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ORIGINAL ARTICLE

DPP-4 Inhibition Leads to Decreased Pancreatic Inflammatory Profile and Increased Frequency of Regulatory T Cells in Experimental Type 1 Diabetes

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Abstract— Sitagliptin is a dipeptidyl peptidase-4 inhibitor (iDPP-4), which has been used for type 2 diabetes treatment. Recently, iDPP-4 has been described as a promising treatment of type 1 diabetes (T1D) but is still necessary to evaluate immune effects of sitagliptin. C57BL/6 mice were induced by multiple low doses of streptozotocin. Diabetes incidence, insulin, glucagon, glucagon-like peptide-1 (GLP-1) serum levels, and inflammatory cytokine levels were quantified in pancreas homogenate after 30 and 90 days of treatment. In addition, frequencies of inflammatory and regulatory T cell subsets were determined in the spleen and in the pancreatic lymph nodes. iDPP-4 decreased blood glucose level while increased GLP-1 and insulin levels. After long-term treatment, treated diabetic mice presented decreased frequency of $CD4^+CD26^+$ T cells and increased percentage of $CD4^+CD25^{hi}Foxp3^+$ T cells in the spleen. Besides, pancreatic lymph nodes from diabetic mice treated with iDPP-4 presented lower percentage of $CD11b^+$ cells and decreased levels of inflammatory cytokines in the pancreas. Treatment of type 1 diabetic mice with iDPP-4 improved metabolic control, decreased inflammatory profile in the pancreatic microenvironment, and increased systemic regulatory T cell frequency. Therefore, we suggest the long-term use of sitagliptin as a feasible and effective therapy for T1D.

KEY WORDS: DPP-4 inhibitor; sitaglipitin; experimental type 1 diabetes; GLP-1; regulatory T cells.

Júlio César Voltarelli is deceased.

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INTRODUCTION

The incretins, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP), are a class of gut hormones responsible for stimulation of pancreatic β cells. Their endocrine signaling increase insulin release [1, 2], decrease glucagon release, stimulate β cell proliferation, and inhibit β cell apoptosis [3–5]. However, incretins have a short half-life in circulation (3–5 min) due to their inactivation by some proteases, such as dipeptidyl peptidase-4 (DPP-4) and other endopeptidases [6].

In this context, in recent years, the pharmaceutical industry has launched new drugs for treatment of patients with type 2 diabetes. A new class of drugs has emerged based on an enzyme described by Glenner and Hopsu-Havu in 1966 [7]. This molecule is known as DPP-4 or CD26 [8], and it is an enzyme that specifically cleaves peptides with proline or alanine residues at the penultimate amino-terminal position and inactivates target molecules, such as GLP-1 [9, 10]. DPP-4 is expressed in soluble form in plasma and also on the surface of various cell types, including epithelial cells, endothelial cells, and lymphocytes [reviewed by 11].

The new class of drugs based on DDP-4 activity is generically called DPP-4 inhibitors or gliptins. Gliptins is a diverse group of compounds which was approved for clinical use, namely sitagliptin, saxagliptin, linagliptin, alogliptin, vildagliptin, anagliptin, and teneligliptin [12–15]. Despite the same mode of action, the different gliptins diverge in their pharmacodynamic and pharmacokinetic properties, which might be clinically relevant for some patients [16–20]. These drugs are filling an important place for the management of hyperglycemia in type 2 diabetes (T2D) [21].

Preclinical studies have described DPP-4 inhibitors acting in the promotion of renal protection [22, 23] and decreasing cardiovascular complication and mortality [24, 25]. Furthermore, gliptins have also contributed to decrease migration of mononuclear cells [26, 27] and reduced adipose tissue inflammation [28] in patients with T2D. Some mechanisms involving the anti-inflammatory effects of gliptins treatment were elucidated in experiments with obese mice in which they promote a decrease of M1 macrophages and inflammatory cytokine production [29] and also in type 2 diabetes model resulting in NLRP3 inflammasome attenuation [30].

