

Increased Levels of AIM2 and Circulating Mitochondrial DNA in Type 2 Diabetes

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ABSTRACT

Background: Chronic inflammation has critical role in Type 2 diabetes (T2D), in which IL-1 β contributes in insulin resistance and beta cell dysfunction. The activation of NLRP3 and AIM2 by endogens ligands, such as mtDNA can lead to the release of active form of IL-1 β . **Objective:** To evaluate AIM2 expression and activation as well as circulating mtDNA levels in T2D patients. **Methods:** AIM2 expression was analyzed by flow cytometry, it's activity was assessed by measuring in vitro release of IL-1 β induced by Poly (dA:dT), and mtDNA copy number was determined by quantitative real-time polymerase chain reaction. **Results:** Increased percent of AIM2+ cells were detected in monocytes from patients with T2D. Moreover, increased levels of IL-1 β in monocytes cultures from T2D patients compared to healthy controls were observed. Also, association between AIM2+ cells and hyperglycemia ($r=0.4385$, $P=0.0095$) and triglycerides levels ($r=0.5112$, $P=0.002$) and waist-hip ratio ($r=0.4710$, $P=0.0049$) were detected. Likewise, the mtDNA copy number was augmented in T2D patients compared to control group. The mtDNA copies number was associated with body mass index ($r=0.4231$, $P=0.0008$) and TNF- α levels ($r=0.5231$, $P=0.0005$). In addition, increased levels of IL-12p70, TNF- α , IL-10, IL-6, IL-8 and IL-1 β were detected in a serum from T2D patients. **Conclusion:** These results suggest the involvement of AIM2 and mtDNA in the inflammatory process seen in T2D.

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Keywords: AIM2, Cell-free mtDNA, IL-1 β , Inflammasome, Type 2 Diabetes

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INTRODUCTION

Type 2 diabetes (T2D) is a major public health problem in Mexico, which, with a prevalence of 11.92%, ranks sixth among the countries with the highest number of patients (1). T2D is characterized, among others, by hyperglycemia and defects in insulin secretion and action along with a low-grade inflammatory process characterized by an increased synthesis of pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF- α . These cytokines conduce to insulin resistance by promoting an inhibitory phosphorylation of the insulin receptor substrate (IRS-1) (2-4) and inhibiting insulin gene expression (5). Research has elucidated the role of an increased synthesis of IL-1 β as a risk factor for T2D (6). Moreover, it has been reported that the neutralization of IL-1 β improves the glycemic control and enhances function of pancreatic β cells in patients with T2D (7,8). The secretion of IL-1 β by monocytes and neutrophils is dependent on the activation of the NLRP3 and AIM2 inflammasomes (9). Accordingly, when activated, these inflammasomes form a multi-protein platform that induce the activation of caspase-1, which, in turn, cleaves the immature form of IL-1 β (pro-IL-1 β), promoting the secretion of the active form of the cytokine. NLRP3 inflammasome assembly requires the interaction of ASC, pro-Caspase-1 and NLRP3 subunits, which is mediated by pyrin and CARD domains (10). Similarly, the assembly of AIM2 inflammasome requires the polymerization of AIM2 and ASC subunits and their interaction with Caspase-1 (11). Both NLRP3 and AIM2 can be activated by endogenous ligands, including DNA, islet amyloid polypeptide, cholesterol crystals and saturated fatty acid (palmitate)(12,13). In addition, activating innate immunity is a result of the release into circulation of mitochondrial endogenous damage-associated molecular patterns (MTD) which include formyl peptides and mitochondrial DNA (mtDNA); these molecular patterns induce the phosphorylation of p38 and p44/p42 MAPK and generate pro-inflammatory cytokines such as interleukin (IL)-6, IL-8 and tumor necrosis factor (TNF)- α through their interaction with formyl peptide receptor-1 and Toll-like receptor (TLR)-9, respectively. The participation of cell-free mtDNA has been demonstrated in such inflammatory diseases as rheumatoid arthritis (14); however, its contribution to the pathogenesis of T2D is an interesting subject yet to be elucidated. Delving deeper into the mechanism involved in the pathogenesis of T2D, it becomes clear that a significant increase occurs in the expression of the NLRP3 and the secretion of IL-1 β by monocytes from T2D patients cultured under hyperglycemic conditions (15). The role of AIM2 in inflammatory diseases such as systemic lupus erythematosus has already been explained (16); on the other hand, its function in the inflammatory state of the T2D remains unknown.

Given the central role of IL-1 β in the inflammatory process of T2D, and the fact that the activation of AIM2 and NLRP3 inflammasomes by endogenous ligands triggers IL-1 β production, this research assesses the expression and activation of AIM2, and cell-free mtDNA levels in patients with T2D and healthy subjects.

