and the *D*-value for the two ears was calculated, where *D*-value = the weight of ear (left) – the weight of ear (right).

Cell Culture RAW264.7 cells were purchased from Shanghai Institutes for Biological Sciences (Shanghai, China) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (P/S) in a constant temperature incubator with 5% CO₂ at 37°C. Bone marrow-derived macrophages (BMDMs) were generated from fresh bone marrow cells and maintained in RPMI1640 containing 10% FBS, P/S and 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) in a constant temperature incubator with 5% CO₂ at 37°C for 7 d. GM-CSF had to be added to the cells every 2 d during the differentiation process.

Transfection RAW264.7 cells were seeded on coverslips in 6-well plates and transfected with mCherry-eGFP-LC3B adenovirus following the manufacturer's standard protocols. Briefly, cells at 60–70% confluence were transduced overnight. Twenty-four hours after transfection, the cells were treated with metformin (320 μ M) for 2 h prior to LPS (1 μ g/mL) stimulation for 24 h. The eGFP and mCherry fluorescence were measured under Leica SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany). Red (mCherry) fluorescence indicated autolysosomes and yellow fluorescence, due to merging of both green and red fluorescence, indicated autophagosomes.¹⁴⁾

Nitrite Assay The amount of nitric oxide (NO) was assessed by determining the nitrite concentration in the supernatant with Griess reagent according to the manufacturer's protocol.

Immunohistochemistry The ear specimens were fixed in 10% formalin and embedded in paraffin. Tissue sections (4 μ m) were stained with hematoxylin and eosin (H&E) and observed using a microscope. For immunohistochemistry, anti-LC3B antibody was added and the samples were incubated overnight at 4°C. The slides were treated with anti-rabbit

antibody for 45 min at 37°C and developed in 0.05% freshly prepared diaminobenzidine solution for 8 min, counterstained with hematoxylin, dehydrated, and mounted on a microscope.

F4/80 Immunofluorescence The sections were treated with 10% normal goat serum for 30 min at room temperature followed by overnight incubation with the anti-F4/80 antibody at 4°C. F4/80-positive staining was visualized with diaminobenzidine as a chromogen and observed using a fluorescence microscope (Olympus, Tokyo, Japan).

Real-Time PCR Total RNA and miRNA was extracted from ear tissues and macrophages and detected using an ABI 7500 Sequencing Detection System (Applied Biosystems, Foster, U.S.A.) as described previously.¹⁵⁾ The sequences of the primers used were as Table 1.

Western Blot Analysis Total proteins in cells and ear homogenates were extracted with RIPA lysis buffer. Western blot analysis was carried out as described previously.¹⁵⁾

Statistical Analysis All data were expressed as the mean $\pm$ standard error of the mean (S.E.M.) of at least three independent experiments. Data were performed by GraphPad Prism 6.0 and using one-way ANOVA followed by *post-hoc* Dunnett's multiple comparison test or Student's *t*-test to evaluate differences between experimental groups with SPSS 21 software, and a *p* value of 0.05 was considered to be statistically significant.

RESULTS

Metformin Inhibits Inflammatory Responses in

RAW264.7 Cells We investigated the effects of metformin in the macrophage cell line, RAW264.7. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) data showed that metformin (0–320 μ M) treatment for 24 h did not alter the RAW264.7 cell (Fig. 1A) and splenic viabilities (our unpublished data), demonstrating that metformin exhibited little cytotoxicity in the macrophages or other immunocytes. We found that metformin decreased the LPS-induced increase

Table 1. Primer Sequences for Real-Time PCR

Gene	Primer (5'–3')
TNF- α -F	CCACCACGCTCTTCTGTCTACTG
TNF- α -R	CGGCTACGGGCTTGTCACCTC
IL-6-F	CTTCCAGCCAGTTGCCTTCTTG
IL-6-R	TGGATGCTCTCATCAGGACAG
IL-1 β -F	GAAATGCCACCTTTTGACAGTG
IL-1 β -R	GGTCTGTTGTGGGTGGTATCCTC
NLRP3-F	TGCGATCAACAGGCGAGACCT
NLRP3-R	CCATCCACTCTTCTCAAGGCTGTC
ASC-F	GGAGTCGTATGGCTTGAGC
ASC-R	ACAAAGTGTCTGTTCTGGCT
IL-10-F	GCTCTTACTGACTGGCATGAG
IL-10-R	CGCAGCTCTAGGAGCATGTG
F4/80-F	GCATCACTGCCTCCACTAG
F4/80-R	ATACCCTCCAGCACATCC
MCP1-F	GTGCTTGAGGTGGTTGTG
MCP1-R	AGGTCCCTGTCATGCTTC
GAPDH-F	CAAGGTCATCCATGACAACCTTG
GAPDH-R	GTCCACCACCCTGTTGCTGTAG
miR-221	CAGCTACATTGTCTGCTGGG
U6	GCTTCGGCAGCACATATACTA