Lee and colleagues proposed the use of DPP-4 inhibitors as new therapeutic alternative for patients with type 1 diabetes (T1D) based on its immunomodulatory and anti-inflammatory properties [31]. The DPP-4 is also known as the lymphocyte cell surface protein CD26 [8]. A subpopulation of lymphocytes characterized by high level of CD26 expression on their surface has been associated with secretion of Th1-cytokine profile [32, 33]. DPP-4 inhibitors also attenuated the inflammatory state [34], reduced insulitis process [35], T $CD8^+/CD4^+$ ratio in pancreatic lymph nodes [36, 37], and renal inflammation [38] of type 1 diabetic mice.

In type 1 diabetic patients, sitagliptin treatment significantly reduced prandial insulin requirements [2], increased C-peptide [39], abrogated insulin requirement for 1 year [40], prolonged clinical T1D remission [34], and attenuated the growth hormone response during late hypoglycemia [41]. In addition, our group reported the metabolic benefit of the association of sitagliptin to autologous hematopoietic stem transplantation in recent-diagnosed type 1 diabetic patients [42].

The therapeutic potential of DPP-4 inhibitors has been widely evaluated in terms of its contribution to the altered metabolic parameter reestablishment during type 1 diabetes. Despite this, it is still necessary to investigate the immunological parameters in a systemic and local manner after type 1 diabetes development. In this context, here we investigated the effects of the DPP-4 inhibitor sitagliptin in the T lymphocyte subsets, inflammatory cytokines, and islet morphology on streptozotocin-induced type 1 diabetes model.

MATERIAL AND METHODS

Induction of Experimental Type 1 Diabetes

Male C57BL/6 mice (8–10 weeks old) were injected i.p. with 42 mg/kg of streptozotocin (STZ, Sigma-Aldrich, St. Louis, MO, USA) during five consecutive days. STZ was diluted in sodium citrate buffer (Sigma-Aldrich, Saint Louis, MO, USA), pH 4.5. Mice were considered diabetic when non-fasted blood glucose levels were above 250 mg/dL after two consecutive determinations in the same week. All animal procedures were approved by the Ethics Committee for Animal Research (CETEA, no. 144/2009) of the School of Medicine of Ribeirão Preto of the University of São Paulo.

Experimental Groups

Diabetic mice (D) received *ad libitum* either a normal chow diet (AIN-93 purified) [43] or a diet (D + iDPP4) containing sitagliptin/MK0431 (AIN-93 purified plus 4 g MK0431/kg; Prag Soluções, Jaú, São Paulo, Brazil) for 30 or 90 days. Each group was composed by five to six mice.

The protocol was performed twice, and only one experiment is described in this article as a representative data.

Blood Glucose and Body Weight Monitoring

Blood samples and body weight were determined every week. Blood samples were collected from the tail vein of non-fasted mice at 9:00 a.m., every week, and glycemia was determined using a portable glucometer (Accu-Chek Go, Roche Diagnostics, Abbott Park, IL, USA). No animals received any exogenous insulin.

Oral Glucose Tolerance Test

The oral glucose tolerance test (OGTT) was performed on 30th and 90th days after initiating the treatment with DPP-4 inhibitor. For the OGTT, mice were fasted for 12 h and glycemia was measured at 0, 15, 30, 60, 90, 120, and 180 min after the oral glucose (Sigma-Aldrich, Saint Louis, MO, USA) challenge (1.5 mg/g, by gavage).

Determination of Serum Insulin, Glucagon, and GLP-1 Concentrations

After 30 or 90 days, diabetic mice and diabetic mice treated with DPP-4 inhibitor were euthanized. Non-fasted blood samples were collected for serum insulin, glucagon, and GLP-1 quantification assays. Commercial ELISA kits were used to measure serum insulin, GLP-1 (Shibayagi Co., Ltd. Japan), and glucagon (Alpco, Diagnostics, Salem, NH, USA) according to the manufacturer's instructions.

