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Metformin improves *in vivo* and *in vitro* B cell function in individuals with obesity and Type-2 Diabetes

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Abstract

Metformin (MET), the first-line medication for Type-2 Diabetes (T2D), has been shown to reduce chronic inflammation indirectly through reduction of hyperglycemia, or directly acting as anti-inflammatory drug. The effects of MET on B lymphocytes is uncharacterized. In the present study, we measured *in vivo* and *in vitro* influenza vaccine responses in 2 groups of T2D patients: recently diagnosed but not taking anti-diabetic drugs, and patients taking MET. Results show that B cell function and vaccine responses, hampered by obesity and T2D, are recovered by MET. Moreover, MET used *in vitro* to stimulate B cells from recently diagnosed T2D patients is also able to reduce B cell-intrinsic inflammation and increase antibody responses, similar to what we have seen in B cells from patients taking MET, who show increased responses to the influenza vaccine *in vivo*. These results are the first to show an effect of MET on B cells.

Keywords

B cells; Inflammation; Metformin

1. Introduction

An important goal of translational aging research is the identification of therapeutic strategies to prevent, delay or treat deleterious age-related effects which may contribute to increased morbidity and mortality in elderly individuals. New opportunities could arise from currently approved drugs for the discovery of optimal novel therapeutic effects. One candidate, Metformin (MET), the best treatment for overweight and obese Type-2 Diabetes (T2D) patients with normal renal function, is the only hypoglycemic drug also influencing cellular processes associated with the development of chronic conditions of old age,

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Disclosures

Conflicts of interest: none.

Author contributions

AD, BBB, DF conceived the experiments. AD, MR, TV, DF carried out the experiments and analyzed data. AD, DF, SL performed statistical analyses. All authors were involved in writing the paper and had final approval of the submitted and published versions.

including inflammation, oxidative damage, increased glycation of proteins, diminished autophagy, cell senescence and apoptosis. Although not fully understood, the pleiotropic effects of MET are thought to be mediated primarily through regulation of the activity of 5'-adenosine monophosphate (AMP)-activated protein kinase (AMPK) and the mammalian target of rapamycin (mTOR). MET has been shown to activate AMPK which is an attractive target because its activity is decreased in the liver, muscle and adipose tissue of obese or Insulin Resistant (IR) animals and humans [1].

Published results have suggested that MET not only reduces chronic inflammation through the reduction of hyperglycemia, IR and atherogenic dyslipidemia, but is also has direct anti-inflammatory effects. For example, it has been shown that MET reduces lipopolysaccharide-induced proinflammatory responses in monocytes and macrophages [2], down-regulates Th17 cells in Rheumatoid Arthritis patients [3], decreases TNF- α receptor signaling and NF- κ B activation in endothelial cells [4,5] and smooth muscle cells [6], and inhibits intestinal inflammation and colitis-associated colon cancer in intestinal epithelial cells [7]. MET has also been suggested to have cardioprotective effects through its reduction of inflammation, improvement of lipid profiles [8] and endothelial cell function [9], and retardation of the process of coronary artery calcification [10].

T2D patients are at risk for infections due to influenza or for complications related to it [11–13] and therefore annual influenza vaccination is highly recommended [11]. Viral and bacterial infections and consequent diseases are associated with increased morbidity and mortality in T2D patients, causing loss of metabolic control leading to an increase of glycosylated serum proteins, ketoacidosis which may result in an increased hospitalization rate and mortality rate, and prolonged complications [14,15]. Previously published results have measured T cell function in vaccinated young [12] and elderly [16] T2D patients and have shown reduced [12] or similar [16] responses in patients *versus* healthy controls. When B cell responses were measured in vaccinated young [17] and elderly [17,18] T2D patients, no differences were found in both age groups. Our interpretation of these results showing no different responses between T2D patients and age-matched healthy controls was that all T2D patients recruited were taking MET or other hypoglycemic drugs, such as sulfonylurea or repaglinide, and it is known that a better T2D control, such as glucose and metabolic-related parameters, positively influence the response to the influenza vaccine [18]. No studies have been conducted so far to evaluate the effects of MET on influenza vaccine responses and on B cell function in T2D patients and this is the topic of our present study.