MATERIALS AND METHODS

Samples. Sixty-eight patients who fulfilled the American Diabetes Association (ADA) diagnostic criteria concerning T2D, including those who were primarily diagnosed with T2D within the last 20 years, were recruited at the Family Medical Unit number 4 of

Mexican Institute of Social Security, Zacatecas, Mexico; and at the Internal Medicine Unit and Endocrinology of the Central Hospital, San Luis Potosi, Mexico. A total of 65 T2D patients including 40 females and 25 males, with a mean of age 50.6 ± 7.9 years, were enrolled. Excluded from the study were T2D patients with microvascular and macrovascular complications treated with corticosteroids and pregnant women. Forty-three healthy subjects were eligible as their plasma glucose levels were ≤ 126 mg/dL. Patients and healthy subjects were excluded if diagnosed with any infectious diseases within fifteen days of sample collection. Weight, height, and hip and waist circumference were obtained in accordance with conventional procedures. It was assumed that the inclusion of approximately 30 T2D patients and 30 controls in each group would suffice to reach statistical differences (with an alpha value 0.049, and a confidence level of 95%, Graph Pad Statmate 2, Graph Pad Inc. 3.0, San Diego, CA). Anthropometric and biochemical parameters of the participants are illustrated in Table 1. As expected, we observed a statistically significant increase in plasma glucose levels and glycated hemoglobin A1c (HbA1c) percentages in patients with T2D compared to healthy controls (Table 1). The present research was approved by the bioethical committee of the National Commission for Scientific Research of IMSS (project R-2011-785-063); patients and healthy volunteers were provided with written informed consent prior to participation in the study.

Table 1. Anthropometric measurements and biochemical parameters of the healthy controls and T2D patients.

	Healthy controls (n=43)	T2D (n=65)	<i>p</i> value
Disease evolution (years)	---	9.39 ± 6.54	
<i>Demographic characteristics:</i>			
Age (years)	47.49 ± 7.54	50.03 ± 7.67	0.0526
Sex (F/M)	33/13	40/25	
<i>Anthropometric measurements:</i>			
Weight (kg)	75.50 ± 13.70	78.15 ± 14.55	0.3527
BMI (kg/m ²)	27.93 ± 6.05	30.04 ± 5.98	0.3607
WHR	0.91 ± 0.07	0.94 ± 0.06	0.0105*
<i>Biochemical parameters:</i>			
Glucose (mmol/L)	9.32 ± 4.19	4.87 ± 0.79	< 0.0001*
HbA1c (mmol/mol)	72.01 ± 29.54	38.49 ± 4.45	< 0.0001*
Triglycerides (mmol/L)	2.68 ± 1.52	2.35 ± 1.19	0.3322
t-Cholesterol (mmol/L)	4.89 ± 1.21	5.06 ± 1.00	0.4589
HDL-c (mmol/L)	1.31 ± 0.33	1.39 ± 0.37	0.1993
LDL-c (mmol/L)	2.42 ± 0.94	2.58 ± 0.70	0.3386
Serum creatinine (mmol/L)	0.074 ± 0.057	0.047 ± 0.016	0.0639

Data correspond to the mean \pm SD. * statistically significant. BMI, body mass index; WHR, waist-hip ratio; HbA1c, glycated hemoglobin; t-Cholesterol, total cholesterol; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol.

Monocyte Cell Isolation. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Histopaque density gradient centrifugation (Sigma-Aldrich Co., St. Louis, MO) and washed twice with a phosphate-buffered saline (PBS) solution containing 0.5%

bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO, USA) and (ethylenedinitrilo)-tetraacetic acid (EDTA) (J.T. Baker, D.F. Mexico). The monocytes were purified through magnetic isolation (Miltenyi Biotec, Auburn, CA, USA) following the manufacturer's instructions. Monocyte purity was verified via flow cytometry with an anti-human CD14 antibody conjugated to fluorescein isothiocyanate (FITC) (eBioscience, San Diego, CA, USA). Purity >85% was considered adequate.

Cell Cultures and Stimuli. Cells were cultured in 24-well plates containing RPMI 1640 medium (GIBCO BRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO BRL, Rockville, MD, USA), 50 U/mL penicillin, and 50 µg/mL streptomycin (Sigma Chemical Co., St. Louis, MO, USA); these plates were maintained at 37°C in a 95% humidified atmosphere of 5% CO₂. The cells were stimulated at a density of 1×10^5 monocytes/well with 10 µg/mL lipopolysaccharide (LPS) (Sigma) and incubated for 2 hours under the above-mentioned conditions. Next, 5 mM adenosine triphosphate (ATP, Sigma) or 20 µM nigericin (Sigma) was added to the cells and incubated for 30 minutes. In a different group of experiments, cells were incubated with 2.5 µg/mL poly (dA:dT) (poly(deoxyadenylic-deoxythymidylic) acid sodium salt) (InvivoGen, San Diego, CA) overnight under similar incubation conditions. Lipofectamine 2000 reagent (Invitrogen) was utilized as a vehicle for Poly (dA:dT). Following the incubations of each experiment, the culture supernatants of monocytes were harvested and stored at -20°C until IL-1β measurements.

Interleukin-1β Measurement. Interleukin-1β (IL-1β) was measured in the culture supernatants of monocytes by ELISA (eBioscience) according to manufacturer's instructions. IL-1β was quantified through measuring the absorbance at 570 nm in a microplate reader spectrophotometer (Multiskan ex. 1.0, Labsystems).