Cell Isolation from Spleen and Pancreatic Lymph Nodes

The animals were euthanized, and the spleen and pancreatic lymph nodes were collected. The spleen was placed into a Petri dish with iced PBS and mashed to obtain a cell suspension. Cells were treated with erythrocyte lysis solution (Tris 0.17 M and NH_4Cl_2 0.16 M buffer). After erythrocyte lysis, RPMI 1640 medium (Gibco, Grand Island, NY, USA), supplemented with 10% FBS, 2 mmol/L L-glutamine, and 100 U/mL penicillin and streptomycin (Gibco, Grand Island, NY, USA), was added to the tube containing splenocytes and centrifuged at 1200 rpm for 10 min at 4 °C. Pancreatic draining lymph nodes (PLNs) were collected and placed in the cell strainer. Pancreatic lymph nodes were mashed through the cell strainer into a Petri dish containing iced RPMI 1640 culture medium. Cell suspension was collected and centrifuged 1200 rpm for 10 min at 4 °C.

Flow Cytometry Analysis

To analyze surface and intracellular markers by flow cytometry, cell suspensions of 2×10^6 cells from spleen or pancreatic lymph nodes were prepared. Cells were incubated with 100 μL of rabbit normal serum 5% for 30 min to block unspecific binding. After this period, the samples were incubated for 20 min with 1 μL of fluorochrome-conjugated primary antibodies CD3 (Fitc), CD4 (PercP), CD8 (APC), CD26 (PE), CD11b (PE), and their isotype controls (BD Pharmingen, San Diego, CA, USA). After extracellular antigen staining, cells were incubated with FACS lysing solution (BD Pharmingen, San Diego, CA, USA) for 10 min. Cells were then washed and resuspended in FACS permeabilizing solution (BD Pharmingen, San Diego, CA, USA) for 10 min. Next, expression of the transcription factor Foxp3 was assessed by incubation of cells with anti-Foxp3 monoclonal antibody (BD Pharmingen, San Diego, CA, USA). Cell suspensions were washed, resuspended, and analyzed by flow cytometry using a FACSCalibur flow cytometer and the CellQuest software (BD Pharmingen, San Diego, CA, USA).

Determination of Cytokine Levels in Pancreatic Tissue

Pancreas were removed and divided into two parts to perform ELISA and histology/immunohistochemistry assays. Pancreatic tissue samples were weighted and placed into a tube containing 700 μL of complete protease inhibitor cocktail (Roche Diagnostics, Abbott Park, IL, USA). The tissue was homogenized using a polytron homogenizer (Brinkmann Instruments Inc., Westbury, NY, USA), and IFN-γ, TNF-α, IL-10, and IL-17 levels were determined by ELISA (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. The results were expressed as mean ± SEM of nanograms per gram of pancreatic tissue.

Histology and Immunohistochemistry

Immunohistochemistry reactions were performed as described previously [44]. The slides were incubated with rabbit monoclonal anti-mouse insulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-mouse glucagon antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The slides were stained with diaminobenzidine (DAB) according to the manufacturer's instructions (DAKO Cytomation, Fort Collins, CO). The sections were counterstained with hematoxylin, mounted, and analyzed.

Islet Morphometry

Quantitative data (counting and measuring) were represented by 10–12 sections per animal, for four animals per experimental group. Each section could present 6 to 20 islets according to the experimental group. We found a great difficulty for count pancreatic islets because diabetic mice presented decreased size islet and poor staining to insulin. Then, staining for glucagon was performed to evaluate the numbers and size of pancreatic islet. Quantitative analysis for all staining was performed in a blinded manner with imaging software (ImageJ, NIH, Maryland, USA).

Statistical Analysis

Data were analyzed by using the GraphPad 5 software (San Diego, CA, USA). Statistical comparisons between diabetic (D) and diabetic treated (D + iDPP4) groups in same period of treatment (30th or 90th day) were performed with two-tailed *t* test. One-way ANOVA and Tukey comparison tests were performed to evaluate the differences between/through D, D + iDPP4, and WT (wild type) mice. All data are presented as mean values ± standard error of the mean. p values < 0.05 were considered to be significant.