We have investigated the effects of obesity and T2D on *in vivo* and *in vitro* B cell responses in 2 groups of patients: those recently diagnosed but not taking anti-diabetic drugs, and patients taking MET. Our *in vivo* model for immune response uses the influenza vaccine. Our results show that B cell function and vaccine responses, hampered by obesity and T2D, are improved by MET. We have used activation-induced cytidine deaminase (AID) as a marker for optimal B cell function in these studies because we have shown that it positively and significantly correlates with the ability of B cells to undergo class switch [19] and somatic hypermutation, necessary for affinity maturation of antibodies [20]. Moreover, MET used *in vitro* to stimulate B cells from recently diagnosed T2D patients is also able to reduce B cell-intrinsic inflammation and increase antibody responses, similar to what we have seen

in B cells from patients taking MET, who invariably show increased responses to the influenza vaccine in vivo. These results may have significant implications for public health.

2. Materials and methods

2.1. Study subjects

Experiments were conducted using blood isolated from individuals with obesity and T2D (age 57–63 years), after appropriate signed informed consent and were approved with IRB protocol #20070481. T2D patients, screened and diagnosed according to the American Diabetes Association guidelines, were divided into 2 groups: (a) recently diagnosed not taking MET (8 individuals), (b) patients taking MET (15 individuals). Patients were taking 1000 mgs of MET (oral tablet), twice/day. Patients were on MET for at least 3 years before recruitment in our study. No one of them had side effects or had to stop MET treatment before completion of the study. Patients in the 2 groups were matched according to age and BMI (their weight was reported to be stable over a period of 12 months) and they were taking the same lipid control medications. T2D patients had very controlled disease and were taking only the oral hypoglycemic drug MET. All were community-dwelling, highly functional, without autoimmune, cancer, cardiovascular or infectious diseases. No participant was taking insulin. No participant smoked. Routine biological parameters (white and red cell counts, glucose, liver and kidney function, HbA1c) were measured. The demographic and clinical characteristics of the participants are in Table 1.

2.2. Influenza vaccination

The study was conducted during 3 consecutive influenza vaccine seasons. T2D MET-naïve patients were recruited during the 2011–2012 (5) and 2012–2013 (3) seasons. Patients taking MET were recruited during the 2011–2012 (3), 2012–2013 (5) and 2013–2014 (7) seasons. Participants with obesity and T2D were vaccinated with the Trivalent Inactivated Vaccine (TIV) during the 2011–2012, 2012–2013 and 2013–2014 seasons, which were characterized by a vaccine containing the same H1N1 strain which was the pandemic (p)2009 H1N1 strain A/California/7/2009. Blood samples were collected before (t0), 1 (t7) and 4–6 (t28) weeks after vaccination. All participants were immunized at least in the 5 previous seasons, and therefore seroprotected, with a titer 1/40 at t0. The peak of the response was at t7, earlier than previously found by us [21,22] and others [23], due to repeated vaccine immunizations. In most cases, peak titers were maintained through t28.

2.3. Hemagglutination inhibition (HAI) assay

The *in vivo* response was measured by the HAI assay which is the most established correlate of vaccine protectiveness, as previously described [17,21,24–27] and results expressed as reciprocal of the titer after vaccination.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Plasma TNF- α , IL-6, CRP were measured by the following ELISA kits: Life Technologies KHC3013, KHC0062, KHA0032, respectively, following manufacturer's instructions.

