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(Citation)
Scientific Reports,15(1):10641

(Issue Date)
2025-03-27

(Resource Type)
journal article

(Version)
Version of Record

(Rights)
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Metformin prevents diabetes development in type 1 diabetes models via suppression of mTOR and STAT3 signaling in immune cells

Haruka Suzuki[1], Shuji Hasegawa[1], Sae Fushimi[1], Kanako Tagami[1], Minaho Nishikawa[1], Yuichi Kondo[1] & Hisafumi Yasuda[1,2]✉

Type 1 diabetes (T1D) is an organ-specific autoimmune disease caused by T cell-mediated pancreatic β cell destruction. To evaluate the effects of metformin on immune cells in autoimmune diabetes, we administered metformin intraperitoneally to two T1D mouse models and analyzed autoimmune diabetes progression. In a cyclophosphamide (CY)-induced T1D model in male non-obese diabetic (NOD) mice, intraperitoneal administration of metformin significantly prevented autoimmune diabetes. Treatment with metformin showed a decrease in activated T cells, $CD44^{hi}CD62L^{lo}$ effector memory cells, macrophages, and dendritic cells (DCs), and an increase in $CD44^{hi}CD62L^{hi}$ central memory cells, B cells, and regulatory T cells (Tregs) in splenocytes. Interestingly, metformin treatment showed a decrease in activated T cells, $CD4^+$ effector memory T cells and Th1-type antigen-specific cells in PLN cells. IL-17 production was significantly suppressed in metformin-treated mice. TNF-α production from DCs in vitro was dose-dependently suppressed by metformin. Activity of mTOR signaling was significantly reduced in $CD4^+$ T cells, $CD8^+$ T cells, and $B220^+$ B cells. In addition, activities of mTOR and STAT3 signaling in DCs were also reduced significantly. Furthermore, metformin treatment in female NOD mice, a spontaneous T1D model, significantly suppressed autoimmune diabetes onset as well and an increase in Tregs was observed. Our results suggest that metformin may suppress autoimmunity and have therapeutic potential in T1D progression as an immunomodulator.

Keywords Metformin, Type 1 diabetes, AMPK, mTOR, STAT3, Immunomodulator

Type 1 diabetes mellitus (T1D) is an organ-specific autoimmune disease caused by T cell-mediated destruction of insulin-producing pancreatic β cells[1]. Non-obese diabetic (NOD) mice spontaneously develop autoimmune insulitis and diabetes and have served as an animal model of human T1D for many years[2]. Diabetes incidence of NOD mice at 30 weeks of age is approximately 80% in females and 10–20% in males, respectively. A panel of studies using NOD mice has demonstrated that autoreactive T cells are activated by antigen presenting cells such as dendritic cells (DCs), leading to development of autoimmune diabetes[3,4]. DCs play a crucial role in the regulation of T cell balance[5]. Polarization of Th1 cells and Th17 cells from Th2 cells, or effector T cells from regulatory T cells (Tregs) is critical in the pathogenesis of T1D[6]. Th1 cells and Th17 cells mainly secrete inflammatory cytokines such as IFN-γ and IL-17, respectively, and in turn contribute to the pathogenesis of T1D[7,8].

Metformin has been widely used for patients with type 2 diabetes as an oral hypoglycemic agent (OHA) and has been shown to reduce cardiovascular events in randomized controlled trials[9,10]. The primary antidiabetic action of metformin mainly depends on suppression of hepatic glucose production (gluconeogenesis in the liver)[11]. In addition to the antidiabetic effect, many beneficial effects have recently been reported. In particular, reduction of cancer incidence and improvement of health span and lifespan are reported in some animal

[1]Division of Health Sciences, Department of Public Health, Kobe University Graduate School of Health Sciences, 7-10-2 Tomogaoka, Suma-ku, Kobe 654-0142, Japan. [2]Department of General Internal Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan. ✉email: yasuda@med.kobe-u.ac.jp

models[12,13]. Regarding the mechanism of metformin, the most commonly accepted mechanism is activation of AMP-activated protein kinase (AMPK) in the liver[14]. Currently, other mechanisms have been reported, including AMPK activation in duodenum, cyclic AMP suppression by AMP inhibition, hepatic glucagon signaling inhibition, gluconeogenesis in mitochondria, increased glucose uptake in muscles, inhibition of glucose absorption in the gut, activation of active GLP-1, and alteration of intestinal microbiota by production of a short-chain fatty acid (SCFA)[14–23].

Metformin has also been reported to have an anti-inflammatory effect, mainly in an AMPK-dependent manner[24]. In addition, metformin has been reported to affect T cell and DC metabolism[25,26]. Immunosuppressive effects of metformin have been reported in animal models of autoimmune diseases such as multiple sclerosis (MS), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and inflammatory bowel disease (IBD)[27–34].

In the T1D model, there are some reports on the effect of metformin, but the effect remains controversial. In the setting of NOD mice treated orally with metformin dissolved in drinking water, one study showed no protective effect on the development of diabetes[35], and another study showed suppression of autoimmune insulitis in 12-week-old NOD mice, but nothing on T1D incidence was indicated[36]. A third study showed that oral administration of metformin by gavage in a streptozotocin (STZ) induced model of diabetes ameliorates insulitis without normalizing blood glucose levels[37].

Therefore, the aim of this study was to determine whether metformin administration can suppress autoimmunity in T1D pathogenesis. To investigate the therapeutic potential of metformin on T1D progression, intraperitoneal administration of metformin was performed in two T1D mouse models; cyclophosphamide (CY)-given, male NOD mice referred to as a CY-induced T1D model[38] and female NOD mice referred to as a spontaneous T1D model. We assessed whether the administration strategy of precise doses of metformin protects against autoimmune diabetes progression.

Results

Protection against development of autoimmune diabetes in CY-given male NOD mice (a CY-induced T1D model) treated with metformin

To examine the effect of metformin on autoimmune diabetes progression in a mouse model of T1D, metformin or vehicle was administered intraperitoneally to CY-given male NOD mice as a CY-induced T1D model[38] (Fig. 1A). The majority of the CY-given NOD mice treated with metformin did not develop diabetes (2/19), whereas approximately 70% of the CY-given male control NOD mice untreated with metformin developed overt diabetes (11/16) within 30 days after the initial CY injection. Thus, the development of autoimmune diabetes was significantly prevented in the metformin-treated NOD mice compared with metformin-untreated controls (Fig. 1B).

Suppression of autoimmune insulitis by metformin treatment

To assess the effects of metformin, pancreatic sections from CY-given male NOD mice treated or untreated with metformin were examined. As shown by the representative histological appearance, only ~ 20% of islet was infiltrated by mononuclear cells and almost no insulitis was observed in metformin-treated NOD mice at 15 weeks of age (day 56 after the initial CY injection) (Fig. 2A, at middle and right panels), whereas CY-given male control NOD mice untreated with metformin developed severe insulitis (Fig. 2A, at left panel). The severity of insulitis was accessed by insulitis score at approximately 13 weeks of age, and we found that the severity of insulitis in metformin-treated NOD mice (0.37 ± 0.06) was significantly decreased, compared with the control, metformin-untreated NOD mice (3.76 ± 0.03) (Fig. 2B).

Immunohistochemical analysis was also performed to examine the population of infiltrating cells into the pancreatic islets of CY-given diabetic NOD mice. Immunohistochemical analysis revealed that $CD3^+$ T cells predominantly infiltrated to the islets, but not macrophages, and that the infiltrating $CD3^+$ T cells were both $CD4^+$ T cells and $CD8^+$ T cells around at day 30 after the initial CY injection (Fig. 2C). These results suggest that $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells are effector cells of beta cell destruction and may primarily cause β cell destruction in CY-given NOD mice. and indicate that metformin treatment may suppress T cell infiltration into pancreatic islets.