RESULTS

Treatment with DPP-4 Inhibitor Reduces Hyperglycemia in Experimental Type 1 Diabetes

Treatment of diabetic mice with DPP-4 inhibitor significantly reduced blood glucose levels on 47th (434.8 ± 60.12 *vs.* 580.0 ± 20.0 mg/dL, $p < 0.05$), 78th (353.0 ± 46.6 *vs.* 598.7 ± 4.9 mg/dL, $p < 0.05$), and 90th (369.7 ± 60.69 *vs.* 582.7 ± 13.33 mg/dL, $p < 0.05$) days after starting treatment, compared with untreated diabetic animals (Fig. 1a). Body weight was not significantly different in treated diabetic mice compared to untreated diabetic mice, except on day 40 after treatment has started (Fig. 1b).

Diabetic mice treated with DPP-4 inhibitor (D + iDPP4) showed attenuated glycemia response to OGTT compared with untreated diabetic animals (D) at both 30 and 90 days after starting treatment (Fig. 1c, d).

GLP-1 serum levels were significantly higher in diabetic mice treated with DPP-4 inhibitor than in untreated diabetic animals at both 30 (46.9 ± 2.2 *vs.* 7.423 ± 1.9 pg/mL, respectively) and 90 days (37.0 ± 11.0 *vs.* 4.2 ± 0.4, pg/mL, respectively) after the beginning of the treatment (Fig. 1e). The increase of serum GLP-1 concentration was approximately 4–5-fold.

Serum insulin levels were not different in treated diabetic mice compared to untreated diabetic mice at 30 days of treatment. However, after 90 days of treatment with DPP-4 inhibitor, treated diabetic animals presented significantly higher insulin serum concentration (2.1 ± 0.7 pg/mL) than untreated diabetic animals (0.2 ± 0.1 pg/mL) (Fig. 1f). Serum glucagon levels of DPP-4 inhibitor-treated and untreated diabetic mice were not different after at 30 days and 90 days (Fig. 1f).

DPP-4 Inhibitor Improves Morphology of Pancreatic Endocrine Area

After 90 days, the untreated diabetic animals showed a scarce number of pancreatic islets (Fig. 2a, b). Then, staining for glucagon was performed to evaluate the numbers and size of pancreatic islet. Some treated diabetic mice showed an increase of small pancreatic islets, especially after 90 days, although this increase was not statistically significant (Fig. 2g, i). The small islands distributed around the vessels (Fig. 2g, black arrows) staining for glucagon increased in treated diabetic mice after 90 days (Fig. 2i, j).

DPP4 Inhibitor Reduces Splenic Inflammatory T Cells and Increased Regulatory T Cell Subsets in Type 1 Diabetic Mice

Treatment of diabetic animals with GLP-1 analogues has been associated with significant changes in immune cell numbers in various lymphoid organs [5, 45]. After 30 days of iDDP-4 treatment, there was a significant increase in the percentage of $CD3^+CD4^+$ T cells in the spleen of treated ($16.39 \pm 0.56\%$) compared with untreated diabetic mice ($13.86 \pm 0.66\%$) (Fig. 3a). The percentage of $CD3^+CD8^+$ T cells in the spleen was also significant increased on treated diabetic mice at 30 days, ($9.67 \pm 0.52\%$) when compared with untreated ones ($7.22 \pm 0.11\%$) (Fig. 3a). After 90 days of treatment, no significant differences between groups were observed.