2.5. Flow cytometry

One hundred μ l of blood were stained for 20 min at room temperature with the following antibodies: anti-CD19 (BD 555415), anti-CD27 (BD 555441), anti-IgD (BD 555778) to measure naive (IgD+CD27-), IgM memory (IgD+CD27+), switched memory (IgD-CD27+), late/exhausted memory (IgD-CD27-) B cells. After staining, red blood cells were lysed using the RBC Lysing Solution (BD 555899), according to the manufacturer's instructions. Up to 10^5 events in the lymphocyte gate were acquired on an LSR-Fortessa (BD) and analyzed using FACS Diva (BD) software. Single color controls were included in every experiment for compensation.

2.6. B Cell culture

PBMC were collected by density gradient centrifugation using Vacutainer CPT tubes (BD 362761) and cryopreserved. PBMC (1×10^6 /ml) were thawed and B cells isolated from PBMC using anti-CD19 Microbeads (Miltenyi Biotec), according to the Mini-Macs protocol (Miltenyi Biotec), including incubation for 20 min at 4 °C with 20 μ l of beads/ 10^7 cells. Cells were then purified using magnetic columns. At the end of the purification procedure, cells were found to be almost exclusively (>97%) CD19+ by cytofluorimetric analysis. B cells were stimulated with CpG at the concentration of 1 μ g/ 10^6 cells for 5 days. To measure the *in vitro* response of B cells to the influenza vaccine, PBMC were cultured for 5 days with the vaccine at the concentration of 2 μ l/ 10^6 cells.

2.7. In vitro treatment of B cells with MET

B cells at the concentration of 10^6 cells/ml were pre-incubated with MET (1 mM) for 60 min, before RNA extraction or CpG stimulation.

2.8. RNA extraction and quantitative (q)PCR

After sorting with magnetic beads, cells were resuspended in TRIzol (Ambion) (10^6 cells/100 μ l), then RNA extracted for quantitative (q)PCR to measure TNF- α , micro-RNA (miR)-155, miR-16 and U6 as control. Total RNA was isolated according to the manufacturer's protocol, eluted into 10 μ l distilled water and stored at -80 °C until use.

The μ MACS mRNA isolation kit (Miltenyi Biotec) was used to isolate mRNA from cultured B cells, to measure AID. GAPDH was used as control. Reactions were conducted in MicroAmp 96-well plates and run in the ABI 7300 machine. Calculations were made with ABI software. Results from TRIzol or μ MACS extraction show 2^{-Ct} of sample RNA normalized to GAPDH (TNF- α and AID) or to U6 (miR-155 and miR-16). Reagents and primers (Taqman) were from Life Technologies.

2.9. Preparation of total cell lysates and Western blotting (WB)

B cells were harvested, centrifuged and the pellet resuspended in 30 μ l of a solution containing Hepes 10 mM pH 7.9, KCl 10 mM, EDTA 1.0 mM, DTT 1 mM, MgCl₂ 1.5 mM, PMSF 1 mM, 1 tablet of protease inhibitor cocktail (Boehringer Mannheim, Germany) (per 20 ml) and Nonidet P-40 (0.1%), briefly vortexed and centrifuged (8000 rpm, 5 min, 4 °C). The

supernatant containing the cytoplasmic extract was removed and stored at -80°C . Protein content was determined by Bradford assay.

Cytoplasmic protein extracts at equal protein concentration were denatured and then electro-transferred onto nitrocellulose filters which were incubated with the following primary antibodies: anti-phospho-AMPK (#2535, Cell Signaling), anti-phospho-PI3K (#3821, Cell Signaling), anti-AMPK (#5831, Cell Signaling), anti-UBC9 (#617048, BD), in PBS-Tween 20 containing 5% milk. After overnight incubation, immunoblots were incubated with HRP-conjugated polyclonal IgG antibodies (Jackson ImmunoRes Labs) for 1.5 h at 4°C . Membranes were developed by enzyme chemiluminescence and exposed to CL-XPosure Film (Pierce). Films were scanned and analyzed using AlphaImager Enhanced Resolution Gel Documentation & Analysis System (Alpha Innotech) and images were quantitated using the AlphaEaseFC 32-bit software.

