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Original Article

Metformin mitigates autoimmune insulitis by inhibiting Th1 and Th17 responses while promoting Treg production

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Abstract: Type 1 diabetes mellitus (T1DM) is still one of the major threats on global public health. This autoimmune condition is mainly caused by the imbalance of auto-reactive inflammatory effector T cells (Teffs) and protective regulatory T cells (Tregs). Therefore, inhibiting the development of Teffs and/or promoting Tregs provides a therapeutic strategy for preventing the development of T1DM. Pathways of energy metabolism have been shown to play a pivotal role in dictating the activation, differentiation and immune function of T cells. Studies have shown that inhibition of glycolysis suppresses the development of Th1 and Th17 cells, but promotes Treg production. AMP-activated protein kinase (AMPK) is a master sensor and regulator of cellular energy metabolism in mammals, which has also been demonstrated to interfere with T cell differentiation and effector function through inhibiting mammalian target of rapamycin (mTOR) and subsequent inhibition of glycolysis, and enhancement of lipid oxidation. In this study, we found that AMPK activator metformin suppresses T cell proliferation and inhibits the differentiation of Th1 and Th17 cells while promoting the development of Tregs in vitro in a dose-dependent manner. Treatment of NOD mice with metformin significantly mitigated autoimmune insulitis and substantially decreased the number of pro-inflammatory IFN-γ+ as well as IL17+ CD4 T cells in the spleens of NOD mice. However, a significantly increased percentage of regulatory IL-10+ and Foxp3+ CD4 T cells were seen. We provided a novel potential therapeutic method--by regulating T cell metabolism through targeting AMPK, to reduce the severity of autoimmune insulitis.

Keywords: T1DM, insulitis, NOD, T cell metabolism, AMPK, metformin

Introduction

Epidemiological studies have demonstrated that type 1 diabetes mellitus (T1DM) is still one of the major threats in global public health. Children under 5 years of age are at greatest risk of this condition and represent the population with greatest incidence [1]. Evidence from research has shown T1DM is a chronic disease caused by autoimmune destruction of functional insulin-producing β cells of pancreatic islets. In T1DM, β-cells are progressively destroyed by islet-infiltrating auto-reactive T cells, which cause pathogenic inflammatory immune response (insulitis) [2]. Non-obese diabetic (NOD) mouse, which spontaneously develop destructive autoimmune insulitis and progresses to overt diabetes in the adult, can mimic the human T1DM [3, 4].

During the onset of T1DM, CD4+ T cells in the draining lymph nodes proliferate and differentiate into autoreactive effector T cells (Teffs) after being presented β-cell antigen from antigen presenting cells (APCs). These Teffs further migrate into pancreatic islets and release inflammatory cytokines such as interferon (IFN)-γ and interleukin (IL)-2, resulting in recruitment of

cytotoxic CD8+ T cells and ultimately causing insulitis through perforin/granzyme-mediated toxicity [5]. Furthermore, IL-17-producing CD4 T cells (Th17), another population of Teffs that are able to induce potent inflammatory responses, have been suggested to be involved in the pathogenesis of autoimmune diabetes [6, 7]. On the other hand, regulatory T cells play an essential role in the maintenance of immune periphery tolerance regulating inflammatory responses and preventing the development of autoimmune diseases [8, 9]. Tregs confer protective effects during the development of T1DM [10], and adoptive transfer of in vitro expanded antigen-specific Tregs can eliminate signs of diabetes even after disease onset [11].

In the periphery, immune tolerance is achieved by a fine balance between effector and regulatory T cells. T1DM is an autoimmune disease caused by the imbalance between Teff and Tregs. Therefore, inhibiting the development of Teff and/or promoting Tregs provides a therapeutic strategy for preventing T1DM. The generation of different subsets of Teffs and Tregs are mainly determined by the environmental cues, such as cytokine milieu and expression of co-stimulatory molecules. Apart from that, more recently, energy metabolism pathways have been also shown to play a pivotal role in the activation, differentiation and immune function of T cells [12-14]. For example, Th1 and Th17 cells were found to rely on glycolysis to develop and maintain their own survival as well as functional characteristics. Alternatively, Tregs appear to be dependent on lipid oxidation. During naïve T cell differentiation into different effector cell subsets in various local cytokine milieu, inhibition of glycolysis dampers the development of Th1 and Th17 cells, but promotes Treg production [15, 16].