Western Blot Analysis. Cells (2×10^6) were lysed with cell lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, NP-40 1%, 1 mM EDTA, 1 mM PMSF, Complete Protease Inhibitor 1X, and 50 mM NaF and 2 mM Na₃VO₄). Supernatants were collected and the protein concentration was specified. Prior to separation on 10 or 12% SDS-PAGE, samples were denatured at 95°C for 5 min in Laemmli's sample buffer, and transferred onto nitrocellulose membranes which were blocked for 1 h with Tris-buffered saline containing 5% dried fat-free milk and 0.1% Tween-20. These membranes were then incubated overnight at 4°C with the respective antibody anti-AIM2 (1:1000) (Abcam, Cambridge; USA) or anti NLRP3 (1:2000) (Abcam, Cambridge; USA). They were further washed and incubated with HRP-conjugated secondary antibody (1:10000) for 1 h at room temperature. Ultimately, immunoreactive proteins were detected through chemiluminescence by use of an ECL western blot detection kit (Amersham Biosciences).

Flow Cytometry Analysis. To specify the percentage of cells expressing intracellular proteins NLRP3 and AIM2, PBMCs were washed with a staining buffer containing PBS plus 1% FBS and 0.09% (w/v) sodium azide. Cells were then permeabilized using a cytofix/cytoperm solution supplied by BD Biosciences and incubated for 20 minutes at 4°C. Next, 1 µg anti-human NLRP3 monoclonal antibody (Abcam, Cambridge, MA) or 1 µL anti-human AIM2 polyclonal antibody (dilution of 1:10) (Thermo Scientific, Meridian Road Rockford, IL) was added to the solution and incubated for 30 minutes at 4°C. Finally, 1 µL of goat anti-mouse IgG polyclonal secondary antibody conjugated with DyLight 488 (dilution of 1:20), or 1 µL of goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC) (dilution of 1:20) was added, and the cells were incubated for 30 minutes at 4°C. The PBMCs were fixed with 2% paraformaldehyde

and analyzed in a flow cytometer (FACS Canto II, DB Biosciences).

DNA Isolation. Total DNA was extracted from 200 μ L of plasma using a QIAamp DNA Blood Mini Kit (Qiagen, Ca, EUA), according to the manufacturer's instructions. The final DNA was eluted with 70 μ L of distilled water and stored at -20°C until use.

Plasmid Construction and Assay Calibration. A 455-bp mitochondrial DNA segment was amplified using the primers MIT-6356-Fw and MIT-6810-Rv. The PCR product was inserted into a pGEM T-easy vector following the recommendations of the manufacturer (pGEM®-T Easy Vector System, Promega). After bacterial transformation, ten colonies were picked and grown overnight in a Luria-Bertani growth medium containing 100 $\mu\text{g}/\text{mL}$ carbenicillin as a selection agent. The plasmid DNA was extracted using the method delineated by Sambrook and Rusell (17). Four clones were selected and sequenced via T3 and T7 primers in order to confirm the identity of the cloned insert and the fact that a single copy was inserted into the recombinant plasmid. One confirmed clone was utilized as a calibrator for mtDNA quantification. The 455-bp mitochondrial DNA copy number was calculated following the procedure in 'Creating Standard Curves with Genomic DNA or Plasmid DNA Templates for Use in Quantitative PCR (Applied Biosystems). Serial dilutions of the selected clone, ranging from 10^9 – 10^2 copies of the insert / 4 μ L, were prepared to generate a standard curve.

Quantitative Real-time PCR. The quantitative real-time PCR was performed as follows: The 10 μ L PCR amplification reaction mixture contained 2X SYBR® Green Master Mix (Bio Rad), 200 nM of primers MitQt-6451-Fw (5'-CTTCGTCTGATCCGTCCTAATC-3') and MitQt-6549-Rv (5'-TTGAGGTTGCGGTCTGTTAG-3'), amplifying a 99-bp fragment of the cloned insert, and 4 μ L of each plasma sample or each calibrator as a template DNA. Each experiment included a no template control (NTC). The PCR reactions were run in a CFX96 Touch™ Real-Time PCR Detection System under the following cycling conditions: 95°C for 3 minutes followed by 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds for annealing and extension. The specificity of the PCR products was confirmed through melting-curve analysis. Each sample was analyzed in triplicate, the standard curve was included in each run, and data were collected and analyzed using CFX Manager Software 2.1 (Bio Rad). Mitochondrial DNA concentrations in the plasma samples were calculated as previously described (18).

Interleukins Measurements in Serum. A cytometric bead array (CBA, BD Biosciences San Jose, CA) was used following the supplier's instructions so as to measure the levels of Interleukin-12p70 (IL-12p70), Tumor Necrosis Factor (TNF), Interleukin-10 (IL-10), Interleukin-6 (IL-6), Interleukin-1 β (IL-1 β) and Interleukin-8 (IL-8) in the serum from T2D patients and healthy controls. The acquisition and analysis were carried out in a FACSCanto II Flow Cytometer through the use of DIVA software (DB Biosciences San Jose, CA U.S.A.).