Flow cytometric analysis of the phenotypes of splenocytes and pancreatic lymph node (PLN) cells

The phenotypes of splenocytes and PLN cells isolated from CY-given male NOD mice treated or untreated with metformin and diabetic female NOD mice without CY injection were analyzed using flow cytometry (Fig. 3A). The absolute number of splenocytes at diabetes onset in metformin-untreated mice was significantly reduced compared with metformin-treated nondiabetic mice at day 56 after the initial CY injection, which were referred to as "tolerized" mice. In splenocytes isolated from metformin-treated mice, the percentages of $CD4^+$ T cells (at day 56), $B220^+$ cells (at day 56), $CD4^+CD69^+$ T cells (at day 14), and $CD44^{hi}CD62L^{hi}$ central memory cells (at day 14) were significantly increased compared with metformin-untreated mice, respectively, and the percentages of $CD11b^+$ macrophages (at day 56), $CD11c^+$ dendritic cells (at day 14), $CD4^+CD44^{hi}$ T cells (at day 56), $CD8^+CD44^{hi}$ T cells (at day 14 and day 56), and $CD44^{hi}CD62L^{lo}$ effector memory cells (whole, $CD4^+$, $CD8^+$) (at day 14) were significantly reduced compared with metformin-untreated mice, respectively (Fig. 3A–D). Furthermore, the percentage of $CD4^+Foxp3^+$ Tregs was significantly increased in metformin-treated "tolerized" mice at day 56, compared with metformin-untreated diabetic mice (Fig. 3C). Interestingly, significant increased levels of $CD127(IL\text{-}7R)^+$ on $CD4^+$ T cells and $CD8^+$ T cells were observed in metformin-treated "tolerized" mice, whereas PD-1 expression level on $CD8^+$ T cells in metformin-treated "tolerized" mice were significantly reduced rather than in metformin-untreated diabetic mice (Fig. 3E). In PLN cells isolated from metformin-treated mice at day

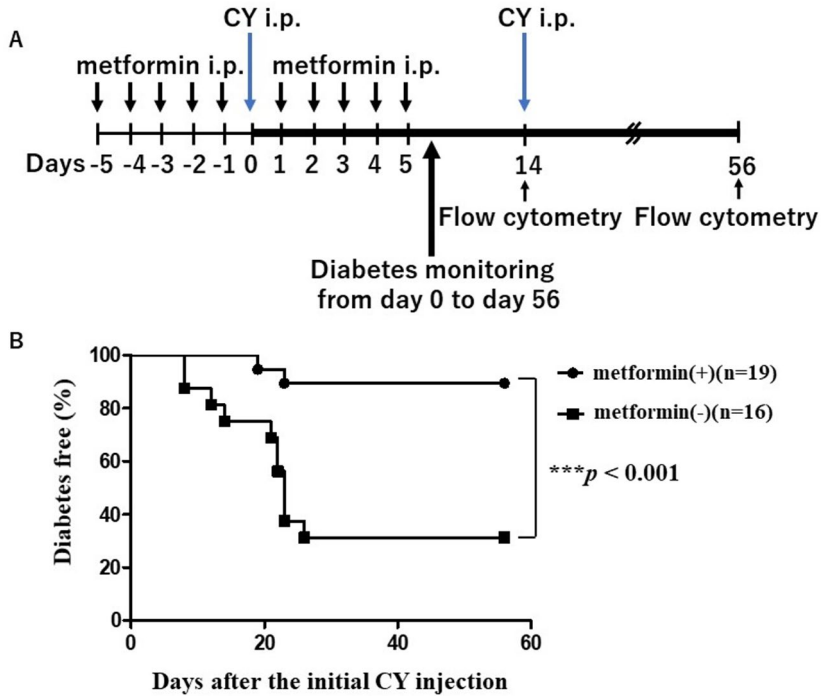


Fig. 1. Protection against development of autoimmune diabetes in CY-given male NOD mice (a CY-induced T1D model) treated with metformin. (**A**) Flowchart of the mouse experiment. Administration of cyclophosphamide (CY) at 7 weeks of age and/or second CY 14 days later to male NOD mice accelerates diabetes onset (a CY-induced T1D model). 100 mg/kg of metformin or vehicle (saline) was given intraperitoneally for 10 days (5 days before and after the initial CY injection), and diabetes incidence was followed up for 8 weeks after the initial CY injection. (**B**) The majority of the CY-given NOD mice treated with metformin ($n = 19$) did not develop diabetes (2/19), whereas approximately 70% of the CY-given male control NOD mice untreated with metformin ($n = 16$) developed overt diabetes (11/16). The development of diabetes was significantly suppressed in the metformin-treated NOD mice compared with metformin-untreated controls (***$p < 0.001$). ***$p < 0.001$ vs. no metformin by a log-rank test.

14, there were no differences in the absolute cell number and the frequencies of $CD4^+$ T cells and $CD8^+$ T cells (Fig. 3H). However, the percentages of $CD4^+CD44^{hi}$ T cells and $CD44^{hi}CD62L^{lo}CD4^+$ effector memory cells were significantly decreased in metformin-treated mice, compared with in metformin-untreated mice, respectively (Fig. 3I,J). The decreased level of PD-1 expression on $CD4^+$ T cells was significantly shown in metformin-treated mice, compared with metformin-untreated mice (Fig. 3K). In addition, we examined the population of PD-1^+CD44^+ cells in splenocytes (Fig. 3F) and PLN cells (Fig. 3L) as an activated T cell population. Furthermore, we also evaluated the population of $CD11a^{hi}CD49d^{hi}$ cells in splenocytes (Fig. 3A,G) and PLN cells (Fig. 3M), which was reported to be associated with the activation of Th1-type antigen-specific cell population in infectious disease[39]. Interestingly, the proportion of PD-1^+CD44^+ cells (whole, $CD4^+$, $CD8^+$) and $CD11a^{hi}CD49d^{hi}$ cells (whole, $CD4^+$, $CD8^+$) in PLN cells were significantly decreased in metformin-treated mice, compared with in metformin-untreated mice, respectively (Fig. 3L,M). These results suggest that metformin treatment may affect various immune cells and play the potential roles in reducing pathogenic cells and increasing regulatory cells.

Cytokine production from splenocytes in mice treated with metformin in vivo

To examine the effect of metformin on cytokine production from splenocytes, splenocytes from CY-given male NOD mice treated or untreated with metformin were stimulated with anti-CD3ε Ab in vitro and were analyzed for cytokine production. In CY-given male NOD mice treated or untreated with metformin, IFN-γ production from splenocytes stimulated with anti-CD3ε Ab was similar at day 14 after the initial CY injection and, surprisingly, was significantly increased in metformin-treated mice at day 56 compared with in metformin-untreated diabetic mice (Fig. 4A). In contrast, IL-17 production from splenocytes stimulated with anti-CD3ε Ab in metformin-treated mice was significantly increased at day 14 and was significantly decreased at day 56, compared with in metformin-untreated mice at day 14 and at diabetes onset, respectively (Fig. 4B). Furthermore, IL-10 production from splenocytes stimulated with anti-CD3ε Ab in metformin-treated mice at day 14 and at day 56 was similar to metformin-untreated mice at day 14 and at diabetes onset, respectively (Fig. 4C). Taken together, the duration of metformin treatment in this study was short, lasting only ten days and it is likely that the inhibitory effect of metformin treatment on IFN-γ production may not be sustained for the long term. Therefore, these results suggest that metformin treatment may, at least, affect cytokine production from T cells.

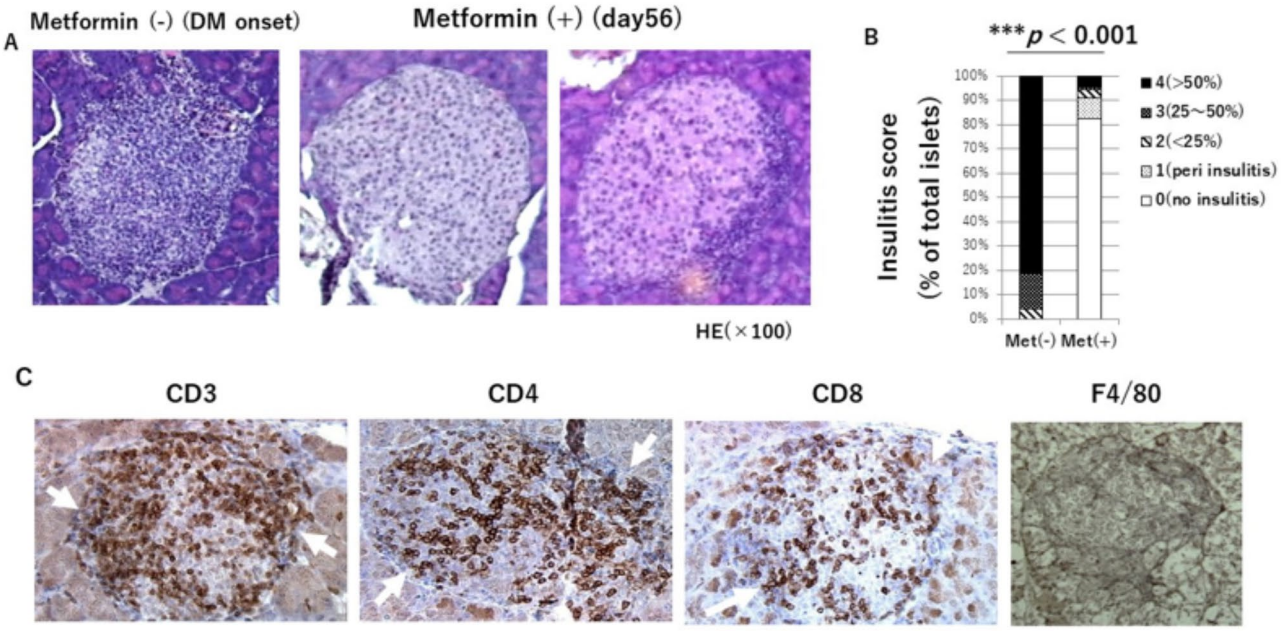


Fig. 2. Suppression of autoimmune insulitis by metformin treatment. (**A**) Representative histological appearances are shown for CY-given male NOD mice treated or untreated with metformin. (**B**) The severity of islet inflammation was quantified by the degree of insulitis at approximately 13 weeks of age. Islets were categorized as: 0 (within normal limits, absent), 1 (lymphocyte infiltration around islets, peri-insulitis), 2 (the percentage of islet lymphocyte infiltration, < 25%), 3 (25–50%), or 4 (50–100%). Insulitis score was calculated for each mouse ($n = 3$ mice/group, approximately 100 islets were blind scored). The score was significantly suppressed in metformin-treated mice, compared with metformin-untreated mice (***$p < 0.001$). (**C**) Immunohistochemical analysis in CY-given diabetic control NOD mice untreated with metformin revealed that $CD3^+$ T cells predominantly infiltrated to the islets but not macrophage, and that the infiltrating $CD3^+$ T cells were both $CD4^+$ T cells and $CD8^+$ T cells. ***$p < 0.001$ vs. no metformin by Mann–Whitney *U* test.