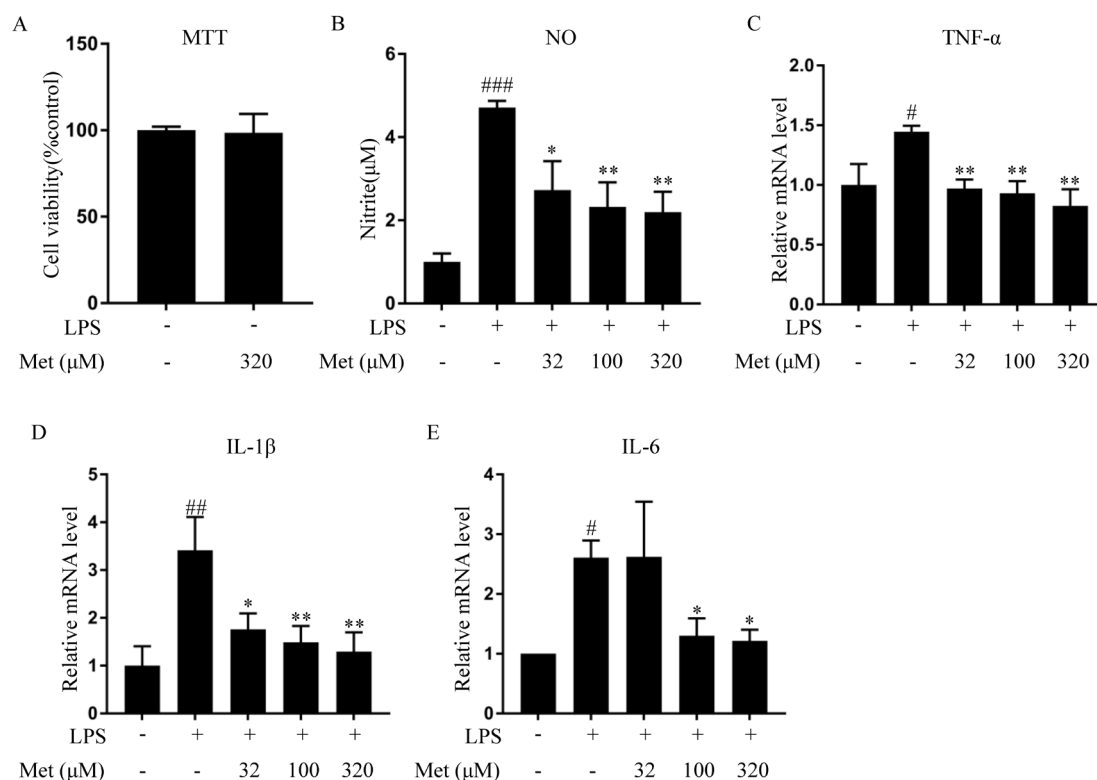


Fig. 1. Metformin Inhibits the LPS-Induced Inflammatory Responses in RAW264.7 Cells

(A) MTT assay was performed to detect the cell viability of RAW264.7 cells treated with metformin. RAW264.7 cells were treated with or without metformin (32, 100, 320 μM) for 2 h prior to LPS (1 μg/mL) stimulation for 24 h; (B) The NO release in the culture media was assayed by Griess reagent. mRNA levels of TNF-α (C), IL-1β (D), and IL-6 (E) were analyzed by real-time PCR. The data are presented at least three independent experiments. The data are presented as the mean ± S.E.M., # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. normal group; * $p < 0.05$, ** $p < 0.01$ vs. control group.