Statistical Analysis. Statistical analysis was performed using Graph Pad Prism 5.0 software (San Diego, CA, USA). And data distribution was specified employing the Kolmogorov–Smirnov test. Nonparametric analysis via Kruskal–Wallis, or the Mann–Whitney U test, determined the differences in the expression percentages of NLRP3 or AIM2 or their activity among groups. The association between the expression of AIM2 and biochemical parameters was assessed by use of Spearman's correlation analysis, where $p < 0.05$ was considered significant. Nonparametric analysis using the Mann–Whitney U test determined the differences in the serum levels of cytokines and the number of mtDNA copies.

RESULTS

The Increase in the AIM2 Inflammasome Expression of the Monocytes from T2D Patients.

The specificity of the AIM2 and NLRP3 antibodies was firstly tested by Western blot on PBMC lysates. Western blot analysis of NLRP3 showed a strong band of approximately 118-kDa, which is the usual mobility predicted for NLRP3 (Figure.1a). As far as AIM2 is concerned, a 40-kDa band was detected that was augmented based on the amount of the loaded protein (Figure. 1b).

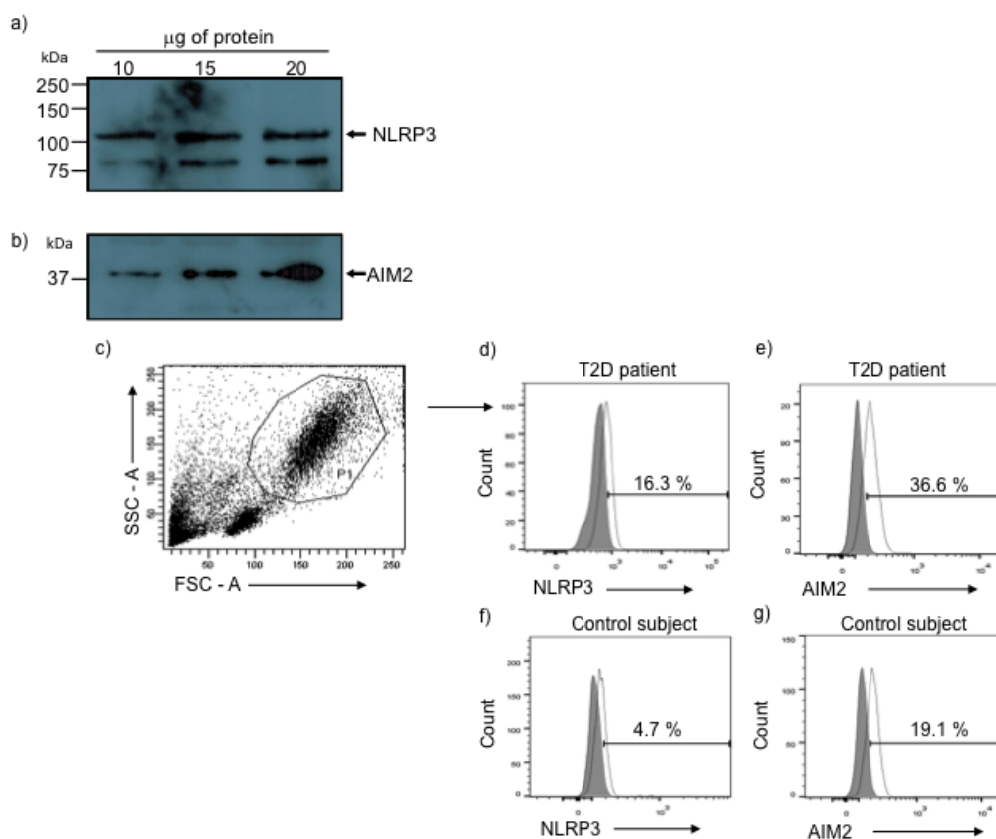


Figure 1. Expression of NLRP3 and AIM2.(a) NLRP3 and (b) AIM2 expression by Western blot. Lysates from PBMC were prepared for the immunoblotting with the indicated antibodies as depicted in the material and methods section. (a-b) The protein molecular weight marker is shown in the left and the µg of protein on the top. One representative blot is shown from three independent experiments. Flow cytometry analysis and gating strategy of peripheral blood mononuclear cells (PBMC). (c) The gate was allocated on the monocyte region according to cell-size (FCS) and granularity (SSC) parameters. (d-e) Histograms show the percentage of NLRP3 positive cells and AIM2 positive cells from the monocyte gate (respectively) for one T2D patient. (f-g) Histograms show the percentage of NLRP3 positive cells and AIM2 positive cells (respectively) from the monocyte gate for one control subject.

Further examined via flow cytometric analysis was the expression of NLRP3 and AIM2 in monocytes from the patients and healthy subjects. Figures 1c-g illustrate the flow

cytometry results and gating strategy of peripheral blood mononuclear cells. Similar percentages of NLRP3+ cells were observed in both groups (Figure.2a). Compared with healthy subjects, on the contrary, the frequency of AIM2+ cells was higher in T2D patients (particularly those with elevated percentages of HbA1c) (Figure. 2(b,c)).