Altered activity of mTOR signaling in T and B cells upon metformin treatment in vivo

To investigate the effect of metformin on the activity of mTOR signaling in splenocytes, intracellular staining to detect p-$S6^{235/236}$ activity in splenocytes from NOD mice treated or untreated with metformin was performed and the activity of mTOR signaling was analyzed by flow cytometry at day 5 after the initial CY injection. In metformin-treated mice, p-$S6^{235/236}$ activity in splenocytes was significantly reduced in $CD4^+$ T cells, $CD8^+$ T cells, and $B220^+$ B cells (Fig. 5) and p-$S6^{235/236}$ activity was also reduced in PLN cells (data not shown). These results suggest that metformin treatment may affect the metabolism of T and B cells via suppression of mTOR signaling as an AMPK activator.

Cytokine production from DCs in the presence of metformin in vitro

Bone marrow-derived dendritic cells (BMDCs) from 7–9-week-old NOD mice were stimulated with lipopolysaccharide (LPS) after 16 h exposure to various concentrations of metformin (0–16 mM) and were cultured for 24 h. To examine the effect of metformin on cytokine production from DCs, the concentration of TNF-α and IL-10 in the culture supernatant were measured. TNF-α production from BMDCs was suppressed by metformin in dose-dependent manner (Fig. 6A). BMDCs exposed to metformin showed a significant reduction in both TNF-α and IL-10 production compared with those unexposed to metformin (Fig. 6B, C). BMDCs in the presence of metformin may have the phenotypes of immature or semimature DCs, because the BMDCs and splenic DCs show the lower expression levels of costimulatory markers and the BMDCs also show the higher phagocytosis of the FITC-dextran (data not shown). Taken together, these results suggest that metformin may at least reduce cytokine production from DCs.

Altered activity of mTOR and STAT3 signaling in DCs in the presence of metformin in vitro

To further investigate the effect of metformin on the activity of mTOR and STAT3 signaling in DCs, intracellular staining to detect p-$S6^{235/236}$ and p-$STAT3^{Y705}$ activities in BMDCs was performed and analyzed by flow cytometry. In BMDCs stimulated with LPS after exposure to metformin in vitro, p-$S6^{235/236}$ and p-$STAT3^{Y705}$ activities were significantly reduced, compared with in BMDCs stimulated with LPS alone (Fig. 7A,B). These results suggest that metformin may affect DC metabolism via suppression of mTOR and STAT3 signaling as an AMPK activator.

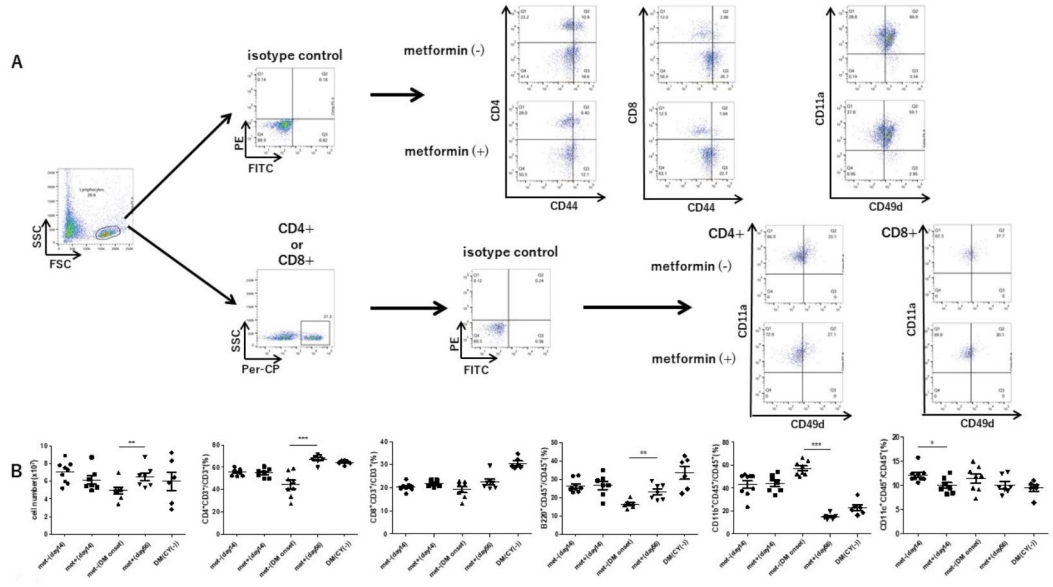


Fig. 3. Flow cytometric analysis of the phenotypes of splenocytes and PLN cells. (**A**) A gating strategy and some representative flow cytometric figures. The phenotype of splenocytes (**B–G**) and PLN cells (at day14) (**H–M**) isolated from CY-given male NOD mice treated or untreated with metformin and diabetic female NOD mice were analyzed by flow cytometry. (**B**-**G**) (**B**)The absolute number of splenocytes at diabetes onset in metformin-untreated mice was significantly decreased compared with in metformin-treated "tolerized" nondiabetic mice at day 56 after the initial CY injection (**$p < 0.01$). In metformin-treated mice, the percentages of $CD4^+$ T cells (**B**), $B220^+$ cells (**B**), $CD4^+CD69^+$ T cells (**C**) and $CD44^{hi}CD62L^{hi}$ central memory cells (**D**) were significantly increased compared with in metformin-untreated mice, respectively (***$p < 0.001$ at day 56, **$p < 0.01$ at day 56, *$p < 0.05$ at day 14 and *$p < 0.05$ at day 14, respectively), and the percentages of $CD11b^+$ macrophages (**B**), $CD11c^+$ dendritic cells (**B**), $CD4^+CD44^{hi}$ T cells (**C**), $CD8^+CD44^{hi}$ T cells (**C**) and $CD44^{hi}CD62L^{lo}$ effector memory cells (whole, $CD4^+$, $CD8^+$) (**D**) were significantly decreased compared with in metformin-untreated mice, respectively (***$p < 0.001$ at day 56, **$p < 0.01$ at day 14, ***$p < 0.001$ at day 56, *$p < 0.05$ at day 14 and *$p < 0.05$ at day 56, and whole; **$p < 0.01$, $CD4^+$, $CD8^+$;*$p < 0.05$ at day 14, respectively). The percentage of $CD4^+Foxp3^+$ Tregs (**C**) was significantly increased in metformin-treated "tolerized" mice at day 56, compared with in metformin-untreated diabetic mice (**$p < 0.01$). (**E**) The increased levels of CD127 expression on $CD4^+$ T cells and $CD8^+$ T cells at day 56 (*$p < 0.05$ and *$p < 0.05$, respectively) and the decreased levels of PD-1 expression on $CD8^+$ T cells at day 56 (*$p < 0.05$) were significantly shown in metformin-treated "tolerized" mice, compared with metformin-untreated diabetic mice. No differences were observed in the percentages of $PD\text{-}1^+CD44^{hi}$ cells (**F**) and $CD11a^{hi}CD49d^{hi}$ cells (**G**). (**H–M**) (**H**) No differences were observed in the absolute cell number and the frequencies of $CD4^+$ T cells and $CD8^+$ T cells. The percentages of $CD4^+CD44^{hi}$ T cells (**I**) and $CD44^{hi}CD62L^{lo}$ $CD4^+$ effector memory cells (**J**) were significantly decreased compared with in metformin-untreated mice, respectively (**$p < 0.01$). The decreased level of PD-1 expression on $CD4^+$ T cells (**K**) was significantly shown in metformin-treated mice, compared with metformin-untreated mice (*$p < 0.05$). $PD\text{-}1^+CD44^{hi}$ cells (whole, $CD4^+$, $CD8^+$) (**$p < 0.01$, **$p < 0.01$ and *$p < 0.05$, respectively) (**L**) and $CD11a^{hi}CD49d^{hi}$ cells (whole, $CD4^+$, $CD8^+$) (**$p < 0.01$, *$p < 0.05$ and **$p < 0.01$, respectively) (**M**) were significantly decreased compared with in metformin-untreated mice, respectively. Data are represented as mean ± SEM for at least five mice per group in one of the three independent experiments. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. no metformin by Mann–Whitney *U* test.