AMP-activated protein kinase (AMPK) is a master sensor and regulator of cellular energy metabolism in mammals, which is activated when the ratio of AMP/ATP increases due to a shortage of cellular nutrients or to be under other physiological stress. The activation of AMPK accordingly restores the energy balance by inhibiting anabolism that consumes energy while stimulating catabolism that produces energy [17]. Irrespective of its role in cellular energy metabolism, recent studies demonstrate that AMPK activation also interferes with T cell activation, differentiation and effector function by controlling T cell 'metabolic plasticity', primarily by inhibiting mammalian target of rapamycin (mTOR) and subsequently inhibiting glycolysis and enhancing lipid oxidation [18, 19].

Thus, strategies designed to activate AMPK to inhibit glycolysis during the development of T1DM to prevent naïve T cells from differentiating into Teffs and/or promoting them to become regulatory T cells might provide an alternative path to prevent the occurrence of insulitis. Metformin is a widely used biguanide antidiabetic drug for treating type 2 diabetes mellitus. Studies have shown that it may also exert anti-inflammatory effects by activating AMPK [20]. Based on the role of AMPK signaling in T cell metabolism, the potential therapeutic role of metformin on the development of autoimmune insulitis in female NOD mice is delineated here.

Materials and methods

Cell culture

CD4+CD25- naïve T cells were purified from spleens and lymph nodes of male C57BL/6 mice using an isolation kit (Miltenyi Biotec, Germany) by negative selection. The purity of each cell preparation used in this study was of greater than 95% according to FACS analysis. T cells were activated with 5 μg/ml anti-CD3 (BioLegend, CA, USA. Clone: 145-2C11), 1 μg/ml anti-CD28 (BioLegend, CA, USA. Clone: 37.51), and 20 ng/ml human IL-2 (R&D Systems, MN, USA). For Th1 differentiation, 20 ng/ml IL-12 (R&D Systems, MN, USA) and 10 ng/ml anti-IL-4 (BioLegend, CA, USA. Clone: 11B11) were added. For Th17 differentiation, 10 ng/ml anti-IL-4 (BioLegend, CA, USA. Clone: 11B11), 10 ng/ml anti-IFN-gamma (BioLegend, CA, USA. Clone: R4-6A2), 1 ng/ml TGF-β (R&D Systems, MN, USA), 100 ng/ml IL-6 (BioLegend, CA, USA) were added into the medium. All cells were cultured for 5 days and medium was changed on the third day.

Mice

6 to 8-week old C57BL/6 male mice and 3-week old NOD/LtJ female mice were obtained from the Beijing HFK Bioscience CO., Ltd. (Beijing, China). Mice were bred and maintained under specific pathogen-free (SPF) conditions

at the Tongji Medical College Facilities for Animal Care and Housing. Cages were placed in environmentally controlled rooms (25°C; 50% to 55% humidity) under a standard 12 h dark-light cycle. All procedures involving animal use in this study were approved by the Institutional Animal Care and Use Committee of the Tongji Medical College in accordance with the National Institutes of Health (NIH) guidelines.

Drugs

The experimental group NOD mice were administered with metformin (Biovision, CA, USA) at 200 mg/kg body weight dissolved in drinking water every day starting from 3-week old until the end of the study. Water consumption measured showed that NOD mice drank water at about 30 ml/100 g of body weight everyday 24 hours. Thus, mice were supplied with 0.67 mg/ml of metformin in the drinking water.

H&E and Immunofluorescent staining

Pancreas from control and metformin-treated NOD mice were excised at 12-week of age. After fixation in 4% paraformaldehyde and paraffin embedding, tissues were cut into 4-μm sections and stained with hematoxylin and eosin (H&E) for histological analysis. For Immunofluorescent staining the paraffin-embedded sections were deparaffinized and stained with primary antibodies against CD3 (ab16669, 1:200, Abcam, Cambridge, UK) and insulin (A0564, 1:500, Dako Cytomation, Glostrup, Denmark) overnight at 4°C. After washing in PBS, sections were visualized by Alexa Fluor-conjugated secondary antibodies (ab150077, 1:300, Abcam) or Cy3-conjugated secondary antibody (ab102370, 1:300, Abcam). Nuclear counterstaining was performed using 4,6-diamidino-2-phenylindole (DAPI) and the sections were viewed with epifluorescence microscope (Nikon, Tokyo, Japan).