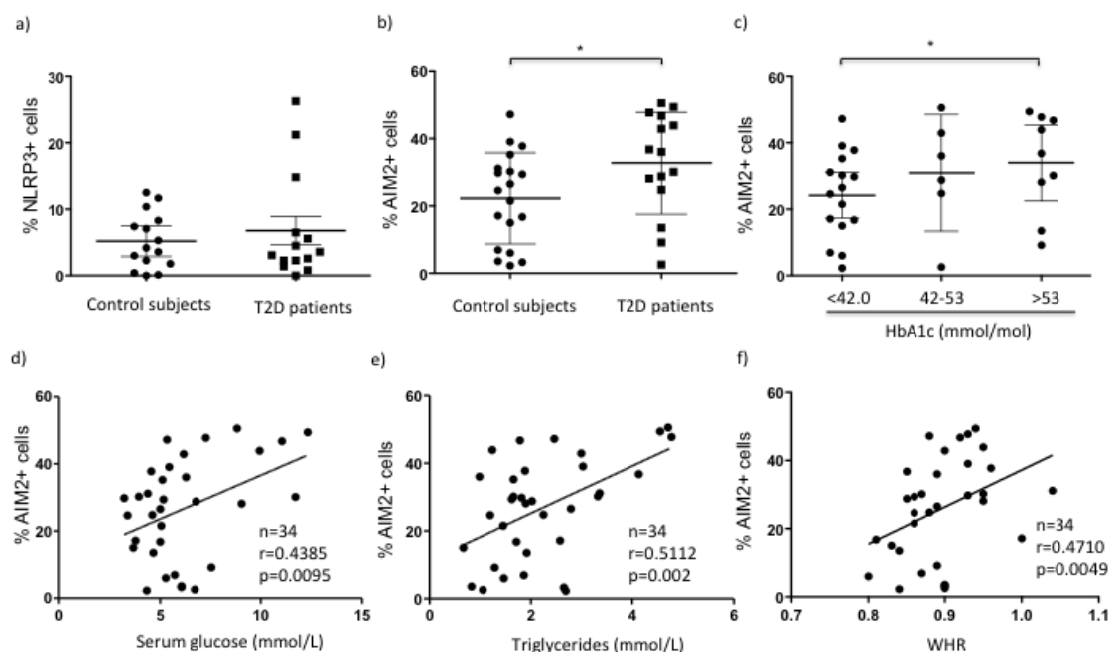


Figure 2. Expression of NLRP3 and AIM2 in monocytes from T2D and healthy controls. (a) PBMC from T2D patients (n=15) and healthy controls (n=20) were immunostained for the detection of NLRP3 and AIM2 by flow cytometry. (b) Percentage of NLRP3 positive cells in monocytes. (c) Percentage of AIM2 positive cells in monocytes. (d) Percentage of AIM2 positive cells in monocytes from control subjects and T2D patients classified according the HbA1c. Correlation analysis between AIM2 positive cells and (e) serum glucose levels (f) triglycerides levels waist-hip ratio. (b-d) Arithmetic mean and SD are indicated. Statistical analysis was performed using the Kruskal–Wallis, or the Mann–Whitney U test. *P<0.05 was considered significant. (d-f) Correlation analysis was performed using the Spearman's correlation test. Correlation coefficient (r), n and p values are indicated.

Correlation between AIM2+ Cells and Biochemical Parameters.

Also analyzed was the correlation between the percentages of NLRP3+ cells or AIM2+ cells and the biochemical parameters of patients with T2D. A significantly positive correlation was observed between the percentage of AIM2+ cells and the serum levels of glucose (Figure. 2d), triglycerides (Figure. 2e) and the waist-hip ratio (WHR) values (Figure. 2f). On the other hand, no relationship was found between the percentage of NLRP3+ cells and the evaluated biochemical parameters (data not shown).

IL-1 β Levels in Monocyte Cultures from T2D Patients and Healthy Controls.

To assess the activity of inflammasomes in the monocytes of patients and healthy individuals, monocytes isolated from peripheral blood mononuclear cells (PBMCs) were stimulated with synthetic DNA (poly (dA:dT)), ATP or nigericin, and the IL-1 β release was measured by ELISA assay. In the presence of a poly (dA:dT), increased

levels of IL-1 β were observed in monocyte cultures of patients comparisons with healthy controls (Figure. 3a). However, in monocyte cultures treated with stimuli specific to NLRP3 inflammasome activation, such as ATP or nigericin, similar levels of IL-1 β were seen in both groups (Figure. 3c). It has already been elucidated that glyburide, a drug used for T2D treatment, is able to inhibit NLRP3 inflammasomes; moreover, no differences were detected between the groups concerning the expression of this molecular complex; therefore, we decided to classify the patients according to their therapy, with or without glyburide, observing a decrease in the release of IL-1 β in the monocytes of patients receiving the drug (Figure. 3d). In a previous report (19), similar levels of AIM2 activation were observed in patients either receiving glyburide or not (Figure. 3b).