Protection against development of autoimmune diabetes in female NOD mice (a spontaneous T1D model) treated with metformin

To further investigate the effect of metformin on autoimmune diabetes progression in female NOD mice, a spontaneous T1D model, metformin or vehicle (saline), was administered intraperitoneally three times a week from 4 to 15 weeks of age, and diabetes incidence was followed until 40 weeks of age. Diabetes onset was significantly delayed and suppressed in the metformin-treated NOD mice compared with metformin-untreated controls, and a significant increase in Tregs in splenocytes was observed in metformin-treated mice compared with metformin-untreated controls (Fig. 8A–C). Taken together with our results from the CY-induced T1D model, this finding suggests that metformin may affect the function of various immune cells such as T and B cells and DCs, delaying and suppressing the onset of autoimmune diabetes as an immunomodulator.

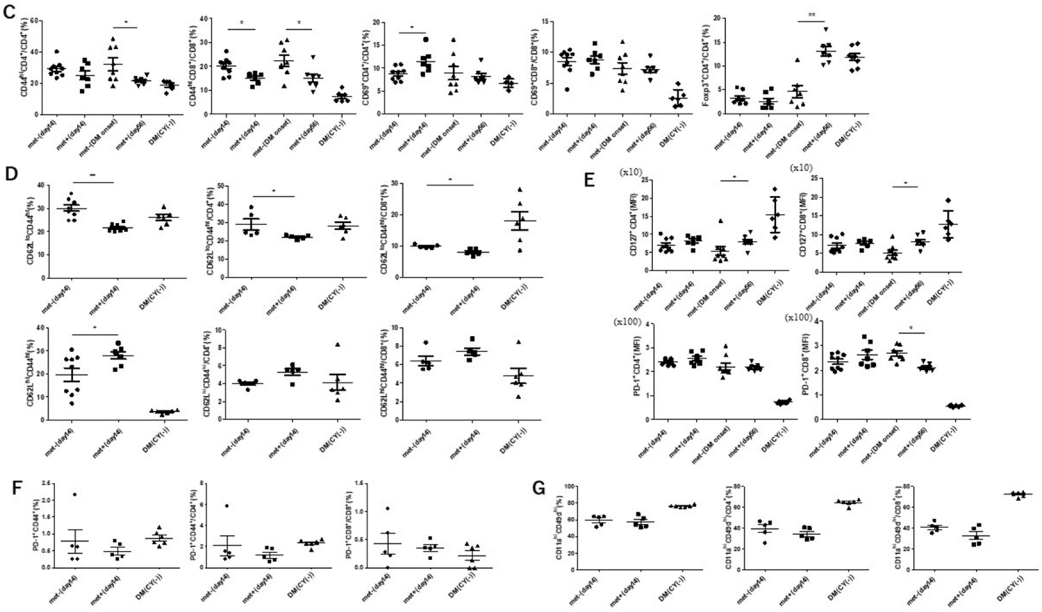


Figure 3. (continued)

Suppression of diabetes by splenocytes from metformin-treated NOD mice in adoptive transfer of diabetes into NOD.Scid mice

We tested the regulatory function of splenocytes isolated from metformin-treated NOD mice in vivo for their capacity to protect NOD, Scid mice from diabetes development by adoptively transferring splenocytes isolated from newly diabetic NOD mice into NOD.Scid mice. Splenocytes (1.5×10^7) from newly diabetic NOD mice with splenocytes (1.5×10^7) from metformin-treated non-diabetic NOD mice or metformin-untreated non-diabetic NOD mice were injected into 5 to 6-week-old NOD.Scid recipients. Splenocytes from newly diabetic NOD mice with splenocytes from metformin-treated non-diabetic NOD mice significantly suppressed diabetes development in NOD.Scid recipients, suggesting that splenocytes from metformin-treated non-diabetic NOD mice may have the regulatory function (Fig. 8D).

Discussion

To elucidate the effects of metformin on immune cells in the development of autoimmune diabetes, metformin was administered intraperitoneally to a mouse model of autoimmune diabetes; CY-given male NOD mice (a CY-induced T1D model)[38] and female NOD mice (a spontaneous T1D model). We found that intraperitoneal administration of metformin significantly suppressed the development of autoimmune diabetes. In splenocytes in the CY-induced T1D model, the decrease in $CD44^{hi}CD8^+$ T cells, $CD44^{hi}CD62L^{lo}$ effector memory cells, and $CD11c^+$ DCs, and the increase in $CD44^{hi}CD62L^{hi}$ central memory cells were demonstrated at early stages of diabetes progression, followed by the decrease in $CD44^{hi}$ effector T cells and $CD11b^+$ macrophages, and the increase in $Foxp3^+CD4^+$ Tregs at later stages of diabetes progression. IL-17 production from splenic T cells was also decreased at the late stage of the progression. TNF-α production from BMDCs was significantly suppressed by metformin in a dose-dependent manner, suggesting that metformin treatment may affect DC function in addition to DC numbers. In PLN cell, the percentages of $CD4^+CD44^{hi}$ T cells and $CD44^{hi}CD62L^{lo}CD4^+$ effector memory cells were significantly decreased in metformin-treated mice, compared with in metformin-untreated mice, respectively. The decreased level of PD-1 expression on $CD4^+$ T cells was significantly shown in metformin-treated mice, compared with metformin-untreated mice. In addition, we evaluated the population of $PD\text{-}1^+CD44^+$ cells as an activated T cell population and the population of $CD11a^{hi}CD49d^{hi}$ cells, which was reported to be associated with the activation of Th1-type antigen-specific cell population in infectious disease[39]. Interestingly, we found that the proportion of $PD1^+CD44^+$ cells (whole, $CD4^+$, $CD8^+$) and $CD11a^{hi}CD49d^{hi}$ cells (whole, $CD4^+$, $CD8^+$) in PLN cells were significantly decreased in metformin-treated mice, compared with in metformin-untreated mice, respectively. In the spontaneous T1D model, metformin treatment also significantly suppressed the development of autoimmune diabetes, and the increase in Tregs was observed in metformin-treated mice. Taken together, these results suggested that metformin administration might induce the decrease in DCs and effector memory cells and the increase in central memory cells, resulting in a significant decrease in effector T cells and IL-17-producing Th17 cells and a significant increase in Tregs.

Our key observation was the therapeutic efficacy of metformin in T1D and its association with the reciprocal balance between effector and regulatory T cells and reduced numbers and function of DC. These findings were consistent with the previous reports that DCs were one of the first infiltrating cells and major producer of TNF-α, and that CY-induced T1D would be associated with a reduction of $CD4^+CD25^+Foxp3^+$ Tregs[4,40]. IL-10 is produced by numerous immune cell types including T cells, B cells, macrophages, and DCs. Mutations in IL-10 or its receptor (IL-10RA) cause severe autoinflammatory conditions such as colitis[41]. IL-10 is one of the

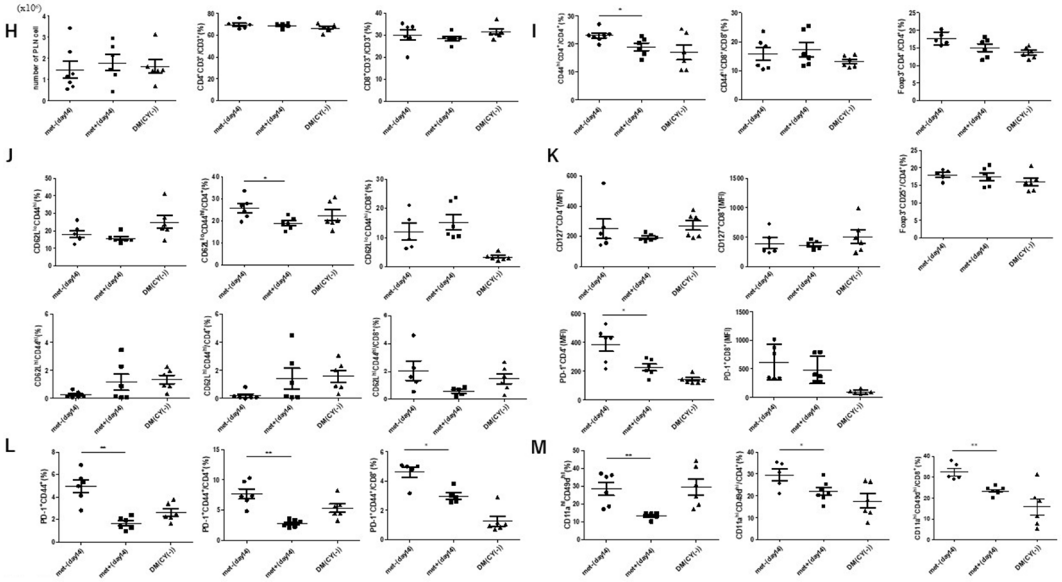


Figure 3. (continued)