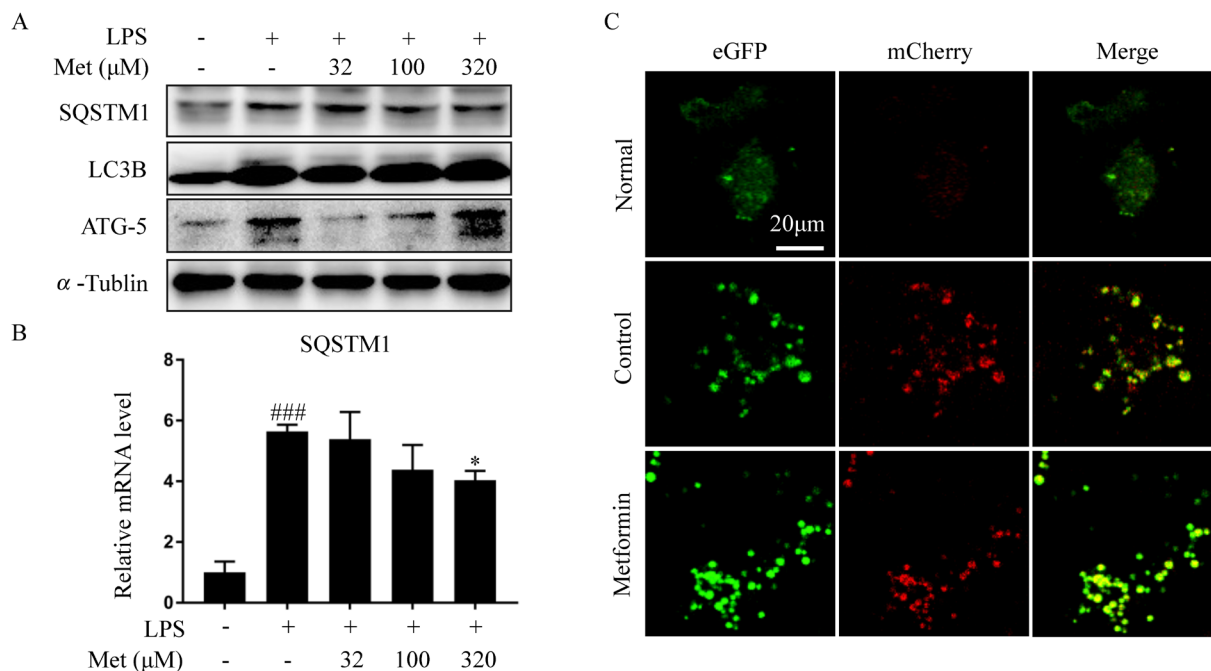


Fig. 2. Metformin Enhances Autophagy in RAW264.7 Cells

RAW264.7 cells were treated with or without metformin (32, 100, and 320 μM) for 2 h prior to LPS (1 μg/mL) stimulation for 24 h; (A) The proteins were analyzed by Western blotting for the expression of ATG5, LC3B and SQSTM1, and α-tubulin was used as a loading control. (B) The mRNA levels of SQSTM1 were analyzed by real-time PCR. (C) RAW264.7 cells were transiently transfected with mCherry-eGFP-LC3B and cultured for 24 h; then, the cells were treated with or without metformin (320 μM) for 2 h prior to LPS stimulation for 24 h. The colocalization of eGFP and mCherry signals was analyzed. Scale bar, 20 μm. The data are presented at least three independent experiments. The data are presented as the mean ± S.E.M., ### $p < 0.001$ vs. normal group; * $p < 0.05$ vs. control group. (Color figure can be accessed in the online version.).