2.10. Statistical analyses

Mean comparisons in Fig. 1 were performed by unpaired Student's *t* test (two-tailed). Also in the analysis of Fig. 2, we compared two groups using *t*-test (where the groups are *in vivo* MET-treated *versus in vivo* MET-naïve) on the dependent variables. We made no a priori hypotheses about how other possible groupings might affect the dependent variables, and therefore ANOVA was not used. In Figs. 3–5, three-group ANOVA (multiple comparisons with no pairing) were conducted to examine *in vivo* MET-treated *versus in vivo* MET-naïve *versus in vitro* MET-stimulated B cells on the dependent variables AID (Fig. 3), TNF- α , miR-155, miR-16, BAFF (Fig. 4) p-p85-PI3 K, p-AMPK (Fig. 5). GraphPad Prism version 5 software was used to perform the analyses and construct all graphs.

3. Results and discussion

3.1. MET improves the *in vivo* and *in vitro* antibody response to the influenza vaccine

We evaluated the effect of taking MET on the *in vivo* antibody response to influenza vaccination measured by the HAI assay. Fig. 1A shows that vaccine-specific titers after vaccination are significantly higher in patients taking MET as compared with patients MET naïve, indicating better overall immune cell function.

Then, we wanted to test the effect of taking MET on the *in vitro* antibody response to the vaccine. To do this, we stimulated PBMC from the same individuals as above, before or after vaccination, with the influenza vaccine for 5 days to induce optimal AID mRNA expression. Results in Fig. 1B also show that the *in vitro* AID response to the influenza vaccine was significantly higher in individuals taking MET. These results show for the first time that MET has a strong immunoenhancing effect on influenza vaccine responses in individuals with obesity and T2D.

3.2. MET increases the percentages of circulating switched memory B cells and decreases that of the pro-inflammatory late/exhausted memory B cell subset

We measured the composition of the peripheral B cell pool in this cohort by flow cytometry at t_0 , as we have previously shown that this influences the individual's immune/vaccine

response with higher percentages of switched memory B cells associated to a better response [17,21,28,29]. Switched memory B cells carry the immunization history of the subjects, and can rapidly secrete high affinity antibodies upon antigen encounter, leading to an effective clearance of the influenza infection [30]. Moreover, late/exhausted memory B cells are highly inflammatory [24] and have been associated with impaired responses to influenza and other viral infections [31]. Late/exhausted memory B cells increase with age, they are characterized by high expression of the RNA for inflammatory mediators such as pro-inflammatory cytokines (TNF- α /IL-6/IL-8), inflammatory miRs (miR-155/16/93), and senescence-associated markers (p16^{INK4}, NF- κ B) [32], all of which have been negatively associated with protective immune responses in elderly individuals. The late/exhausted memory B cells, previously identified in healthy individuals [33] and in patients with SLE [34,35], have also been called double negative [36]. Fig. 2 shows significantly higher percentages of switched memory B cells, and significantly lower percentages of late/exhausted memory B cells in the blood of patients taking MET *versus* those MET naïve. No significant differences were found for IgM memory and naïve B cell subsets. Representative dot plots, one for a MET-naïve patient and one for a MET-treated patient, are also shown in Fig. 2 (top).