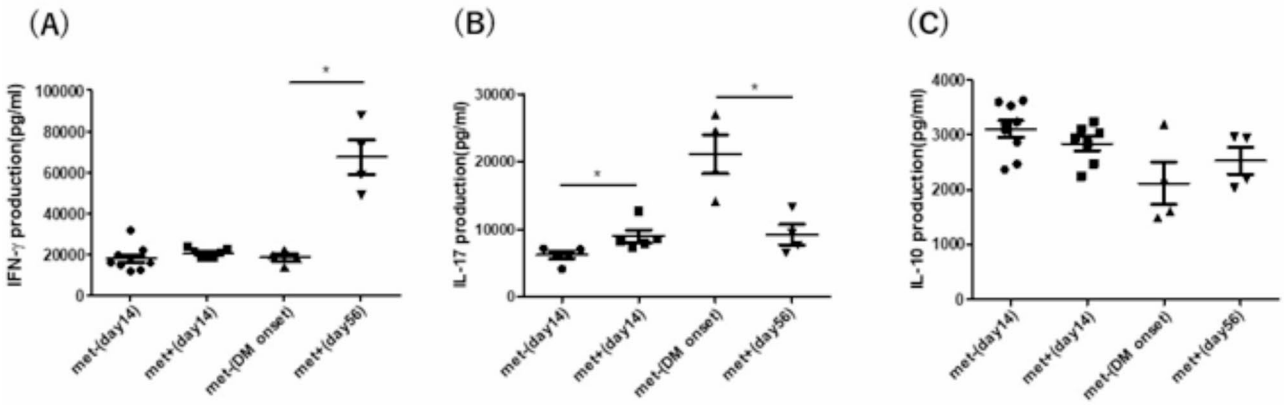


Fig. 4. Cytokine production from splenocytes in mice treated with metformin in vivo. Cytokine production from splenocytes stimulated with anti-CD3εAb in CY-given male NOD mice treated or untreated with metformin were analyzed. In metformin-treated mice, (**A**) IFN-γ production from splenocytes was similar at day 14 after the initial CY injection and was significantly increased at day 56 compared with in metformin-untreated diabetic mice ($n = 4–9$/group) ($^*p < 0.05$). (**B**) IL-17 production from splenocytes was significantly increased at day 14 and was significantly decreased at day 56, compared with in metformin-untreated mice at day 14 and at diabetes onset, respectively ($n = 4–5$/group) ($^*p < 0.05$ at day 14 and day 56, respectively). (**C**) IL-10 production from splenocytes was similar to metformin-untreated mice at day 14 and at diabetes onset, respectively ($n = 4–9$/group). Data are represented as mean ± SEM for at least four mice per group in one of the three independent experiments. $^*p < 0.05$ vs. no metformin by Mann–Whitney *U* test.

STAT3-activating cytokines and IL-10 production is also induced by pSTAT3. IL-17 is produced by T cells and induced by pS6. STAT3 has also been reported to increase IL-17 transcription and IL-17 production[42]. Our study suggests that metformin may reduce the IL-10-STAT3 axis via the STAT3 pathway, resulting in no increased IL-10 production from DCs. Metformin treatment also decreased IL-17 production from splenocytes at late stages. This result may coincide with the reports that AMPK inhibits mTOR activation leading to upregulation of pSTAT3[43,44].

Increased glycolysis is associated with activation of mTORC1, which has been reported to be essential for Foxp3 downregulation and decreased Treg generation[45]. mTOR is one of the downstream targets of AMPK and metformin is known to activate AMPK, leading to downregulation of mTORC1 and STAT3 activity, decreased glycolysis, and increased oxidative phosphorylation[46].

In this study, we demonstrated that metformin plays a pivotal role in inhibiting mTOR signaling in T and B cells, and mTOR and STAT3 signaling in DCs. We found a significant reduction in $S6^{235/236}$ phosphorylation

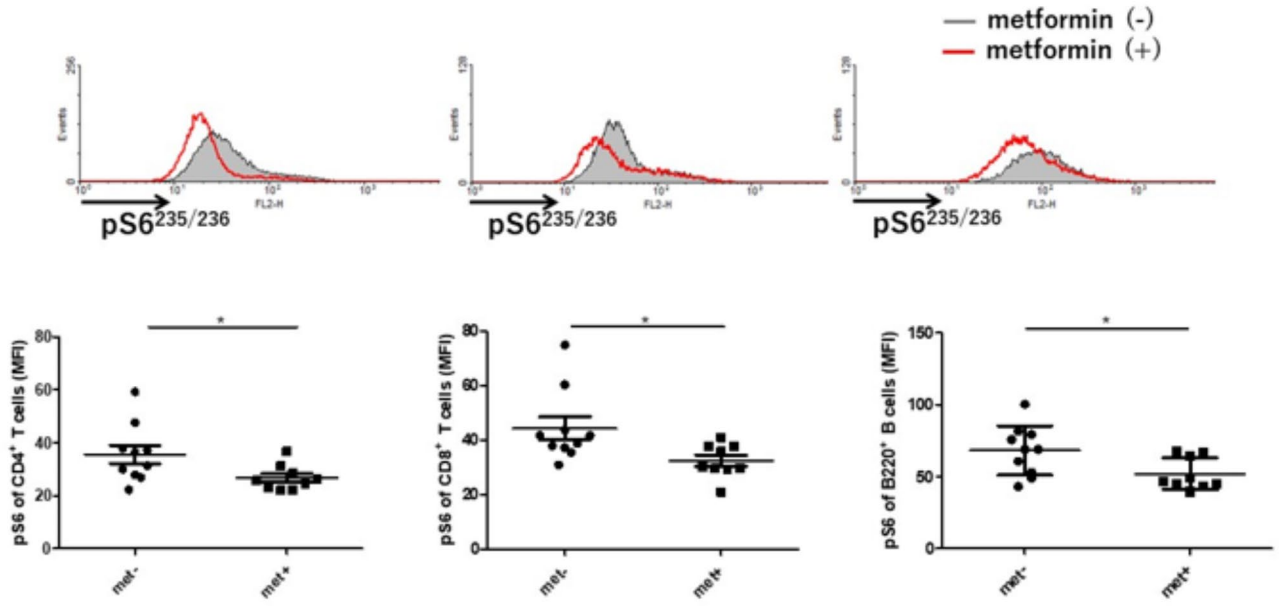


Fig. 5. Suppression of mTOR signaling in T and B cells upon metformin treatment in vivo. Intracellular staining to detect p-S6$^{235/236}$ activity in splenocytes from NOD mice treated or untreated with metformin was performed and analyzed for the activity of mTOR signaling by flow cytometry at day 5 after the initial CY injection. In metformin-treated mice, p-S6$^{235/236}$ activity of splenocytes was significantly decreased in CD4$^+$ T cells, CD8$^+$ T cells, and B220$^+$ B cells ($n = 9$–10) (*$p < 0.05$, *$p < 0.05$ and *$p < 0.05$, respectively). Data are represented as mean ± SEM for at least nine mice per group. Three independent experiments were performed. *$p < 0.05$ vs. no metformin by Mann–Whitney U test.