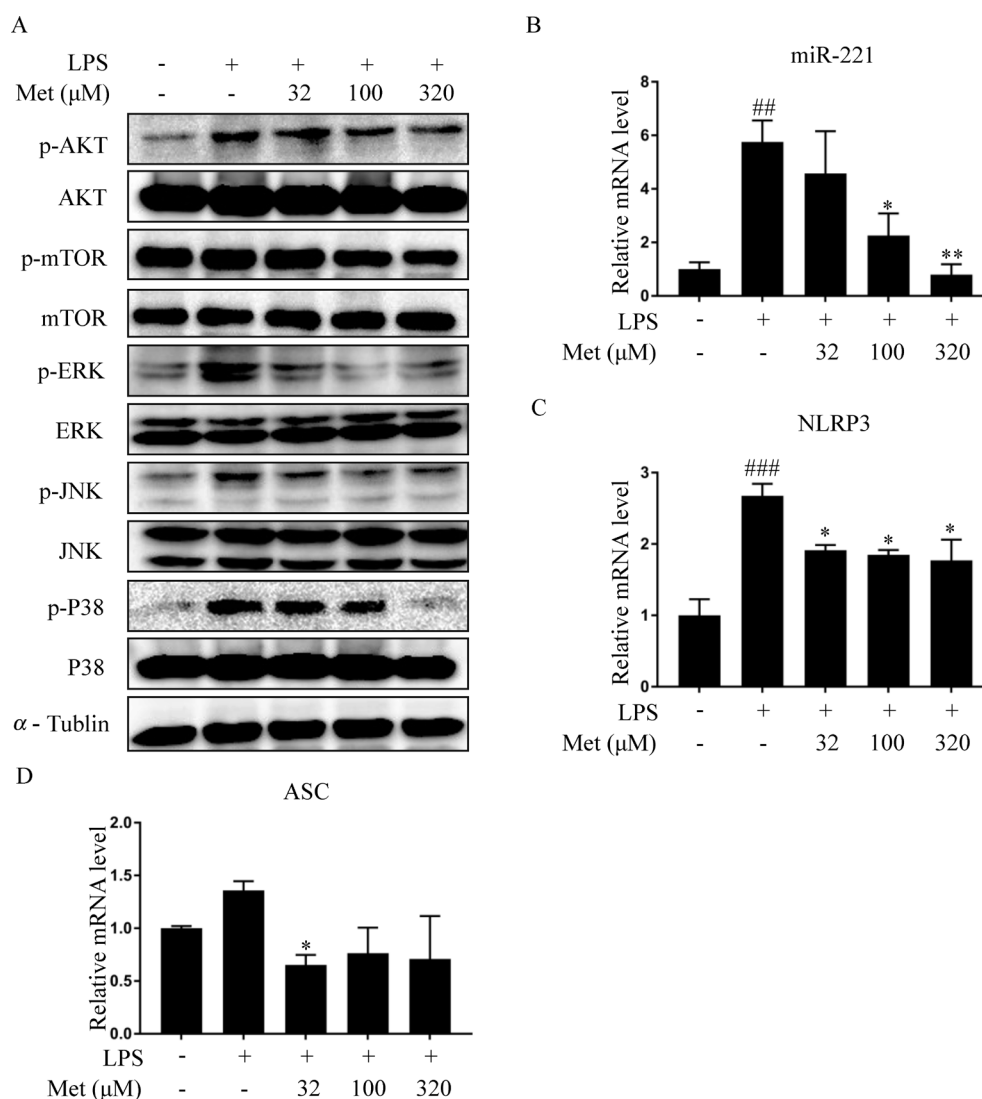


Fig. 3. Metformin Inhibits the AKT/mTOR, MAPK Pathways in RAW264.7 Cells

RAW264.7 cells were treated with or without metformin (32, 100, and 320 μM) for 2 h prior to LPS (1 μg/mL) stimulation for 24 h; (A) The proteins were analyzed by Western blotting for the phosphorylation of AKT, mTOR, ERK, JNK, and p38. The relative levels of miR-221 (B) and mRNA levels of NLRP3 (C), ASC (D) were analyzed by real-time PCR. The data are presented at least three independent experiments. The data are presented as the mean ± S.E.M., ^{###} $p < 0.001$ vs. normal group, ^{***} $p < 0.001$; ^{*} $p < 0.05$, ^{**} $p < 0.01$ vs. control group.

of NO in a dose-dependent manner (Fig. 1B). In addition, the increased mRNA levels of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 in LPS-induced RAW264.7 cell were significantly reduced by metformin treatment for 24 h (Figs. 1C–E), suggesting that metformin can inhibit inflammatory responses of macrophage.

Metformin Enhances Autophagy in RAW264.7 Cells

When autophagy is induced, a cytosolic form of LC3-I is converted to LC3-II which is recruited to autophagosomal membranes. Thus the amount of LC3-II positively correlates with the number of autophagosomes.¹⁶ Both suppression and induction of autolysosomal maturation result in increased numbers of autophagosomes. SQSTM1 serves as a link between LC3 and ubiquitinated substrates and is considered a marker of autophagic flux.¹⁶ Western blot analysis showed that metformin treatment resulted in the dose-dependent accumulation of LC3 II in RAW264.7 cells (Fig. 2A), suggesting that metformin enhanced the conversion of LC3 I to LC3 II. Consistent with this, the expression of ATG5, which is essential for autophagosome formation, was increased in the dose-dependent manner

after metformin treatment. Furthermore, the expression of SQSTM1 was increased after LPS induction, implying that the initial accumulation of LC3 II after LPS induction likely reflected a decrease in autophagic flux. In contrast, metformin treatment attenuated the accumulation of SQSTM1. Consistent with this, a remarkable decrease in SQSTM1 mRNA levels was also detected after metformin treatment of RAW264.7 cells (Fig. 2B), suggesting that metformin may enhance autophagic flux to inhibit the activation of RAW264.7 cells. In addition, to confirm these results we examined the localization of autophagosome-specific protein LC3 in RAW264.7 cells treated with metformin for 24 h using mCherry-eGFP-LC3B adenovirus. As shown in Fig. 2C, LPS-induced cells exhibit more double-tagged fusion proteins and red dots than normal cells, however, metformin-treated only increased yellow LC3 puncta than LPS-induced cells indicating metformin induced the formation of autophagosome rather than autolysosomes.

Metformin Inhibits the AKT/mTOR, Mitogen-Activated Protein Kinase (MAPK) and NOD-Like Receptor Protein 3 (NLRP3) Pathways in RAW264.7 Cells It has been re-