Insulitis score

Histological analysis was performed by using sections of H&E-stained pancreas from control and metformin-treated NOD mice at 12-week of age. A score from 0 to 3 was assigned based on islet infiltration [21]. Insulitis was analyzed by two observers and a minimum of 30 islets per animal. The pancreases were isolated from at least 4 mice per group. Insulitis score was graded as follows: grade 0, normal islets; grade 1, mild mononuclear infiltration involving <10% at the periphery; grade 2, 10-50% of the islets infiltrated; grade 3, more than 50% of the islets infiltrated. An average insulitis score was calculated by adding up the score of each islet and dividing it by the total number of islets counted.

Flow cytometry

In vitro cultured cells were obtained after application of differentiation protocol (see cell culture in methods). Single-cell suspension was prepared from spleens of NOD mice at 12-week old. To analyze the intracellular staining for IFN-γ, IL-10 and IL-17, cells were stimulated with Cell Stimulation Cocktail (eBioscience, CA, USA) containing phorbol myristate acetate, ionomycin and brefeldin A for 4 h. Cells were then fixed and permeabilized for 30 min at 4°C using BD Fixation/Permeabilization Solution kit (BD Biosciences, Jose, CA, USA) according to the provided instructions, and then incubated with antibodies specific for IFN-γ (clone XMG 1.2), IL-10 (clone JES5-16E3) and IL-17 (eBio17B7) at 4°C for 30 minutes before acquisition. For staining FOXP3 (clone FJK-16S) and ROR-γt (AFKJS-9), cells were fixed and permeabilized with Treg staining kit (eBioscience, CA, USA). All antibodies were purchased from eBioscience (San Diego, CA, USA). Flow cytometric analysis was performed with a FACS Caliber system (BD Biosciences), and data was analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Proliferation assay

Single cell suspension was obtained from mesenteric lymph node and spleen of male C57-BL/6 mice by gradient centrifugation using lymphocyte separation medium (Dakewe Biotech, Guangdong, China). Cells were set up in 96-well round-bottomed plates using standard 3-day cultures and stimulated by anti-CD3/CD28 antibody in the presence of different concentrations of metformin (Biovision, CA, USA). Cell Counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) was used for cell proliferation assays according to the manufacturer's instructions. Statistical comparisons were performed using one-way ANOVA. Data are represented as counted OD value means ± SEM.

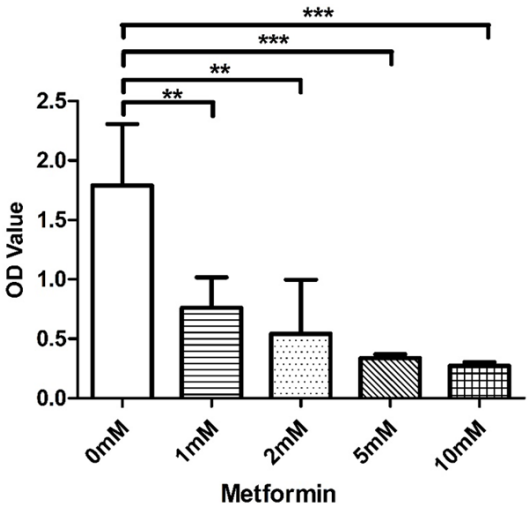

Figure 1. Metformin dose-dependently suppresses T cell proliferation in vitro. Single cell suspensions from mesenteric lymph nodes and spleens of male C57BL/6 mice were cultured using a standard 3-day culture and stimulated by anti-CD3/CD28 antibody in the presence of different concentrations of metformin. Cell Counting Kit-8 was used for cell proliferation assays according to the manufacturer's instructions. Statistical comparisons were performed using one-way ANOVA. Data are represented as counted OD value means ± SEM. (***P*<0.01, ****P*<0.001; n=4 for each group).

Western blot

Total proteins from cultured cells were prepared using a cell lysis buffer (Beyotime, Shanghai, China) with a protease inhibitor mixture (Sigma-Aldrich, MO, USA). Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay. An equal amount of protein extracts from each sample was separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene fluoride membrane (Millipore, CA, USA). Non-specific binding was then blocked in 5% (w/v) milk at room temperature. After that, the membrane was incubated with the primary antibodies against AMPKα (1:1000, Cell Signaling Technology, USA), phospho-AMPKα (1:1000, Cell Signaling Technology, USA), mTOR (1:1000, Cell Signaling Technology, USA), phospho-mTOR (1:1000, Cell Signaling Technology, USA), hif-1α (1:500, Novus, USA), and α-Tubulin (1:2500, Santa Cruz, CA, USA). The blots were then probed with a goat anti-rabbit IgG or an anti-mouse secondary horseradish peroxidase-conjugated antibody (Santa Cruz, CA, USA). The membranes were developed using enhanced chemiluminescence reagents (Pierce Chemical, IL, USA). The relative amount of the protein was normalized to α-Tubulin and analyzed with Labworks image acquisition and analysis software (UVP, Upland, USA).