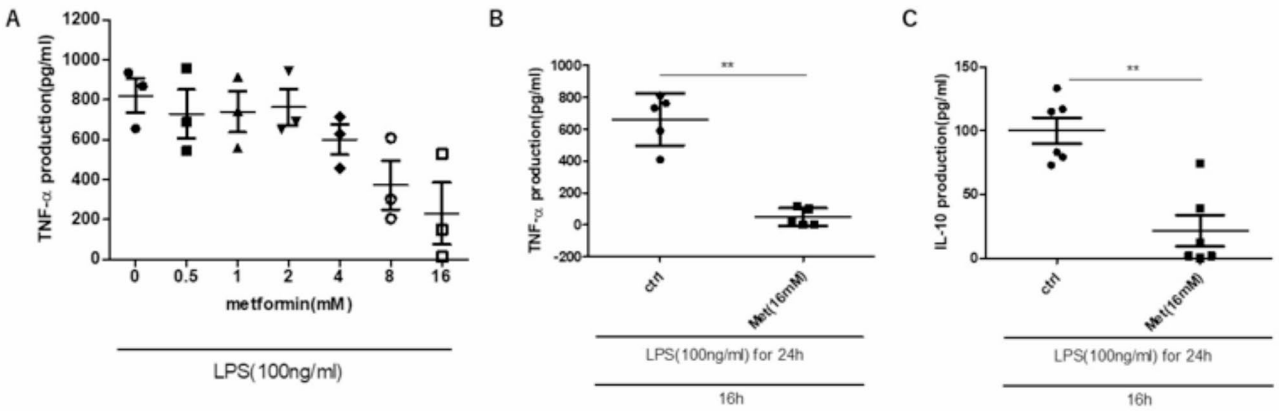


Fig. 6. Cytokine production from DCs in the presence of metformin in vitro. Bone marrow-derived dendritic cells (BMDCs) from 7–9-week-old NOD mice were stimulated with LPS after 16 h exposure to metformin (0–16 mM). After 24 h stimulation with LPS, the concentration of TNF-α and IL-10 in the culture supernatant were measured by ELISA. (**A**) TNF-α production from BMDCs was dose-dependently suppressed by metformin ($n = 3$/group). (**B**, **C**) BMDCs exposed to metformin showed a significant decrease in both TNF-α (**B**) and IL-10 (**C**) production compared with those unexposed to metformin (**$p < 0.01$ for TNF-α and IL-10, respectively). Data are represented as mean ± SEM ($n = 5$ for TNF-α and $n = 6$ for IL-10 in one of the three independent experiments). **$p < 0.01$ vs. no metformin by Mann–Whitney U test.

and STAT3 phosphorylation at Tyrosine 705 (pSTAT3 Y705). Altered activity of the mTOR signaling pathway in immune cells is likely related to AMPK-mediated activity by metformin. Moreover, metformin also suppressed STAT3 activation in DCs. Taken together, the decrease in effector memory T cells and effector T cells, and the increase in central memory T cells and Tregs may be related to changes in T cell metabolism due to metformin inhibition of mTOR signaling. Decreased cytokine production such as TNF-α and IL-10 from DCs may also be related to alterations in DC metabolism due to inhibition of mTOR and STAT3 signaling by metformin.

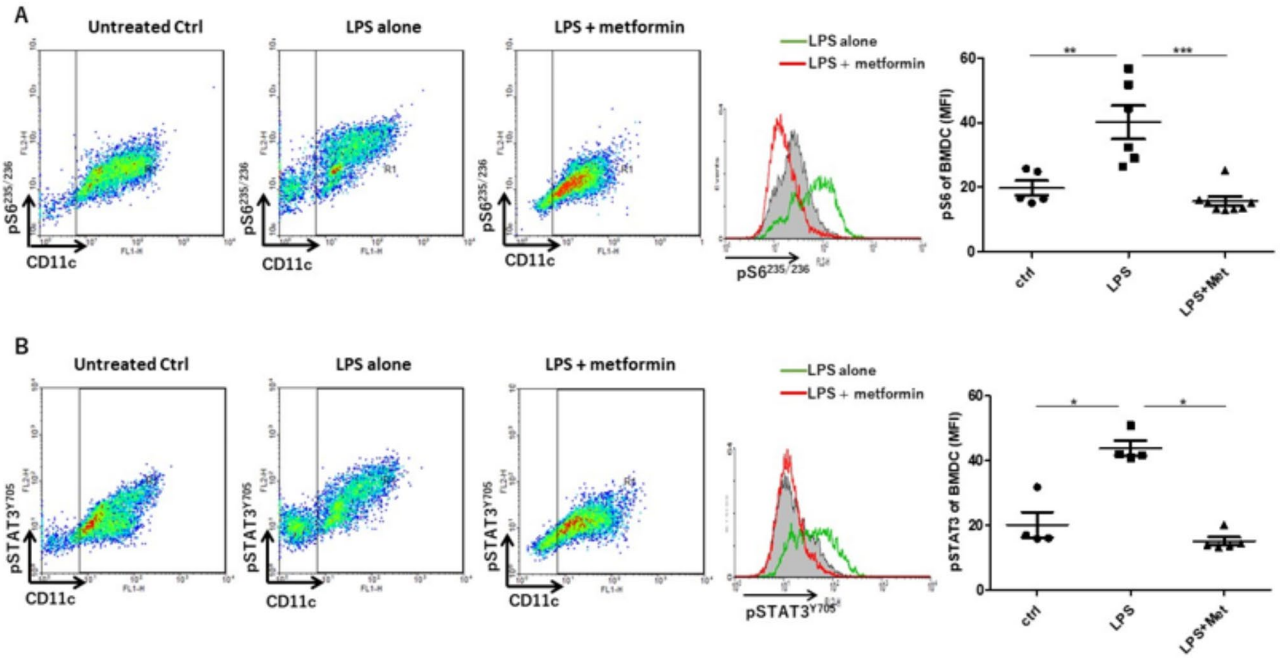


Fig. 7. Suppression of mTOR and STAT3 signaling in DCs in the presence of metformin in vitro. (**A**, **B**) BMDCs from 7–9-week-old NOD mice were stimulated with LPS after 16 h exposure to metformin (0 or 16mM). After 24 h stimulation with LPS, intracellular staining to detect p-S6$^{235/236}$ (**A**) and p-STAT3^{Y705} (**B**) activities in DCs were performed and analyzed for the activity of mTOR and STAT3 signaling by flow cytometry. In BMDCs stimulated with LPS after exposure to metformin in vitro, p-S6$^{235/236}$ and p-STAT3^{Y705} activities of BMDCs were significantly decreased compared with in BMDCs stimulated with LPS alone, respectively ($n = 5$–8 for p-S6$^{235/236}$ activity and $n = 4$–6 for p-STAT3^{Y705} activity. Three independent experiments were performed.) (**$p < 0.01$ for LPS alone vs. control, ***$p < 0.001$ for LPS alone vs. LPS + metformin in p-S6$^{235/236}$ and *$p < 0.05$ for LPS alone vs. control, *$p < 0.05$ for LPS alone vs. LPS + metformin in p-STAT3^{Y705}, respectively). Data are represented as mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ by Mann–Whitney U test.

In clinical settings, oral metformin treatment can cause adverse gastrointestinal effects, including nausea, vomiting, and diarrhea. However, we cannot exclude the possibility that oral doses of metformin (750–1500 mg/day) in clinical use might be insufficient for previously reported pleiotropic beneficial effects such as anticancer activity and prolonged survival in animal models[12,13]. We therefore intraperitoneally administered metformin (100 mg/kg) at doses approximately 4–8 times higher than the clinical use as an OHA in humans, and no adverse events were observed in treated mice.

In contrast, rapamycin also inhibits the mTOR pathway and has been tested in NOD mice. Rapamycin has been reported to enhance CD4$^+$Foxp3$^+$ Treg proliferation and function in vitro and to expand CD4$^+$Foxp3$^+$ Tregs in vivo in pre-diabetic NOD mice. However, it has been reported that the dose of rapamycin and the length of treatment should be considered carefully because higher doses and/or repeated treatments of rapamycin have detrimental effects in vivo, and that rapamycin exerted a detrimental effect on the disease outcome in NOD mice whose diabetes was reversed and cured by anti-CD3 therapy, when combined with anti-CD3 Ab[47]. Another report also indicates that rapamycin may cause upregulation of autophagy and impair islets function both in vitro and in vivo[48].

Some studies reported that metformin has anti-inflammatory effects, mainly in an AMPK-dependent manner[26]. Anti-inflammatory effects of metformin have been reported in animal models of autoimmune diseases such as MS, SLE, RA, and IBD[27–34]. It has been reported that metformin attenuated the autoimmunity and/or reduced Th17-mediated inflammation in these models.

Previous studies on T1D reported controversial effects of metformin. In the setting of NOD mice given metformin orally, dissolved in drinking water, one study showed no protective effect on the development of diabetes[35,] and another study showed suppression of autoimmune insulitis in 12-week-old NOD mice but did not show diabetes incidence[36]. The other study showed that oral administration of metformin by oral gavage to a model of STZ-induced diabetes ameliorates insulitis without normalizing blood glucose levels[37]. Our study revealed that metformin might have a protective effect on T1D pathogenesis through modulation of the AMPK-mTOR and AMPK-STAT3 pathways, leading to suppression of autoimmunity and diabetes incidence in both a CY-induced T1D model and a spontaneous T1D model. The inhibitory properties of metformin on mTOR and STAT3 signaling were associated with reciprocal regulation of T cells and DCs. In our opinion, high doses are important, and may be achieved by routes of administration other than the oral route. Our observations suggest that metformin may have anti-inflammatory and anti-autoimmune effects on immune cells, as well as