The frequency of inflammatory $CD3^+CD4^+CD26^+$ T cells was evaluated after 30 days of treatment, and no significant differences were observed between groups (Fig. 2b). However, at 90 days after starting iDDP-4 treatment, the percentage of $CD3^+CD4^+CD26^+$ T cells significantly decreased in the treated animals ($10.50 \pm 12.51\%$) compared to untreated animals ($14.81 \pm 2.3\%$) (Fig. 3b). No significant differences between groups in the $CD3^+CD8^+CD26^+$ T cell frequencies were observed, in the analyzed periods (Fig. 3b). Regarding the percentage

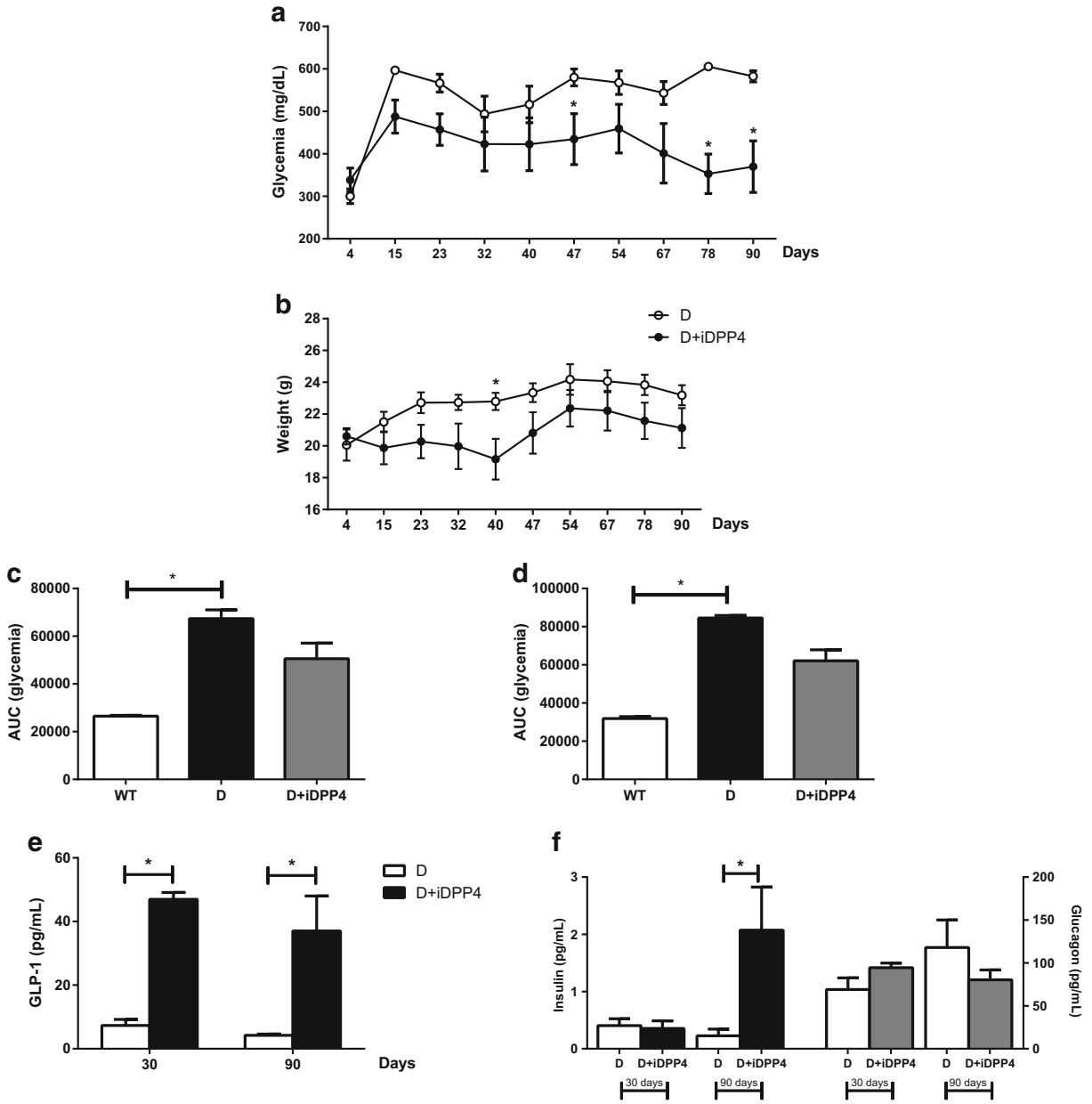

Fig. 1. DPP-4 inhibitor ameliorated metabolic parameters of type 1 diabetic mice. **a** Blood glucose levels. **b** Body weight. Evolution of blood glucose and body weight of diabetic animals treated and untreated with DPP-4 inhibitor incorporated into the diet *ad libitum* for 90 days. After being considered diabetic, the animals received control diet (AIN-93M purified) or diet containing MK0431 (MK0431 4 g/kg added to AIN-93M diet purified) *ad libitum*. Blood glucose levels were assessed weekly in the morning (non-fasting) for 12 weeks. Glucose tolerance oral test. Glucose tolerance oral test was performed after 30 days (**c**) and 90 days (**d**) of treatment with iDPP-4. At the end of treatment and after 12 h of fasting, glucose solution (1.5 mg/g animal) was administered by gavage (oral) and blood glucose levels were monitored before glucose administration, 15, 30, 60, 90, 120, and 180 min after. **e** GLP-1 serum levels. Active GLP-1 serum level of untreated diabetic mice and treated with DPP-4 inhibitor after 30 and 90 days of treatment. **f** Insulin and glucagon levels. Insulin and glucagon serum levels of untreated diabetic mice and treated with DPP-4 inhibitor after 30 and 90 days of treatment were determined. GLP-1 hormone and insulin were quantified by ELISA. $*p < 0.05$ diabetic group compared to the diabetic group treated with iDPP-4 in the same period.