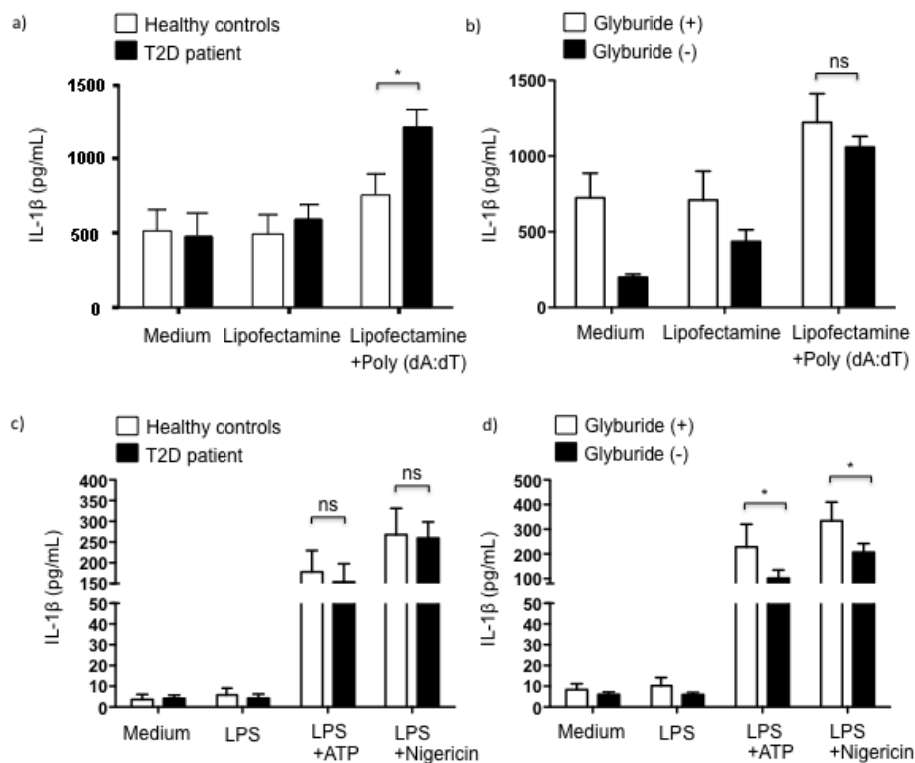


Figure 3. Activity of AIM2 and NLRP3 in monocytes cultures from T2D patients and controls subjects.(a-b) Freshly isolated monocytes were transfected with synthetic DNA, Poly (dA:dT) (2.5 μ g/mL) using lipofectamine (2.5 μ g/mL) overnight at 37°C. Likewise, (c-d) freshly isolated monocytes were primed with 10 μ g/ml LPS and then treated or not with 5mM ATP for 30 minutes at 37°C. At the end of the incubation period, the levels of IL-1 β were measured in the cell culture supernatants using ELISA, as stated in methods section. (a) Effect of Poly (dA:dT) (specific ligand of AIM2) on IL-1 β release in monocytes from healthy controls (white bars) (n=20) and T2D patients (black bars) (n=15). (b) Effect of ATP (5mM) and nigericin (20 μ M) on IL-1 β release in monocytes from healthy controls (white bars) (n=20) and T2D patients (black bars) (n=15). (b and d) The T2D patients were classified according to use of glyburide (or not) as treatment for T2D. Arithmetic mean and SD of the IL-1 β levels in monocyte cultures supernatants are shown. Statistical analysis was performed using the two ways ANOVA with Bonferroni post hoc test. *P<0.05 was considered significant.

Increase in the Cell-Free Mitochondrial-DNA Levels in Serum from T2D Patients.

The serum levels of cell-free mtDNA from patients and controls were quantified via real-time PCR as delineated in the materials and methods section where the number of mtDNA copies/mL in serum was significantly higher in the patients (Figure. 4a). Furthermore, a correlation analysis was carried out between mtDNA copies number and biochemical parameters, as a result of which, a positive association was observed between mtDNA copy number and body weight and body mass index (BMI) (Figure. 4b and 4c, respectively). Further specified was the relationship between pro-inflammatory cytokine levels and mtDNA copy number, where a positive association was only observed between mtDNA copy number and TNF- α levels ($r=0.5231$, $p=0.0005$) (data not shown).