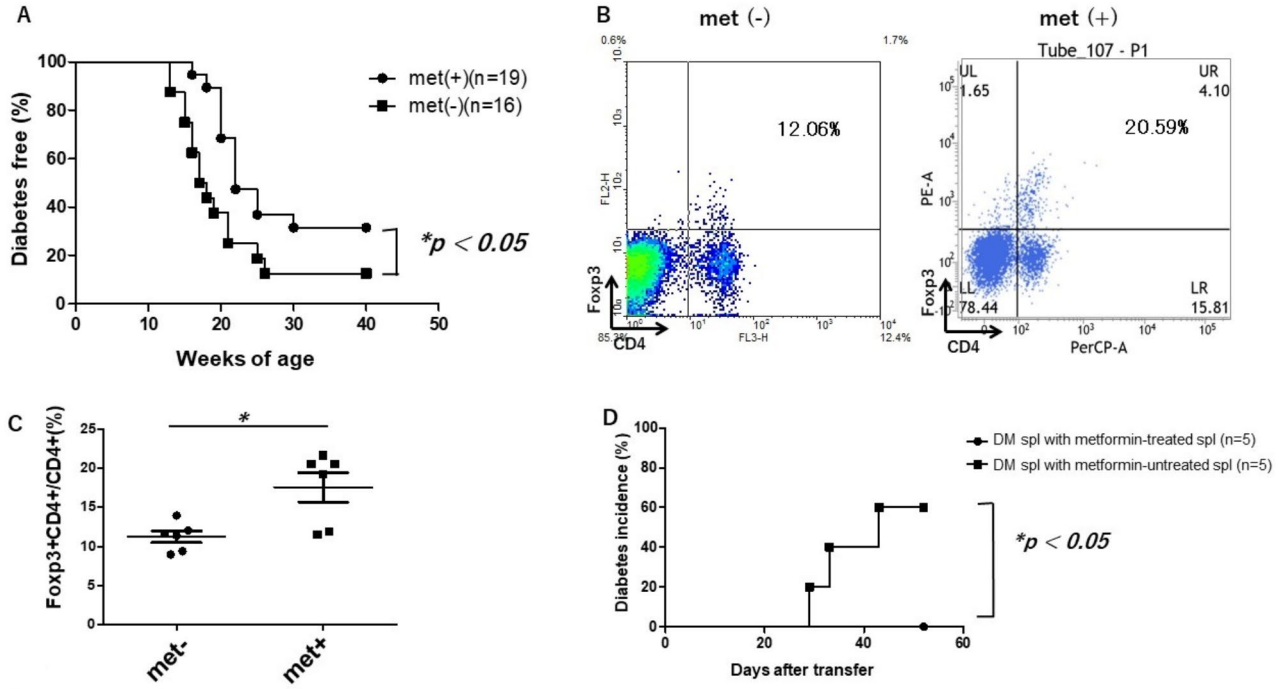


Fig. 8. Protection against development of autoimmune diabetes in female NOD mice (a spontaneous T1D model) treated with metformin. 100 mg/kg of metformin ($n = 19$) or vehicle (saline) ($n = 16$) was given intraperitoneally three times a week from 4 to 15 weeks of age in female NOD mice, and diabetes incidence was followed until 40 weeks of age. (**A**) The development of diabetes was significantly suppressed in the metformin-treated NOD mice compared with metformin-untreated controls ($^*p < 0.05$). $^*p < 0.05$ vs. no metformin by a log-rank test. (**B**) Representative flow cytometry plots show increased Tregs in metformin-treated mice. (**C**) In metformin-treated mice, the percentages of Tregs in splenocytes significantly increased compared with in metformin-untreated mice (15–20 weeks of age)($n = 6$/group) ($^*p < 0.05$). Data are represented as mean ± SEM. $^*p < 0.05$ vs. no metformin by Mann–Whitney *U* test. (**D**) Splenocytes (1.5×10^7) isolated from newly diabetic NOD mice with splenocytes (1.5×10^7) isolated from metformin-treated non-diabetic NOD mice ($n = 5$) or metformin-untreated non-diabetic NOD mice ($n = 5$) were injected into 5 to 6-week-old NOD.Scid recipients. Splenocytes from newly diabetic NOD mice with splenocytes from metformin-treated non-diabetic NOD mice significantly suppressed diabetes development in NOD.Scid recipients ($^*p < 0.05$). $^*p < 0.05$ vs. splenocytes from newly diabetic NOD mice with splenocytes from metformin-untreated non-diabetic NOD mice by a log-rank test.

an antidiabetic effect. Interestingly, significant increased levels of CD127(IL-7R)$^+$ on CD4$^+$ T cells and CD8$^+$ T cells was observed in metformin-treated "tolerized" mice, compared with metformin-untreated diabetic mice. CD127 is an IL-7 receptor and anti-IL-7R antibodies have been previously reported to inhibit the development of autoimmune diabetes in female NOD mice[49,50]. A previous report also showed that CD127 is highly expressed on memory T cells[51]. We speculate that the high expression of CD127 on CD4$^+$ and CD8$^+$ T cells at late stages might be associated with a decrease in effector memory cells (whole, CD4$^+$, CD8$^+$) and an increase in (whole) central memory cells in splenocytes and a decrease in CD4$^+$ effector memory cells in PLN cells at early stages. However, the precise mechanism remains unclear and further exploration is needed to elucidate these findings.

The duration of metformin treatment in the CY -induced T1D model was short, lasting only 10 days. The frequency and duration of metformin treatment in the spontaneous T1D model was three times a week from 4 to 15 weeks of age, but not frequent enough and not throughout the entire follow-up period. Therefore, we speculate that promising candidate strategies such as a longer duration and sufficient frequency of metformin treatment may have further beneficial effects on diabetes prevention. In addition, splenocytes from newly diabetic NOD mice with splenocytes from metformin-treated non-diabetic NOD mice, which represented the higher frequency of Treg cells than those from metformin-untreated NOD mice, significantly suppressed diabetes development in NOD.Scid recipients. These results suggest that splenocytes from metformin-treated non-diabetic NOD mice may have the immunoregulatory activity. However, not only Treg cells but also DCs and B cells might contribute the inhibition of autoimmune diabetes. Since each purified immune cell possibly suppresses T1D, adoptive transfer by each cell deleted or purified from the splenocytes will reveal their actual immunosuppressive activity. Therefore, further studies will provide evidence as to which cell types of splenocytes have the regulatory function in preventing autoimmune diabetes by metformin treatment.

In summary, this study demonstrated that metformin might have the therapeutic potential in T1D. These effects appear to depend on changes in DC and T cell metabolism, followed by a decrease in effector memory T cells and effector T cells, an increase in central memory T cells and induction of Tregs. Taken together, our

findings suggest that pharmacologic effects of metformin may be beneficial for T1D, and that metformin dosing may have important clinical implications applying to the prevention and development of T1D, especially slowly progressive T1D (SPIDDM). AMPK-mediated inhibition of mTOR and STAT3 signaling via metformin treatment led to inhibition of autoimmune diabetes. Our findings indicate that metformin may be useful in the treatment of T1D patients as an immunomodulator that affects the intracellular metabolism of immune cells. Our study suggests that strategies to suppress AMPK-mTOR and AMPK-STAT3 signaling may represent a new therapeutic intervention against T1D as an immunomodulator.

Methods

Mice

NOD/Shi/Kbe mice were maintained at the Institute for Experimental Animals, Kobe University School of Medicine, Kobe, Japan. All animals were housed in specific pathogen-free facilities. NOD.Scid mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). For mouse analysis, mice were anesthetized using inhaled isoflurane (3% v/v) and were euthanized via cervical dislocation. This study was approved by the Institutional Animal Care and Use Committee (Permission number: P181002-R1) and performed in accordance with relevant guidelines and regulations (the Kobe University Animal Experimentation Regulation). Animal experiments were carried out in accordance with the ARRIVE guidelines.

Antibodies and reagents

Fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD3 (145-2C11), CD4 (RM4-4), CD25 (7D4), CD44 (IM7), CD45 (30-F11), CD11c (HL3) and CD49d (R1-2) monoclonal antibodies, phycoerythrin (PE)-conjugated anti-mouse CD4 (GK1.5), CD8 (53–6.7), B220 (RA3-6B2), CD11b (M1/70), CD11c (HL3), CD62L (MEL-14), CD69 (H1.2F3), CD127 (SB/199), PD-1 (J43) and CD11a (M17/4) monoclonal antibodies, and the peridinin chlorophyll protein complex (PerCP)-conjugated anti-mouse CD4 (RM4-5), CD8 (53-6.7) and B220 (RA3-6B2) were purchased from BD Pharmingen (San Diego, CA). PE-conjugated anti-mouse forkhead box P3 (Foxp3) monoclonal antibody (FJK-16s) was purchased from eBioscience (San Diego, CA).

PE-conjugated anti-mouse p-S6$^{235/236}$ monoclonal antibody (N7-548) and anti-mouse p-STAT3^{Y705} monoclonal antibody (4/P-STAT3) were purchased from BD Phosflow and BD Pharmingen, respectively.