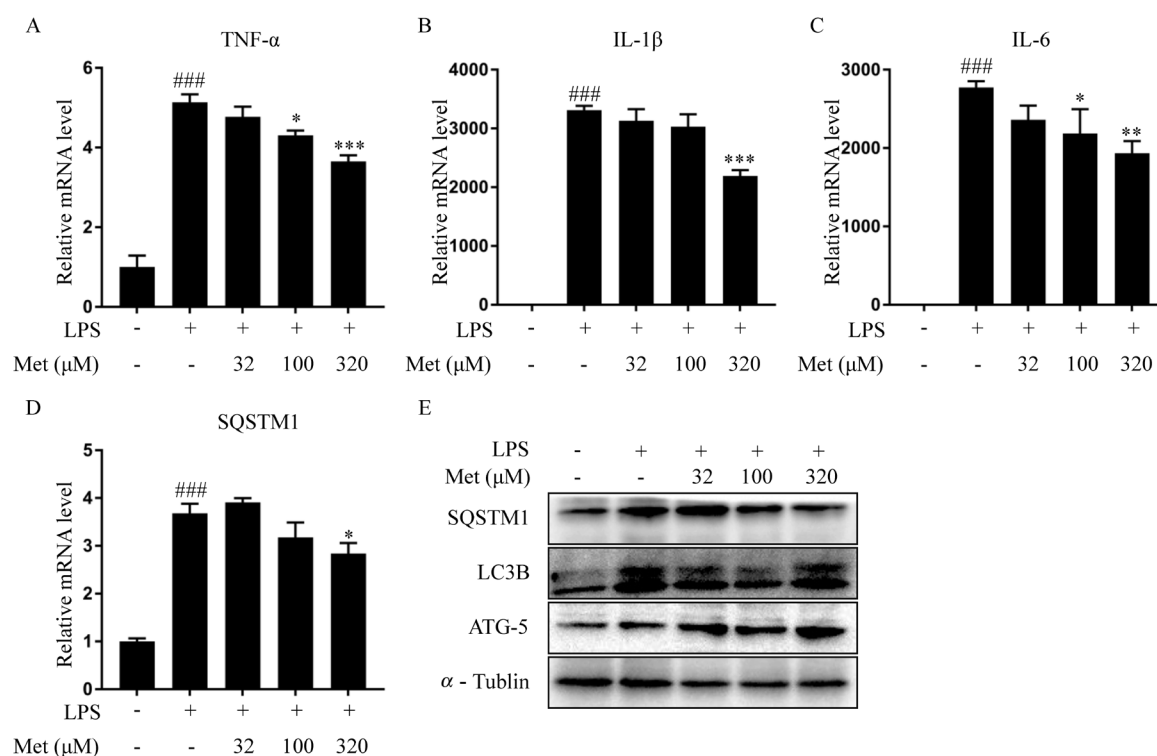


Fig. 4. Metformin Inhibits LPS-Induced Inflammatory Responses in BMDMs

BMDMs were treated with or without metformin (32, 100, and 320 μM) for 2 h prior to LPS (1 μg/mL) stimulation for 24 h, and the mRNA levels of TNF-α (A), IL-1β (B), IL-6 (C), and SQSTM1 (D) were then analyzed by real-time PCR. (E) The proteins were analyzed by Western blotting for the expression of ATG5, LC3B and SQSTM1, and α-tubulin was used as a loading control. The data are presented as the mean ± S.E.M., ###*p* < 0.001 vs. normal group; **p* < 0.05, ***p* < 0.01, ****p* < 0.001 vs. control group.

ported that inhibition of AKT/mTOR signaling pathway stimulates autophagy.¹⁷⁾ As shown in Fig. 3A, metformin inhibited the phosphorylation of AKT and mTOR, suggesting that metformin suppressed macrophage activation partly through the AKT/mTOR signaling pathway. We found that metformin could inhibit the activation of the MAPKs pathways by inhibiting the phosphorylation of the ERK1/2, JNK, and p38 MAPKs (Fig. 3A). Previous study revealed that miR-221 expression plays a significant role in the MAPK and mTOR signaling pathways.^{18,19)} Therefore, we evaluated the expression of miR-221 after metformin treatment. As shown in Fig. 3B, LPS stimulation markedly increased the miR-221 level, and metformin inhibited miR-221 in a dose-dependent manner suggesting that metformin can reduce inflammation *via* the AKT/mTOR and MAPK pathways in RAW264.7 cells. In addition, NLRP3 inflammasome activity is negatively regulated by autophagy.²⁰⁾ As shown in Figs. 3C–D, metformin significantly reduced the mRNA levels of NLRP3 and apoptosis associated speck-like protein (ASC) in LPS induced RAW264.7 cells, but it could not alter the expression of Caspase-1 and IL-18 (data not shown).

Metformin Inhibits Inflammatory Responses in BMDMs

Because the RAW264.7 cell line is from murine leukemia, we investigated the effects of metformin on primary differentiated BMDMs. Flow cytometry analysis illustrates that the purity of the GM-CSF-induced bone marrow-isolated cells at day 7 reached 97.81% using the macrophage markers CD11b and F4/80 (data not shown). We found that LPS induced the increase in cytokines (TNF-α, IL-1β, IL-6) in BMDMs, and metformin inhibited the mRNA levels of these cytokines (Figs. 4A–C). In addition, as shown in Fig. 4D, metformin

inhibited the mRNA level of SQSTM1. Western blot analysis showed that metformin treatment resulted in the accumulation of LC3-II and ATG5, and attenuated the accumulation of SQSTM1 in BMDMs (Fig. 4E), suggesting that metformin may enhance autophagic flux in BMDMs, which is consistent with the results in the RAW264.7 cells.

Metformin Ameliorates DNFB-Induced ACD To explore the potential therapeutic effect of metformin on ACD *in vivo*, we employed a mouse model of ACD by DNFB. We also compared the effects of metformin and methotrexate, an immune system suppressant which has been proven to be effective in the treatment of psoriasis, ACD, and other T cell-mediated skin diseases.²¹⁾ At the end of the experiment, the weights of both ears were evaluated. In the control group, repeated treatment with DNFB obviously elevated ear weight compared with that in the normal group. Metformin (100 and 250 mg/kg) treatment effectively inhibited ear weight gain (Fig. 5A), and the effect of the 250 mg/kg dose is similar to that of methotrexate (1 mg/kg). Metformin and methotrexate did not alter the body weight of the mouse, suggesting that they experienced few side effects. Furthermore, we found that the mRNA levels of TNF-α, IL-1β, and IL-6 (Figs. 5B–D) were significantly increased in the ears of ACD mice; however, metformin and methotrexate inhibited the mRNA levels of these cytokines, suggesting that metformin has an anti-inflammatory effect. Nevertheless, metformin did not inhibit cyclooxygenase 2 expression (data not shown). As shown in Fig. 5E, the ears of control mice showed severe inflammatory cell infiltration, epidermal hyperkeratosis, and marked edema in the dermis and subcutaneous tissue, which were largely mitigated by metformin.