3.3. MET *in vitro* increases AID and reduces B cell-intrinsic inflammation

In order to find a mechanism to explain these significant *in vivo* effects on B cell function, we next evaluated whether *in vitro* treatment of purified B cells from patients taking MET had an effect on the expression of key players of the antibody response and inflammation processes. We hypothesized that MET *in vitro* could potentiate the function of B cells in obese T2D subjects who showed an impaired antibody response to the influenza vaccine (i.e. those not on MET). First, B cells from T2D patients taking MET and from those MET naïve were stimulated with CpG for 30 min. Additionally, B cells from the patients not taking MET were also pre-incubated with MET, before CpG, for 60 min. Fig. 3 shows that patients not taking MET, *versus* those taking MET, had a significantly lower expression of AID in response to CpG. The expression of AID in response to CpG is a measure of the quality of the B cell response, and we have shown that it can also predict the *in vivo* antibody response to the influenza vaccine [20,22,25,37]. Interestingly, pre-treatment of the B cells with MET for 30 min before CpG stimulation significantly increased AID mRNA expression to that seen in patients taking MET. Thus, MET *in vitro* was able to reverse the low responsiveness shown by B cells in T2D subjects which are MET naïve.

This novel and exciting result lead us to ask if MET could also reduce the expression of inflammatory mediators known to have a negative effect on the antibody response. We have previously shown that high TNF- α expression in B cells before stimulation can make them refractory to further stimulation, and this can be reversed by antibodies blocking TNF- α signaling both *in vitro* [24] and *in vivo* in mice [38]. We evaluated the expression of TNF- α in *ex vivo* isolated, unstimulated B cells from T2D patients taking or not taking MET. B cells from T2D patients taking MET and from those not taking MET were isolated and RNA was extracted. B cells from the patients not taking MET were also pre-incubated for 30 min with MET before RNA extraction. Results in Fig. 4A show that patients not taking MET had increased expression of B cell TNF- α RNA as compared to subjects taking MET. More

importantly, treatment of B cells from patients MET naive with MET *in vitro* significantly reduced RNA expression of TNF- α in B cells. Thus, the significant impact of *in vivo* MET of reducing B cell TNF- α can be mirrored by treating B cells *in vitro* with MET.

We went on to evaluate the effects of MET on the expression of miR-155 and miR-16 in B cells. The amounts of these two miRs are elevated in B cells from healthy elderly individuals and associated with a lower antibody response to the influenza vaccine [39]. In particular, miR-16 is known to reduce the expression of the E47 transcription factor [39], which is key to the expression of AID and the quality of the antibody response [40], while miR-155 is known to interact directly with and inhibit the mRNA of AID [39,41,42]. As Fig. 4B and C shows, taking MET significantly reduces the expression of both miR-155 and miR-16 in unstimulated B cells from subjects with T2D. Also, the *in vivo* MET effect could be effectively achieved on B cells of T2D subjects not taking the drug by briefly treating the cells with MET *in vitro* before RNA extraction. *In vitro* incubation with MET did not modify RNA levels of BAFF, the B-cell-activating factor of the TNF family, which is a survival factor for B cells [43], used here as control. BAFF RNA levels were comparable in the 3 groups (Fig. 4D).

Results in Fig. 4 altogether show for the first time that MET, both *in vivo* and *in vitro*, can significantly reduce the amount of inflammatory mediators in B cells associated with decreased antibody responses to influenza vaccination, which in turn could lead to a beneficial effect in potentiating these responses in T2D patients.

In conclusion, similar to what has been shown in non immune cells, MET seems to have a direct anti-inflammatory action on B cells. As a consequence of reduced B cell-intrinsic inflammation, B cell function is improved, as we have previously shown in humans [24] and mice [38]. This effect is parallel to the hypoglycemic action of MET which could also contribute to improve B cell function.