Purified hamster anti-mouse CD3εAb (145-2C11) was purchased from R&D Systems (Minneapolis, MN). Lipopolysaccharide (LPS) *(E.coli O55:B5)*, a ligand for TLR4, was purchased from Merck (Darmstadt, Germany). Cyclophosphamide (CY) was provided by Shionogi & Co., Ltd. (Osaka, Japan). Metformin was provided by Sumitomo Pharma Co., Ltd. (Osaka, Japan).

In vivo experiment designs

Cyclophosphamide (CY) (200 mg/kg body weight) was injected intraperitoneally twice, separated by a 2-week interval into 7- and 9-week-old male NOD mice (starting at 7 weeks of age), if the initial injection at 7 weeks of age induced no diabetes. All mice were nondiabetic prior to CY injection. Most CY-given male NOD mice developed overt diabetes within 8 weeks after the initial CY injection, referred to as a CY-induced T1D model[38]. In the experiment using the CY-induced T1D model, 100 mg/kg of metformin or vehicle (saline) was administered intraperitoneally for 10 days (5 days before and after the initial CY injection).

In the experiment using female NOD mice as a spontaneous T1D model, female NOD mice were intraperitoneally administered metformin 100 mg/kg or vehicle (saline) three times a week from 4 to 15 weeks of age, and diabetes incidence was followed up until 40 weeks of age.

Assessment of diabetes development

In the experiment using a CY-induced T1D model, urine glucose levels were monitored in each mouse twice a week from the day after the initial CY injection, and the monitoring was performed for 8 weeks after the initial CY injection.

In the experiment using female NOD mice, urine glucose levels were monitored in each mouse twice a week from 10 weeks of age, and the monitoring was performed continuing twice a week until 40 weeks of age.

Blood glucose levels were measured in mice with glycosuria with the Glutest Ace blood glucose monitoring system (Sanwa Chemical, Nagoya, Japan). Mice were diagnosed with diabetes when blood glucose level was over 250 mg/dl for 2 consecutive days.

Histological examination

CY-given male NOD mice treated or untreated with metformin were sacrificed for histological analysis. The pancreata were harvested from the mice, fixed in 10% formalin, embedded in paraffin, and sectioned at a thickness of 5 μm. To examine the severity of insulitis in each mouse, the sections were stained with hematoxylin and eosin and examined by light microscopy. The severity of islet inflammation was quantified by the degree of insulitis. Islets were categorized as: 0 (within normal limits, absent), 1 (mononuclear cell infiltration around islets, peri-insulitis), 2 (the percentage of islet mononuclear cell infiltration < 25%, mild insulitis), 3 (25–50%, moderate insulitis), or 4 (> 50%, severe insulitis). In addition, to examine the infiltrating cells to the islets in CY-given diabetic NOD mice, immunohistochemical analysis was performed by staining to examine with monoclonal antibodies used as primary antibodies (including clone/reference, dilution, and antigenic retrieval solution, respectively): anti-CD3 (SP7, 1/100, 10 mM citrate (pH 6.0)), anti-CD4 (4SM95, 1/100, 10 mM citrate (pH 6.0)), anti-CD8 (4SM15, 1/100, 10 mM citrate (pH 6.0)) and anti-F4/80 (T45-2342, 1/100, 1 mM EDTA (pH 9.0)).

Flow cytometric analysis of the phenotypes of splenocytes and PLN cells

CY-given male NOD mice treated or untreated with metformin and diabetic female NOD mice without CY injection were sacrificed for flow cytometric analysis. Various cell populations were identified with specific antibodies. Splenocytes from the mice were stained with antibodies against CD45, CD3, B220, CD4, CD8, CD44, CD62L, CD69, CD11b, CD11c, CD127, PD-1, CD11a, and CD49d. PLN cells from the mice were also stained with antibodies against CD3, CD4, CD8, CD25, CD44, CD62L, CD127, PD-1, CD11a, and CD49d. The fluorescence intensity of the stained cells was analyzed on a FACS 440 flow cytometer (Becton Dickinson, San Jose, CA).

Foxp3 intracellular staining

For intracellular Foxp3 staining, splenocytes and PLN cells were isolated from CY-given male NOD mice treated or untreated with metformin, labeled with PerCP-conjugated anti-mouse CD4 monoclonal antibody, fixed and permeabilized, and then labeled with PE-conjugated anti-mouse Foxp3 monoclonal antibody, according to the manufacturer's instructions. Intracellular staining to detect $Foxp3^+$ cells was performed with a Foxp3 staining set (eBioscience). The percentage of $CD4^+Foxp3^+$ cells was analyzed by flow cytometry.

Cytokine production from splenocytes

Purified splenocytes (1×10^6/200 µ1) from CY-given male NOD mice treated or untreated with metformin were resuspended in culture medium and transferred to each well of a round-bottom 96-well plate. Anti-CD3εAb was then added to each well (final concentration 2.5 µg/mL). No stimulant was added to control wells. The cells stimulated with anti-CD3εAb were cultured for 72 h at 37 °C in a humidified 5% CO_2 atmosphere. The supernatant was collected at the end of culture and frozen at − 30 °C until cytokine assay. IL-17, IFN-γ, and IL-10 production from splenocytes stimulated with anti-CD3εAb were evaluated by enzyme-linked immunosorbent assay (ELISA) as recommended by the assay manufacturer (R&D Systems).

Generation of bone marrow-derived dendritic cells (BMDCs)

DCs were generated from mouse bone marrow as described previously[52,53]. Briefly, bone marrow cells were isolated from 7–9-week-old NOD mice. $CD11c^+$ cells were isolated from cultured cells using an autoMACS magnetic cell sorter (Miltenyi Biotec, GmbH, Bergisch-Gladbach, Germany) and were designated as bone marrow-derived dendritic cells (BMDCs).

Cytokine production from DCs in the presence of metformin in vitro

Purified BMDCs (1×10^6/500 µl, 1 ml) from 7–9-week-old NOD mice were incubated with LPS (100 ng/mL) for 24 h after 16 h exposure or non-exposure to metformin. TNF-α and IL-10 was measured from the 24 h culture supernatant using commercially available ELISA kits from R&D Systems.

Activity of mTOR signaling in splenocytes by pS6 intracellular staining

For intracellular pS6 staining of splenocytes, splenocytes were isolated from CY-given male NOD mice treated or untreated with metformin, labeled with PerCP-conjugated anti-mouse CD4, CD8, or B220 monoclonal antibody, fixed and permeabilized, and then labeled with PE-conjugated anti-mouse p-$S6^{235/236}$ monoclonal antibody, according to the manufacturer's instructions. To examine the activity of mTOR signaling, intracellular staining to detect p-$S6^{235/236}$ activity was performed and analyzed by flow cytometry, because pS6 is a downstream target of mTOR.

Activity of mTOR and STAT3 signaling in DCs by pS6 and pSTAT3 intracellular staining

For intracellular pS6 staining and pSTAT3 staining of BMDCs, BMDCs were generated from bone marrow of 7–9-week-old NOD mice, labeled with FITC-conjugated anti-mouse CD11c monoclonal antibody, fixed and permeabilized, and then labeled with PE-conjugated anti-mouse p-$S6^{235/236}$ monoclonal antibody or anti-mouse p-$STAT3^{Y705}$ monoclonal antibodies, according to the manufacturer's instructions. To examine the activity of mTOR and STAT3 signaling, intracellular staining to detect p-$S6^{235/236}$ and p-$STAT3^{Y705}$ activities were performed and analyzed by flow cytometry, respectively.

Adoptive transfer experiment

To examine the regulatory function of splenocytes isolated from metformin-treated NOD mice, splenocytes (1.5×10^7) isolated from newly diabetic NOD mice with splenocytes (1.5×10^7) isolated from metformin-treated non-diabetic NOD mice ($n = 5$) or metformin-untreated non-diabetic NOD mice ($n = 5$) were injected into 5 to 6-week-old NOD.Scid recipients. All the recipients were monitored for diabetes for 50 days or until diabetes developed.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 5 for Windows (GraphPad Software, San Diego, CA). Survival curves were analyzed with a log-rank test using the Kaplan–Meier method. Mann–Whitney *U* tests were used to compare each group. Data are represented as mean ± SEM. The p values < 0.05 were considered statistically significant.

Data availability

The data presented in this manuscript are available on reasonable request to the corresponding author.

Received: 22 June 2024; Accepted: 7 March 2025
Published online: 27 March 2025

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Acknowledgements
We are grateful to Ms. Atsumi Katsuta for her outstanding assistance.

Author contributions
H.S. performed experiments, analyzed data, generated figures, and wrote the manuscript. S.H., S.F., K.T., M.N. and Y.K. performed experiments and provided mice. H.Y. designed and supervised the study, performed data analysis, and wrote the manuscript.

Funding
This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Science, Sports, and Technology, Japan (19K07912) (HY).

Declarations

Competing interests
The authors declare no competing interests.

Additional information
Correspondence and requests for materials should be addressed to H.Y.

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