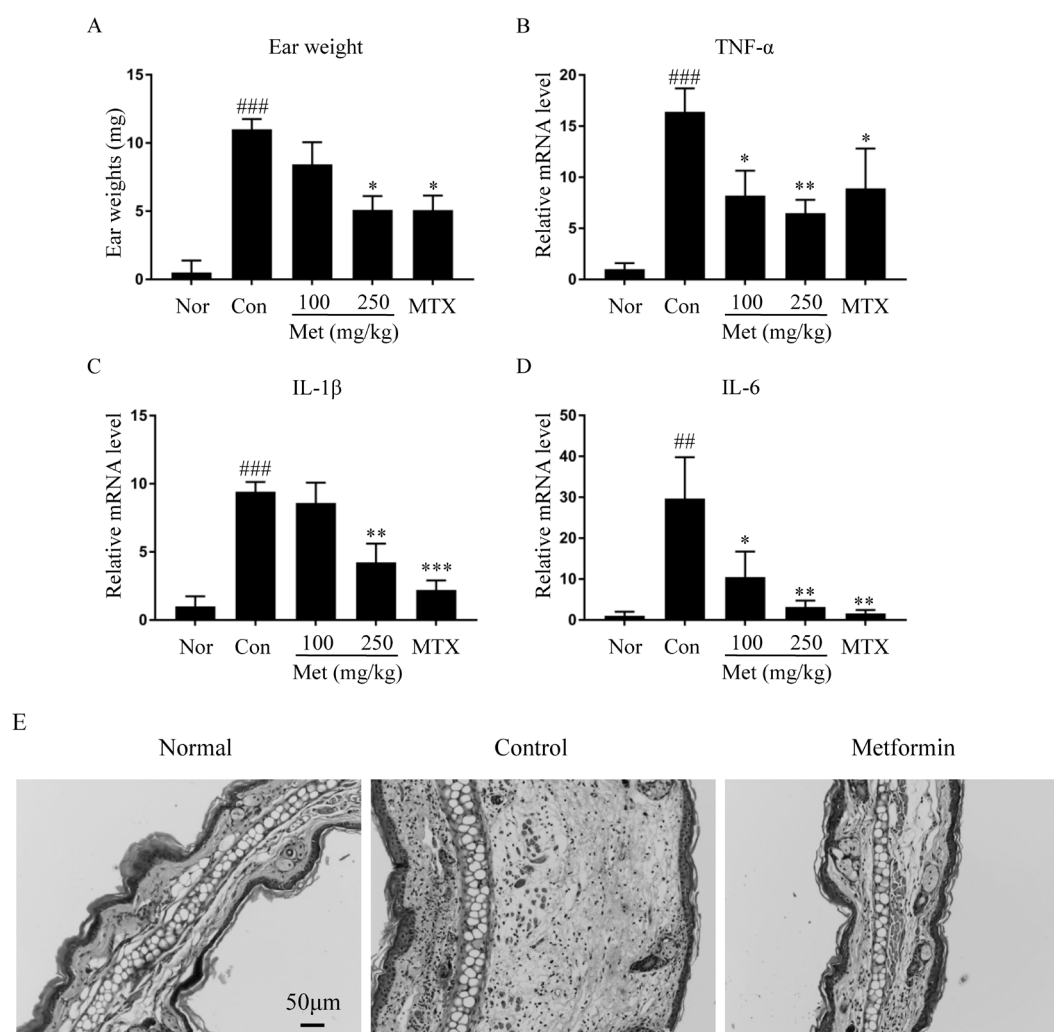


Fig. 5. Metformin Ameliorates DNFB-Induced ACD

(A) The ear weight gains of the normal, control, metformin (100 mg/kg, 250 mg/kg) and methotrexate (MTX, 1 mg/kg) groups were detected. Total RNA was collected from the ear tissues and assayed for the mRNA levels of TNF- α (B), IL-1 β (C), and IL-6 (D) by real-time PCR, $n = 8$. (E) The ear tissues of the normal, control, and metformin (250 mg/kg) treatment groups were stained with H&E. Scale bar, 50 μ m. The data from at least three independent experiments are presented. The data are presented as the mean $\pm$ S.E.M., ^{##} $p < 0.01$, ^{###} $p < 0.001$ vs. normal group, ^{*} $p < 0.05$, ^{**} $p < 0.01$, ^{***} $p < 0.001$ vs. control group.

Metformin Inhibits Macrophage Activation in the Ears of ACD Mice We investigated the whole blood of mice using an automatic blood cell counter and found that metformin did not significantly alter the number of white blood cells, lymphocytes, or neutrophils in ACD mice (data not shown). Macrophages play important roles in ACD. We also found that the macrophage marker F4/80 was increased in the ears of control mice, indicating that macrophages infiltration in the ACD model; however, metformin treatment decreased the numbers of macrophages in the ears of ACD mice (Figs. 6A, B). In addition, metformin treatment significantly decreased the mRNA level of monocyte chemoattractant protein (MCP-1) (Fig. 6C). As shown in Fig. 6D, the mRNA of IL-10 was expressed at low levels in the DNFB-induced ACD mice; however, metformin reversed this effect, suggesting that metformin ameliorated ACD partly through the inhibition of macrophage activation.