3.4. MET induces AMPK phosphorylation in B cells

To investigate more thoroughly the possible mechanisms of these effects of MET on B cells in obese T2D patients, we set up experiments to evaluate the expression of molecules shown to be key players in MET signaling in different cell types. We focused our attention on one of the best characterized MET targets, AMPK, and its upstream signaling protein PI3K. Results in Fig. 5 shows that MET *in vitro* increases the phosphorylation of AMPK and of its upstream signaling protein PI3K in B cells from recently diagnosed T2D patients, similar to what is observed in B cells from T2D patients taking MET. AMPK is a heterotrimeric enzyme composed of a catalytic subunit α and two regulatory subunits, β and γ [44,45]. AMPK becomes activated by decreases in the ratios of ATP/ADP and phosphocreatine (PCr)/creatine through mechanisms involving phosphorylation by one or more upstream AMPK kinases. The increase in AMPK activity results in the stimulation of glucose uptake in muscle, fatty acid oxidation in muscle and liver, and the inhibition of hepatic glucose production, cholesterol and triglyceride synthesis, and lipogenesis [46,47]. MET also significantly increases AMPK activity in cultured rat hepatocytes and isolated muscles [48]. Importantly, the activation of the enzyme is associated with reduced glucose production by

the hepatocytes and higher muscle glucose uptake [49]. It has not been shown so far that AMPK is also a target of MET in B cells.

In conclusion, our results herein show that MET improves B cell responses through reduction in B cell-intrinsic inflammation and increased AMPK function. Based on this evidence, we can hypothesize that MET may influence fundamental aging factors that underlie multiple age-related conditions. Numerous controlled clinical trials have been completed or are in progress to examine effects of MET on specific outcomes in different patient populations. One of these clinical trials is using MET to target aging and age-related diseases (i.e. “Targeting Aging with Metformin”, TAME study). The clarification of the effects of MET on immune function will open the possibility of novel therapeutic applications of this drug, which may improve immune responses through a therapeutic manipulation of systemic metabolism also in different inflammatory-based conditions. Moreover, the mechanistic overlap of metabolic changes in different disorders suggests that AMPK may be a therapeutic target for the treatment of multiple disorders, holding significant therapeutic promise. A better understanding of AMPK regulation will also help to identify novel AMPK activators, which may act in concert with AMPK activators to ameliorate IR.

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Fig. 1.

The use of MET by T2D patients ameliorates their *in vivo* and *in vitro* antibody response to the influenza vaccine. (A) Participants (all obese T2D) were recruited during the 2011–2012, 2012–2013 and 2013–2014 influenza vaccine seasons. Results are expressed as reciprocal of the titer after vaccination at t7. Mean comparisons between groups were performed by Student's *t* test (two-tailed). **p* < 0.05. (B) PBMC isolated from the blood of the same participants in Fig. 1A before and after vaccination were stimulated with the influenza vaccine for 5 days. Results show fold-increase in AID mRNA expression [qPCR values (2^{-C_t}) of AID mRNA normalized to GAPDH] after vaccination calculated as follows: qPCR values after vaccination/qPCR values before vaccination. **p* < 0.05.

Fig. 2.

T2D patients on MET have increased switched memory (SM) B cells and less late/exhausted memory (LM) B cells. Participants (all obese T2D) are the same as in Fig. 1. One hundred μ l of blood were stained to measure switched memory (SM, IgD-CD27+), IgM memory (IgD+CD27+), naïve (IgD+CD27-), late/exhausted memory (LM, IgD-CD27-) B cells. One hundred μ l from the same individuals were also stained to measure transitional B cells (CD24^{bright}CD38^{bright}). *Top.* Representative dot plots, one for a MET-naïve patient and one for a MET-treated patient, are shown. Gates are set based on isotype controls. *Bottom.* Results are expressed as percentages of CD19 + B cells. * $p < 0.05$. ** $p < 0.01$.

Fig. 3.

MET *in vitro* increases the expression of AID mRNA. Participants (all obese T2D) are the same as in Fig. 1. B cells from T2D patients taking MET and those not taking MET were stimulated with CpG for 5 days. The mRNA was extracted, RT reactions performed in the presence of random primers for AID and GAPDH (control) evaluation. Then qPCR were performed. B cells from patients not taking MET were also pre-incubated with MET, before CpG stimulation, for 60 min. Results show qPCR values (2^{-C_t}) of AID (normalized to GAPDH) RNA expression. * $p < 0.05$. ** $p < 0.01$.