Statistical analysis

Data, given as means ± SEM, were obtained from at least three individual experiments. Statistical analysis used student's t-test or one-way ANOVA, as appropriate. Differences were considered significant if $P<0.05$.

Results

Metformin suppresses T cell proliferation in vitro in a dose-dependent manner

We first examined the effect of metformin on CD4 T cell proliferation in vitro. Naïve CD4 T cells were first isolated from naïve C57BL/6 mice and equal numbers of CD4 T cells were then stimulated with plate-bound anti-CD3/CD28 antibodies in the presence of media control or different concentrations of metformin. 3 days later, cell numbers from each well of different groups were counted with a CCK-8 cell-counting kit. As shown in **Figure 1**, metformin significantly inhibits the proliferation of activated T cells in a dose-dependent manner.

Metformin inhibits the differentiation of Th1 and Th17 cells while promoting Treg development in vitro

Given the critical role of glycolysis in determining Th1 and Th17 differentiation, we next sought to address the role of metformin on their differentiation. To this end, naïve T cells isolated from C57BL/6 mice were cultured under Th1 and Th17 polarizing conditions in the presence of media or 5 mM of metformin. 5 days later, the expression of IFN-γ was examined in Th1 polarizing cells by intracellular staining. Expression of transcription factors RORγt and Foxp3 were examined in Th17-polarized cells. As shown in **Figure 2**, the data suggest that metformin significantly inhibits the development of Th1 (**Figure 2A**, **2D**) and Th17 (**Figure 2B**, **2E**) cells. The decrease of ROR-γt expression was associated with an increase of Foxp3 expression (**Figure 2B**, **2F**), suggesting that metformin promotes the production of Tregs.