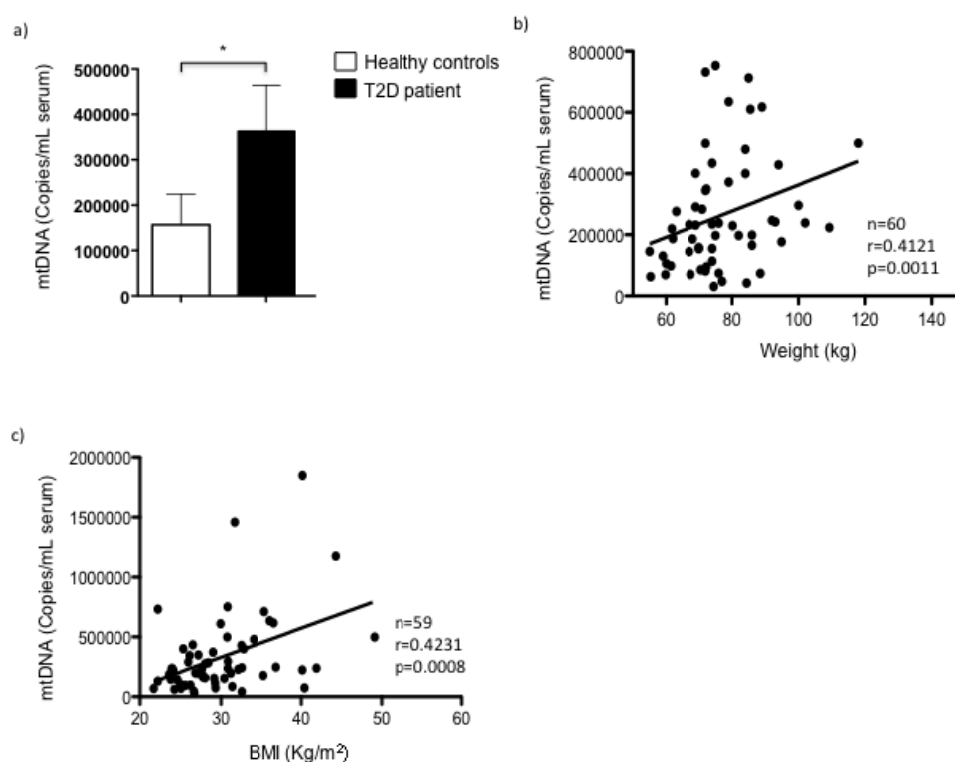


Figure 4. Levels of cell-free mtDNA in serum of T2D patients and healthy controls.(a) Serum levels of cell-free mtDNA from T2D patients and healthy controls were performed using real time PCR as depicted in material and methods section (a) Correlation analysis between mtDNA copies number and (b) weight and (c) body mass index (BMI) values. Arithmetic mean and SD are indicated. Statistical analysis was performed using the Mann–Whitney U test. * $P<0.05$ was considered significant. Horizontal brackets show statistically significant differences ($p<0.05$) between the means of the indicated groups. (c-d) Correlation analysis was performed using the Spearman's correlation test. Correlation coefficient (r), n and P values are indicated.

Levels of Pro-Inflammatory Interleukins in Serum.

Finally, patient and control serums were tested for the presence of IL-12, IL-6, IL-10, TNF- α , IL-1 β , and IL-8. It was observed that the levels of IL-12, TNF- α , IL-10, IL-6,

IL-8 and IL-1 β were higher in patients with T2D (Figure. 5a-f), although only IL-12p70 and IL-8 had significantly elevated levels compared to the controls.

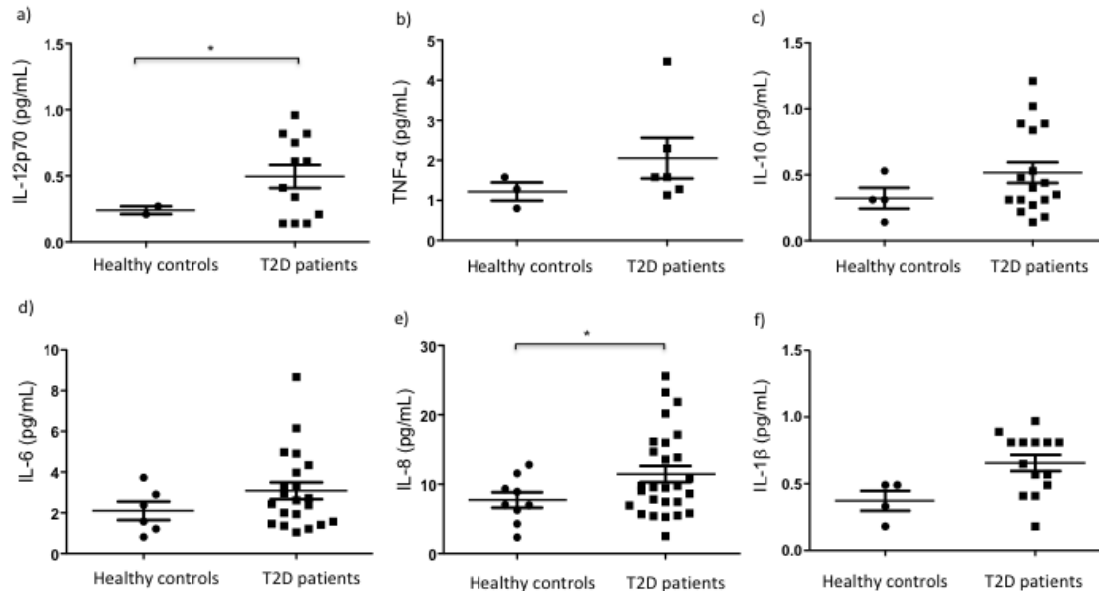


Figure 5. Interleukins levels in serum from T2D patients and healthy controls. A cytometric bead array (CBA) measurement of (a) Interleukin-12p70 (IL-12p70), (b) Tumor Necrosis Factor (TNF), (c) Interleukin-10 (IL-10), (d) Interleukin-6 (IL-6), (e) Interleukin-1 β (IL-1 β) and (a) Interleukin-8 (IL-8), concentration in serum from healthy control subjects or T2D patients. (a-f) Arithmetic mean and SD are indicated. Statistical analysis was performed using the Mann–Whitney U test. * $P < 0.05$ was considered significant. Horizontal brackets show statistically significant differences ($P < 0.05$) between the means of the indicated groups.