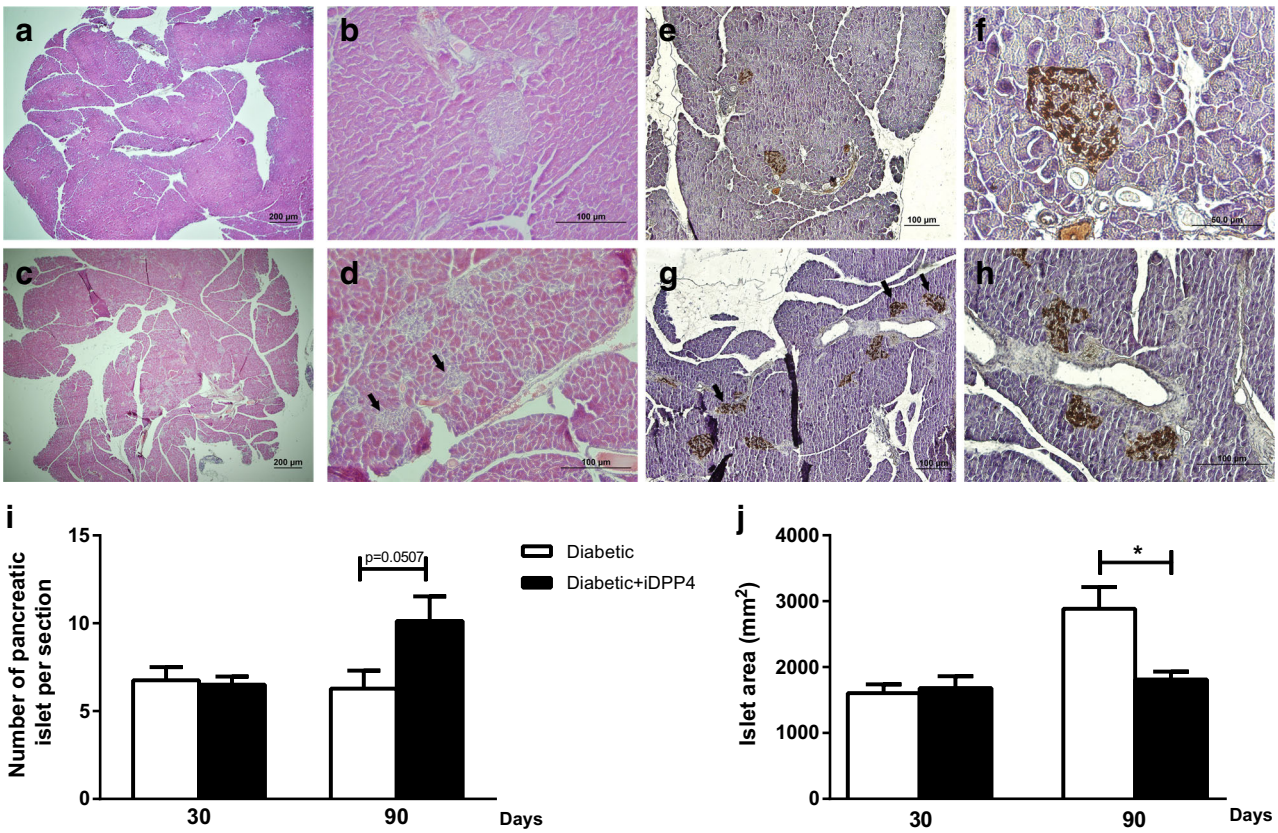

Fig. 2. DPP-4 inhibitor increased the number of islet in type 1 diabetic mice. **a**–**d** Pancreas morphology. **e**–**h** Glucagon staining. **i** Number of pancreatic islet stained for glucagon. **j** Measurement of pancreatic islet stained for glucagon. Pancreas morphology was performed by hematoxylin-eosin staining (**a**–**d**) and glucagon staining (**e**–**h**) after 90 days. After the treatment with iDDP-4, the animals had their pancreas collected, embedded in paraffin, cut into sections of 5 μm, to perform the immunohistochemical reactions to hematoxylin-eosin and glucagon, viewed by optic microscopy. Staining for glucagon was performed to evaluate the numbers and size of pancreatic islet. Quantitative analysis for all staining was performed in a blinded manner with imaging software. **a**, **b**, **e**, and **f** represent diabetic mice after 90 days. **c**, **d**, **g**, and **h** represent diabetic mice treated with iDPP-4 after 90 days. *$p < 0.05$ diabetic group compared to the diabetic group treated with iDPP-4 in the same period.

of $CD3^+CD4^+CD26^{high}$ and $CD3^+CD8^+CD26^{high}$ T cells (with high expression of CD26), no significant differences between groups were observed after different periods of treatment (data not shown).