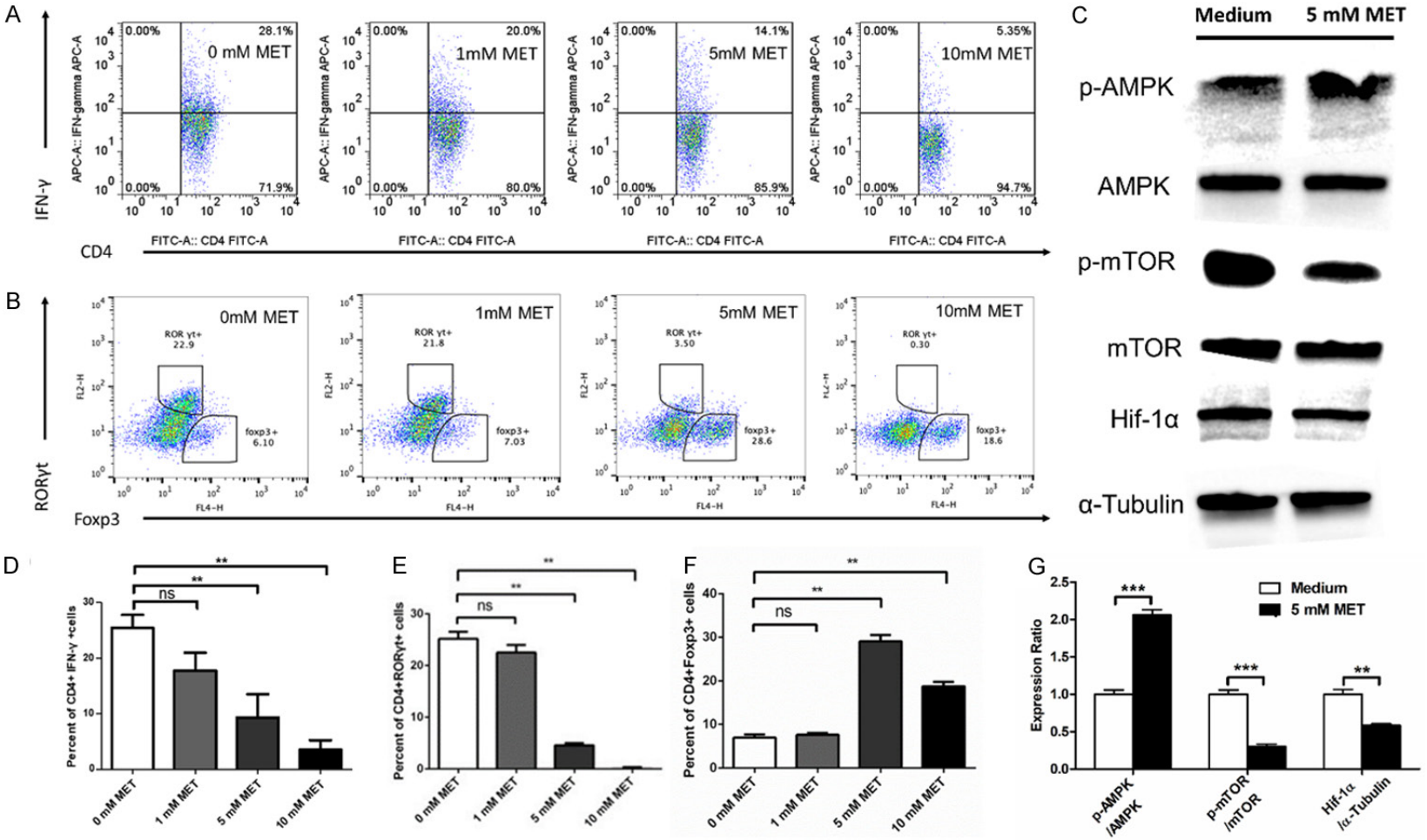

Figure 2. Metformin inhibits the differentiation of Th1 and Th17 cells, whereas promotes Tregs development in vitro. (A) Naïve CD4 T cells from male C57BL/6 mice were differentiated under Th1-inducing conditions for 5 days with different concentrations of metformin, followed by surface staining of CD4 and intracellular staining of IFN-γ. (D) Statistical analysis of the percentage of CD4+IFNγ+ cells among different groups. (B) Naïve CD4 T cells from male C57BL/6 mice were differentiated under Th17-inducing conditions for 5 days with different concentrations of metformin, followed by surface staining of CD4 and intracellular staining of RORγt and Foxp3. (E) Statistical analysis of the percentage of CD4+RORγ+ cells among different groups. (F) Statistical analysis of the percentage of CD4+Foxp3+ cells among different groups. (C) Naïve CD4 T cells from male C57BL/6 mice were differentiated under Th17-inducing conditions with or without 5 mM metformin for 5 days. The expression of p-AMPKα, AMPKα, p-mTOR, m-TOR, Hif-1α and α-Tubulin expression in the cells were determined by Western blot. (G) Statistical analysis of the expression of p-AMPKα, AMPKα, p-mTOR, m-TOR, Hif-1α and α-Tubulin by Western blot. Expression in Medium group cells was assigned the value of 1. Statistical comparisons were performed using one-way ANOVA in (D-F), and student's t-test in (G). Data shown are representative images or expressed as means ± SEM. (**P<0.01, ***P<0.001, n=4 for each group).