DISCUSSION

IL-1 β plays a key role in T2D pathology, damaging β -cells of the pancreas and conducting to insulin resistance. Several mechanisms have been posited regarding IL-1 β release. For example, the activation of NLRP3 and AIM2 inflammasomes is responsible for caspase-1 and L-1 β activation. These inflammasomes can respond to different ligands that are present during diabetes, such as islet amyloid polypeptide, amyloid β , cholesterol crystals, saturated fat acid and endogen DNA. Accordingly, the deregulation of the expression and function of NLRP3 and AIM2 might be conducive to the pathology of T2D. In this study, the expression and activity of NLRP3 and AIM2 were evaluated in the monocytes of T2D patients and healthy subjects. In accordance with the previous studies regarding resting monocytes, low percentages of NLRP3 $^{+}$ cells were seen in both patients and controls (20). Further observed was a great variability in the expression of NLRP3 among individuals, ranging from three patients with T2D who displayed detectable levels and an increased proportion of NLRP3 $^{+}$ cells to those with undetectable data. A plausible explanation is that the expression of NLRP3 is induced varying significantly among individuals (20). A reduction was also found in IL-1 β production by monocytes from patients receiving glyburide therapy, corroborating the

fact glyburide inhibits NLRP3 inflammasome activation (21). In this regard, it is worth remembering that a decrement in K⁺ levels in the cytosol is required for the activation of NLRP3 inflammasome (22) and that glyburide is a K⁺ channel inhibitor that may reduce the IL-1 β release by monocytes from glyburide-receiving patients. Myriad studies have provided evidence as to the participation of AIM2 inflammasome in inflammatory diseases such as systemic lupus erythematosus (SLE) and arthritis (23,24). In the present research, the increase in serum glucose and HbA1c, and the augmented proportions of AIM2⁺ cells in patients with T2D were observed. Hyperglycemia may have promoted inflammation through releasing TNF- α , IL-1 β and IFN- γ (25,26), possibly further inducing an increase in the AIM2 expression in monocytes from T2D patients (27). Likewise, the positive association between AIM2⁺ cells with triglycerides and waist-hip ratio suggests that caspase-1 activation by AIM2 may have a role in the lipid metabolism. The absence of caspase-1 was followed by a decrease in adipose tissue mass in mice fed with a high fat diet (HFD) (28), and caspase-1^{-/-} mice exhibited reduced plasma triglyceride levels and hepatic triglyceride secretion (28). In this context, caspase-1 activation is controlled by the AIM2 inflammasome and the increase in the expression and activity of AIM2 might contribute to the alterations in the lipid metabolism of patients with T2D. In addition, because the activation of AIM2 inflammasome releases IL-1 β which is related to a tendency toward higher abdominal obesity (29), the association of AIM2 expression with waist-hip ratio might reflect an increase in IL-1 β release by activation of AIM2 inflammasome. Taken together, these results suggest that the augmentation in the expression and activity of AIM2 in T2D patients probably leads to caspase-1 activation and IL-1 β release, thereby conducting to metabolic disturbances in T2D. Previous reports have demonstrated an increase in peripheral blood mtDNA content in patients with T2D nephropathy (30), likewise, increased mtDNA content in leucocytes was associated with hyperglycemia (31). However, the present study is the first one to examine circulating cell-free mtDNA in patients with T2D, which may modulate the production of inflammatory cytokines, hence the importance of the finding (32). We detected increased levels of mtDNA in the serum of T2D patients and observed increased levels of pro-inflammatory cytokines, which is in accordance with previous reports where IL-6, IL-8, TNF- α and IL-12p70, IL-1 β levels were higher in patients with T2D (33). Moreover, it was observed that the mtDNA copy numbers was associated with the levels of TNF- α . A possible explanation for this finding is that mtDNA can activate the cell surface Toll-like receptor (TLR)-9 (34) on monocytes and neutrophils, promoting TNF- α synthesis (35,36). On the other hand, an increase was observed in the expression of TLR-9 in T2D patients (37), a question to be addressed in futures studies. Herein, we demonstrated that mtDNA copy number was associated with BMI (marker of adiposity) and body weight, which is consistent with previous reports in which body weight was associated with mtDNA content in mononuclear cells (38). Likewise, ours findings are in agreement with studies demonstrating that the increase in mtDNA content in fat cells is associated with lipogenesis (39) and those in which the mtDNA content of adipose tissue increases in obese subjects compared to the lean controls (40). Together, these results suggest that mtDNA serum levels might be the result of alterations in the content of mtDNA in the cells during obesity.

Overall, the expression and activation of AIM2, and the increase in circulating mtDNA levels may contribute to the inflammatory process in patients with T2D.

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