Fig. 4.

The use of MET *in vitro* decreases the expression of TNF-α, miR-155 and miR-16 in unstimulated B cells from recently diagnosed T2D patients. Participants (all obese T2D) are the same as in Fig. 1. B cells from T2D patients taking MET and those not taking MET were resuspended in Trizol, RNA was extracted, RT reactions performed in the presence of random primers for TNF-α (A), BAFF (D) and GAPDH (control). B cells from patients not taking MET were also pre-incubated with MET, before Trizol, for 60 min. Results show qPCR values (2^{-C_t}) of TNF-α and BAFF (normalized to GAPDH). For miRs evaluation, B cells were resuspended in Trizol, RNA was extracted, RT reactions performed in the presence of specific primers for miR-155 (B), miR-16 (C) or U6 (control) evaluation. Then qPCR were performed. B cells from patients not taking MET were also pre-incubated with MET, before Trizol, for 60 min. Results show qPCR values (2^{-C_t}) of miR-155 and miR-16 (normalized to U6). **p < 0.01. ***p < 0.001. ****p < 0.0001.

Fig. 5.

MET *in vitro* increases the phosphorylation of AMPK and of its upstream activator p85-PI3K in B cells from recently diagnosed T2D patients. Participants (all obese T2D) are among those in Fig. 1. B cells from T2D patients taking MET and those not taking MET were stimulated with CpG for 30 min. B cells from the patient not taking MET were also pre-incubated with MET, before the stimulation with CpG, for 60 min. Cytoplasmic protein extracts were prepared and analyzed in Western blot. Total UBC9 (or total AMPK) was used as loading control. Results are representative of 4 independent experiments done on individuals with sufficient numbers of B cells to perform protein extraction and Western blot experiments. (A) A representative Western blot is shown. Densitometric analyses (arbitrary units) of p-p85-PI3K (normalized to UBC9) (B) and p-AMPK (normalized to total AMPK) (C)

(C) expression are shown and referred to the value in patients taking MET *in vivo* (taken as 1). **** $p < 0.0001$.

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Table 1

Demographic and clinical characteristics of T2D patients.

	No MET	MET
Participants (n)	8	15
Age (mean years $\pm$ SE)	59 $\pm$ 2	61 $\pm$ 3
BMI	33 $\pm$ 2	31 $\pm$ 1
Gender (M/F)	4/4	8/7
Race (W/B)	4/4	8/7
Ethnic Categories (Hispanic/Non Hispanic) ^a	4/4	6/9
Glucose (mg/dL)	130 $\pm$ 4	97 $\pm$ 3 *
HbA1c (%)	6.9 $\pm$ 0.2	5.0 $\pm$ 1.5 *
Total cholesterol (mmol/L)	4.5 $\pm$ 0.3	2.1 $\pm$ 0.2 *
Triglycerides (mmol/L)	1.7 $\pm$ 0.2	1.1 $\pm$ 0.1 *
HDL (mmol/L)	1.3 $\pm$ 0.3	0.9 $\pm$ 0.1
LDL (mmol/L)	3.7 $\pm$ 0.2	1.1 $\pm$ 0.1 **
TNF- α (pg/mL)	9 $\pm$ 2	10 $\pm$ 3
IL-6 (pg/mL)	102 $\pm$ 13	95 $\pm$ 21
CRP (pg/mL)	451 $\pm$ 63	400 $\pm$ 21

Plasma inflammatory cytokines were measured by ELISA and results are means $\pm$ SE. Prevalence of CMV-seropositivity was comparable between the 2 groups. All subjects were CMV IgM-negative, which indicates chronic CMV infection and no viral reactivation.

^a All races. Hispanic are individuals from Mexico, Puerto Rico, Cuba, Central/ South America and from other countries with Spanish culture or origin.

*
p<0.05.

**
p<0.01.