Metformin Enhances Autophagy in the Ears of ACD Mice Interestingly, we found by immunohistochemistry staining that the ears of ACD mice showed more LC3 expression than the ears of normal mice; however, metformin further enhanced the abundance of LC3 (Fig. 6E), indicating that

metformin may alter autophagy. Western blotting data showed that metformin treatment promoted the expression of LC3 II and ATG5, decreased the expression of SQSTM1 in the ears of ACD mice (Fig. 6F), suggesting that metformin may enhance autophagic flux in the ears of ACD mice. Furthermore, metformin treatment decreased the phosphorylation of AKT and mTOR (Fig. 6F). We also found that the oral administration of metformin significantly inhibited the increase in the level of miR-221 in the ears of ACD mice (Fig. 6G), which is consistent with the *in vitro* data. The data suggest that metformin inhibits ACD through autophagy.

DISCUSSION

Metformin, a classic oral hypoglycemic agent, has been reported to alleviate various diseases beyond its hypoglycemic effects, including LPS-induced sepsis²²⁾ and hepatic steatosis,¹³⁾ which implies that metformin has anti-inflammatory effects. However, the protective effects of metformin in ACD remain unknown. From 1985, some researchers reported that ACD is caused by medical devices (such as isobornyl acrylate) for diabetes patients.²³⁾ It is well established that macrophage

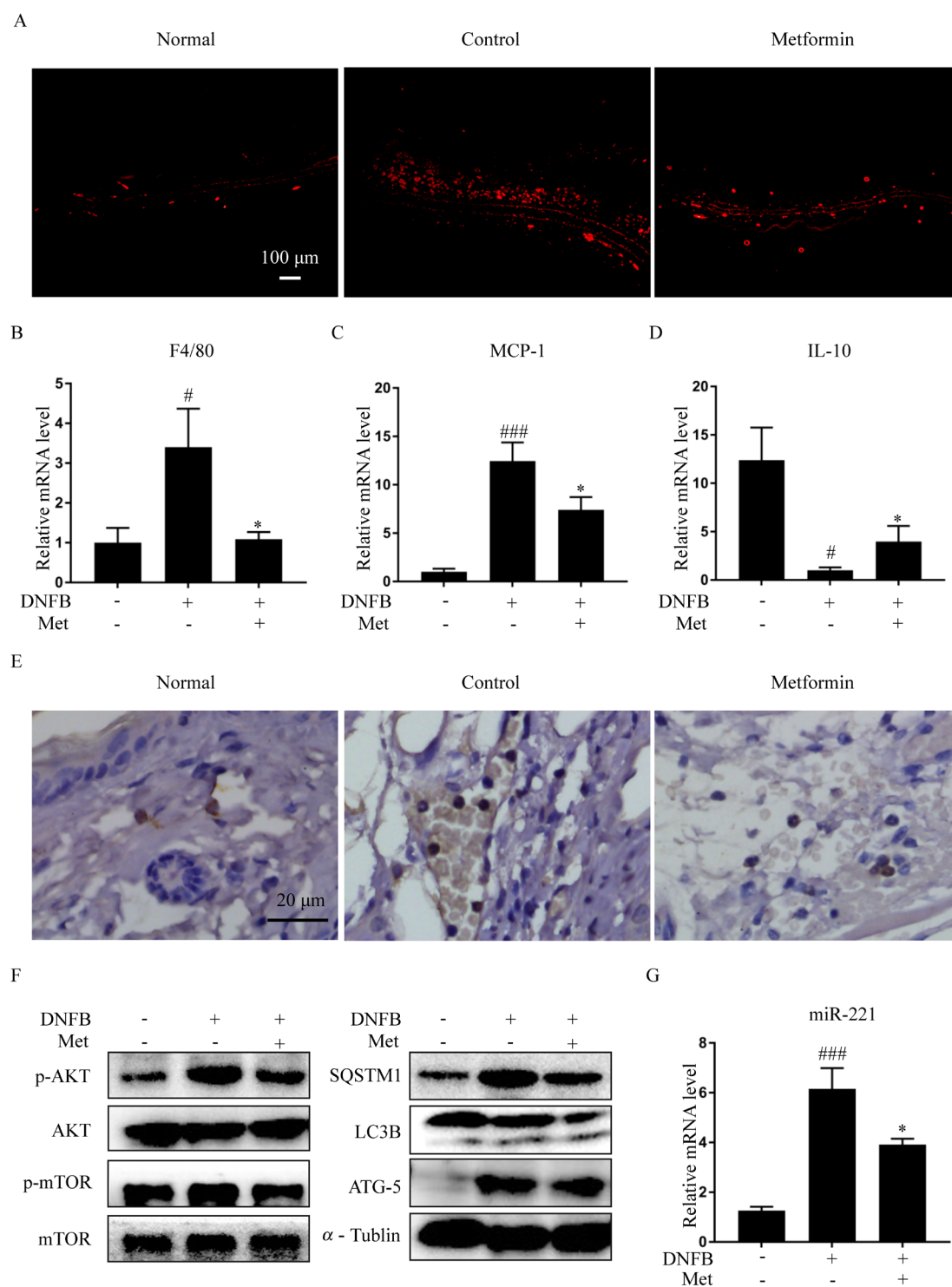


Fig. 6. Metformin Inhibits Macrophage Activation and Enhances Autophagy in the Ears of ACD Mice