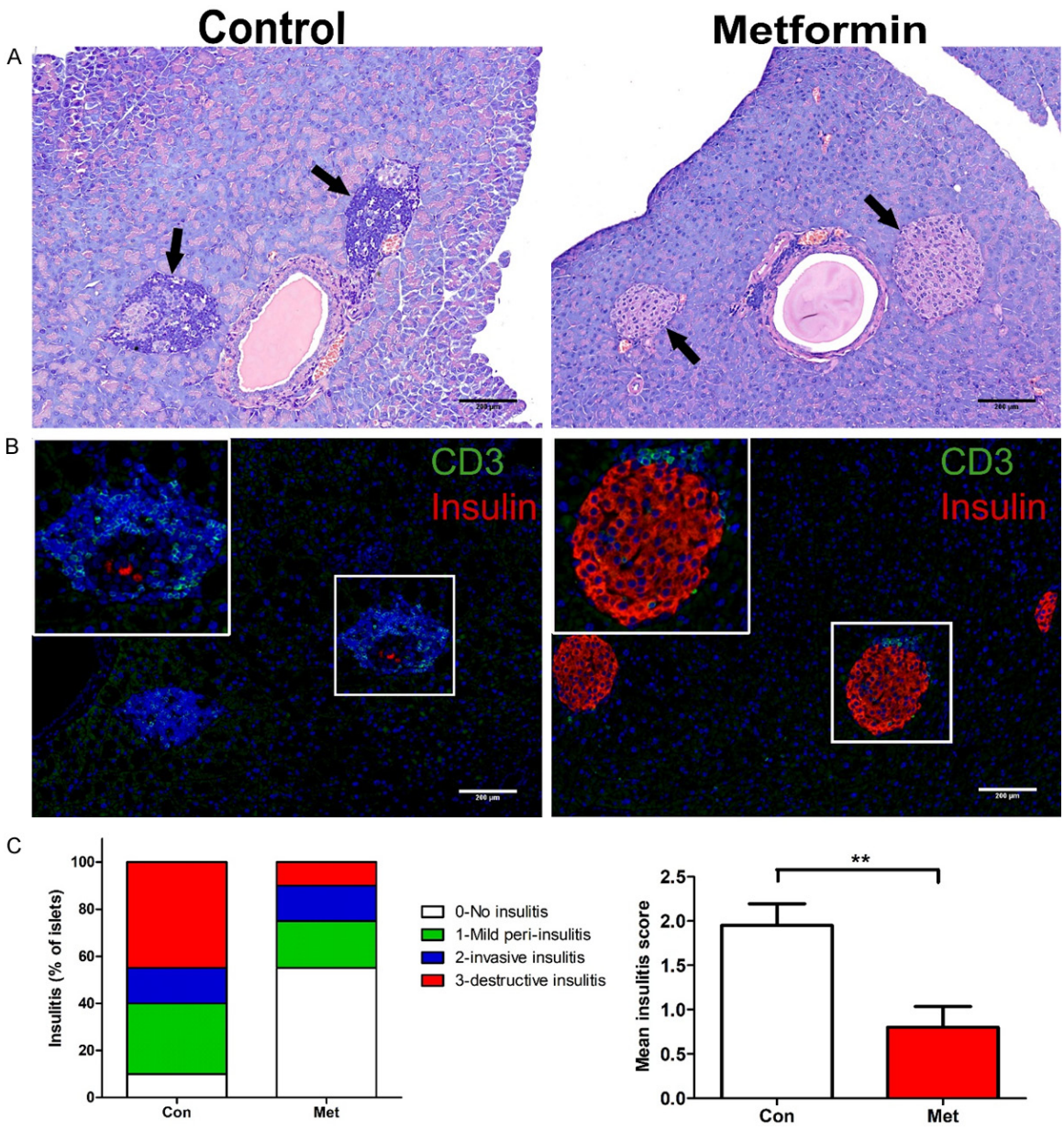

Figure 3. Metformin treatment reduces the severity of insulitis. Representative H&E staining of pancreas from 12-week old untreated (A, left) and metformin-treated (A, right) female NOD mice. Black arrows indicate islets. Immunofluorescent staining of CD3 (red) and insulin (green) from the pancreas of 12-week old untreated (B, left) and metformin-treated (B, right) female NOD mice were also shown. Insulitis scores of pancreas from the two groups were shown in (C), 0= No insulitis (unfilled bars); 1= Mild peri-insulitis (green bars); 2= invasive insulitis (blue bars); 3= Destructive insulitis (red bars); mean insulitis score of each group were calculated for statistics. Scale bar =200 μm. Statistical comparisons were performed using student's t-test. Data are represented as means ± SEM. (***P*<0.01, n=4 for each group).

Previous studies have shown that Hypoxia inducible factor 1a (HIF-1α), whose expression is regulated by mTOR, regulates the development of Th17 and Tregs. To study the role of metformin on AMPK/mTOR/HIF-1α signaling, the expression of phosphorylated AMPK and mTOR, as well as HIF-1α from whole protein lysate of 5 mM metformin or vehicle-treated Th17 polarized cells were determined by western blot analysis. As shown in **Figure 2C**, treatment with metformin significantly activates AMPK and inhibits mTOR. The expression of Hif-1α was further inhibited.

Metformin treatment reduces the severity of insulitis

Having demonstrated the effect of metformin on T cells proliferation and differentiation in vitro, we hypothesized that metformin could be a candidate for preventing the development of T1DM. NOD female mice were treated daily with metformin or vehicle starting from 4 weeks of age. At week 12 normal NOD mice had developed insulitis, but not diabetes, and were sacrificed for insulitis scoring. As shown in **Figure 3**, metformin-treated mice preserved many more functional β cells (demonstrated by the positive staining of insulin), whereas the control group had fewer functional β cells. Metformin-treated mice had significantly lower insulin scores than vehicle treated ones.

We further compared the percentage of different subsets of T cells in the spleen of metformin- or vehicle-treated mice. As shown in **Figure 4**, metformin treatment significantly decreased the number of pro-inflammatory IFN-γ+ as well as IL17+ CD4 T cells in the spleens of NOD mice. Also, a significantly-increased percentage of regulatory IL-10+ and Foxp3+ CD4-T cells, which exhibit immune regulatory function, were seen in the metformin-treated mice.