(A) The ears of the normal, control, and metformin (250mg/kg) treatment groups were stained with anti-F4/80 antibody. Scale bar, 100 μ m. Total RNA was collected from the ear tissues, and assayed for the mRNA level of F4/80 (B), MCP-1 (C) and IL-10 (D) by real-time PCR, $n=8$. (E) The ear tissues were stained with anti-LC3B for immunohistochemical analysis. Scale bar, 20 μ m. (F) Total proteins in ear tissues were analyzed by Western blotting for the phosphorylation of AKT and mTOR and the expression of ATG5, LC3B and SQSTM1. (G) The relative levels of miR-221 were analyzed by real-time PCR, $n=8$. The data from at least three independent experiments are presented. The data are presented as the mean $\pm$ S.E.M., [#] $p<0.05$, ^{###} $p<0.001$ vs. normal group; ^{*} $p<0.05$, vs. control group. (Color figure can be accessed in the online version.).

plays a critical role in dermatitis.^{4,5)} Therefore, we investigated the effects of metformin on the macrophage cell line RAW264.7 and primary differentiated BMDMs. Our data indicated that metformin inhibited the inflammatory responses of LPS-induced macrophages, which is consistent with studies by Tsoyi *et al.*²²⁾ and Kim *et al.*²⁴⁾ We demonstrated the ben-

eficial effects of metformin in ACD using a DNFB-induced animal model. Our results showed that, similar to the positive drug methotrexate, metformin significantly ameliorated DNFB-induced ear injury, suggesting that metformin has an anti-inflammatory effect on ACD. Moreover, we found that metformin decreased the number of F4/80 positive macro-

phages in the ears, decreased the mRNA level of MCP-1 and increased the mRNA level of IL-10, suggesting that metformin ameliorated ACD partly through the inhibition of macrophage activation.

Autophagy serves as a cellular self-defense mechanism to not only prevent the toxic accumulation of damaged or unnecessary components that influence cell growth and development but also sustain metabolic homeostasis.²⁵⁾ It has been reported that autophagy can directly regulate inflammatory responses.²⁶⁾ Because recent reports suggest that metformin inhibits cancers and rheumatoid arthritis by regulating autophagy,¹²⁾ we investigated the expression of LC3 in the ear tissues of ACD mice following metformin treatment. We found that metformin increased the expression of LC3 II and ATG5, and decreased the accumulation of SQSTM1 in LPS-induced macrophages, indicating that metformin may inhibit activation of macrophage through autophagy. We also found for the first time that metformin increased the abundance of LC3 II in the ear of control group. The recruitment of LC3 to the autophagosome membrane in an ATG5-dependent manner and the formation of autophagosome accelerates autophagic flux result in degradation of SQSTM1,²⁷⁾ thus the increase of SQSTM1 suggests defective autophagy. Our results showed that metformin treatment not only increased the expression of LC3 II and ATG5 but also decreased the expression of SQSTM1, suggesting that metformin may enhance autophagic flux in the ears of ACD mice. We also found that metformin slightly inhibited miR-30a, which negatively regulate autophagic activity,²⁸⁾ in LPS-induced RAW264.7 and in the ears of ACD mice (data now shown). All of our data demonstrated that metformin ameliorated ACD through enhanced autophagic flux to inhibit the activation of macrophages.

It has been investigated that autophagosomes can recruit and degrade NLRP3 inflammasome, which contributes to eliminating subsequent expression of IL-1 β .²⁰⁾ We found metformin could reduce NLRP3 and ASC expressions in RAW264.7 cells, indicating that metformin attenuate LPS-induced inflammasome activation to inhibit inflammatory factors expression by autophagy induction in macrophages. The AKT/mTOR signaling axis is a crucial pathway to regulate the formation of autophagosomes, and MAPK pathways are also related to LPS-induced inflammation and autophagy.^{17,29)} We found that metformin inhibited the activation of AKT and mTOR which negatively regulated autophagic flux, and inhibited the expression of miR-221 which plays a significant role in the MAPK and mTOR signaling pathway, indicating that metformin enhanced autophagic flux *via* AKT/mTOR and MAPK signaling to inhibit the activation and inflammatory responses of macrophages.

In addition to macrophages, immune cells such as mast cells, B cells, neutrophils, NK cells and T cells are also important in the early initiation phase of ACD.³⁰⁾ In this study, excessive activation of autophagy to inhibit the activation of macrophages might not be the only effector mechanism of metformin to inhibit inflammatory responses *in vivo*. As shown in Fig. 5C, metformin inhibited the level of miR-221, which is involved in the activation of mast cells,³¹⁾ suggesting that metformin also alters mast cells. Furthermore, toluidine blue staining showed that metformin decreased the number of mast cells in the ears of ACD mice (our unpublished data). Therefore, we will next investigate the effect of metformin on

mast cells in ACD.

In summary, the present study provided evidence that metformin could suppress LPS-induced inflammatory responses of macrophage and ameliorate DNFB-induced ACD, thus indicating the potential of metformin as a novel therapeutic drug for ACD patients. Furthermore, the underlying mechanism of metformin ameliorates ACD may be closely related to autophagy. Therefore, autophagy may be an attractive target for novel drugs to regulate ACD and related hypersensitivity diseases.

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Conflict of Interest The authors declare no conflict of interest.

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