Discussion

In this study, a novel potential therapeutic method is described in regulating T cell metabolism and reducing the severity of autoimmune insulitis prior to the development of type 1 diabetes. AMPK activator metformin dose-dependently suppresses T cell proliferation and inhibits the differentiation of Th1 and Th17 cells while promoting the development of Tregs in vitro. NOD mice treated with metformin had significantly lower insulin scores than vehicle-treated ones at least till week 12.

Aerobic glycolysis refers to the use of glycolysis even in the presence of a sufficient supply of oxygen. This was first described by Ott Warburg in cancer cells in the 1950s and was further found to exist in activated T cells [22]. The explanation of this interesting phenomenon is that aerobic glycolysis can promote the generation of substrates crucial for activation and proliferation [23]. More interestingly, a recent study demonstrated different subsets of T cells have different metabolic characteristics. During naïve T cell differentiation into different effector cell subsets in various local cytokine milieu, inhibition of glycolysis hinders the development of Th1 and Th17 cells but promotes Treg production [15, 16].

AMPK is an evolutionarily conserved Serine/Threonine kinase that regulates cellular metabolism and energy balance in eukaryotic cells including lymphocytes [24, 25]. AMPK influences cellular energy balance by inhibiting anabolic metabolism and enhancing catabolic metabolism. One of the important pathways to conserve energy is to antagonize mRNA translation through the inhibition of the mammalian target of rapamycin complex 1- (mTORC1) signaling. By activating the TSC1-TSC2 complex, AMPK is able to repress mTOR-dependent mRNA translation [18, 26, 27]. mTOR is a regulator of a variety of metabolic pathways including autophagy and protein synthesis. In addition, mTOR also acts as an important regulator of T-cell metabolism. Activation of mTOR upregulates glycolysis, which promotes differentiation and effector function of Th1 and Th17 cells [28, 29]. Conversely, inhibition of mTOR signaling impedes the differentiation of these Teffs [29, 30] while promoting the development of Treg cells [29, 31]. HIF-1α, which has been shown to be able to induce numerous genes that are involved in glycolysis, is one of the target genes of mTOR. Under both normoxic and hypoxic conditions, HIF-1α enhances Th17 development through direct transcriptional activation of ROR-γt but restricts FoxP3 expression by targeting its proteasomal degradation. Therefore, it acts as a regulator of Th17/Treg balance during differentiation [32]. Further study proved HIF-1α-dependent glycolysis dictates the lineage choices between Th17 and Treg cells. Lack of HIF-1 resulted in diminished Th17 development but enhanced Treg cell differentiation [16]. Tregs exhibit higher levels of AMPK activity and rely more on fatty acid oxidation for development and functional maintenance [15]. During lipid metabolism, Acetyl-CoA carboxylase is a rate-limiting enzyme for lipid biosynthesis and oxidation. The activation of AMPK further phosphorylates ACC on Ser-79 and decreases its activity, thus promoting fatty acid oxidation [33].

Given the inhibitory roles of AMPK on mTOR and HIF-1α signaling pathways and the AMPK-mediated promotion of fatty acid oxidation, we postulate that activating AMPK could be an

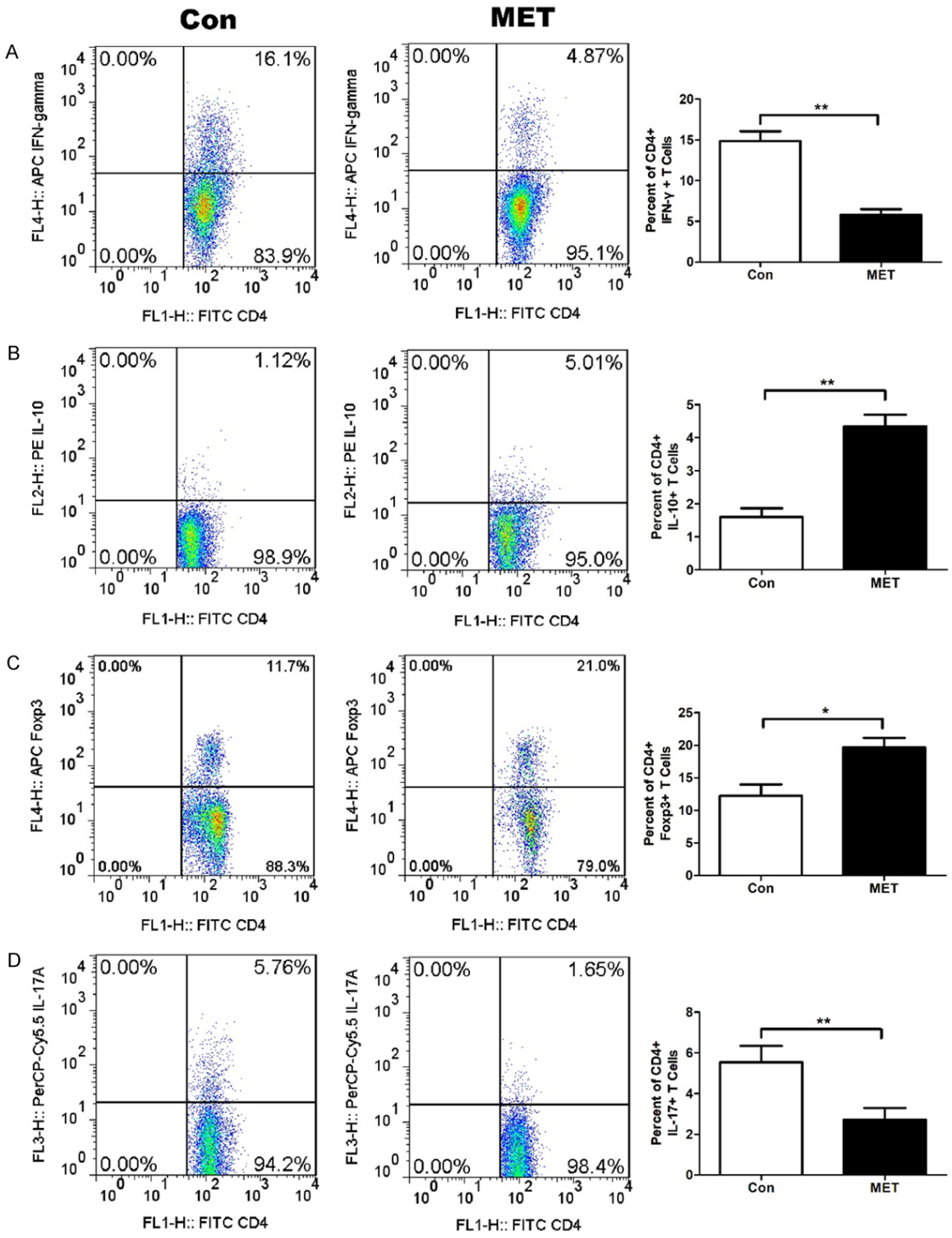

Figure 4. Metformin treatment decreases the numbers of inflammatory T cells whereas increases the numbers of regulatory T cells. The percentage of different T cell subsets in the spleens of 12-week old metformin-treated (MET) and untreated (CON) female NOD mice. Statistical comparisons were performed using student's t-test. Data are represented as means ± SEM. (**P*<0.05, ***P*<0.01, n=4 for each group).

important target for regulating Teff/Treg balance and a promising therapeutic method for treating autoimmune diseases, including T1-DM. Consistent with aforementioned studies,

our data showed that treatment with AMPK activator metformin dose-dependently represses T cell proliferation. During in vitro T-cell-subset-polarizing conditions, metformin treatment significantly decreased Th1 and Th17 development and promoted Treg production. The reduced Th17/Treg balance after AMPK activation is associated with a decreased mTOR/Hif-1α signaling pathway.

A fine balance between Teffs and Tregs is crucial for the development of peripheral tolerance and protective immunity as well as the suppression of inflammatory autoimmune diseases, including T1DM. Previous studies have demonstrated that in vivo treatment with AMPK activators exerts protective effects in different models of inflammatory/autoimmune diseases models [34-37].

In our study, in vivo treatment with metformin mitigates the severity of insulitis in female NOD mice during the early stage of disease development. Analysis of splenic cells revealed a significantly decreased percentage of inflammatory IFN-γ+ and Th17+ CD4 T cells in metformin-treated mice relative to vehicle-treated NOD mice, with the percentage of immune-regulatory IL-10+ and Foxp3+ CD4 T cells found to be significantly elevated as well. An increased ratio between regulatory and inflammatory cells may, at least partially, account for the protective effects of metformin. Although we observed reduced severity of insulitis and lower incidence of diabetes in metformin-treated compared with vehicle-treated mice at an early stage, we did not see a significant difference in diabetic rate after a 30-week observation (data not shown). We speculate that other factors reverse the protective effects of metformin, causing an increase in diabetic incidence in the late stage. Further study needs to be done to address this question.

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Disclosure of conflict of interest

None.

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