It has been shown that treatment with DPP-4 inhibitors, by increasing concentration of GLP-1, may induce higher frequencies of regulatory T cells ($CD4^+CD25^+Foxp3^+$ and $CD4^+CD25^{hi}Foxp3^+$) [5, 46]. After 30 days of treatment, the spleen-treated diabetic mice showed a significant increased percentage of $CD4^+CD25^+Foxp3^+$ ($1.064 \pm 0.071\%$) compared to untreated diabetic mice ($0.745 \pm 0.099\%$) (Fig. 3c). The frequency of $CD4^+CD25^{hi}Foxp3^+$ was also increased in the treated diabetic animals ($0.362 \pm 0.069\%$) compared to diabetic untreated animals ($0.189 \pm 0.036\%$) (Fig. 3c). However, after 90 days of treatment, no significant differences were observed in the regulatory T cell frequencies between groups.

DPP4 Inhibitor Modulates Pancreatic Inflammatory Profile of Type 1 Diabetic Mice

We have then investigated the immunological effects of iDPP-4 treatment in pancreatic microenvironment by evaluating $CD3^+CD4^+$, $CD3^+CD8^+$, and $CD11b^+$ cell frequencies in pancreatic lymph nodes (PLNs) and inflammory cytokine concentrations in pancreatic tissue. The percentage of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells in 30 and 90 days after treatment was not altered in PLNs (Fig. 4a); otherwise, percentage of $CD11b^+$ macrophages was decreased after 90 days of treatment (Fig. 4b).

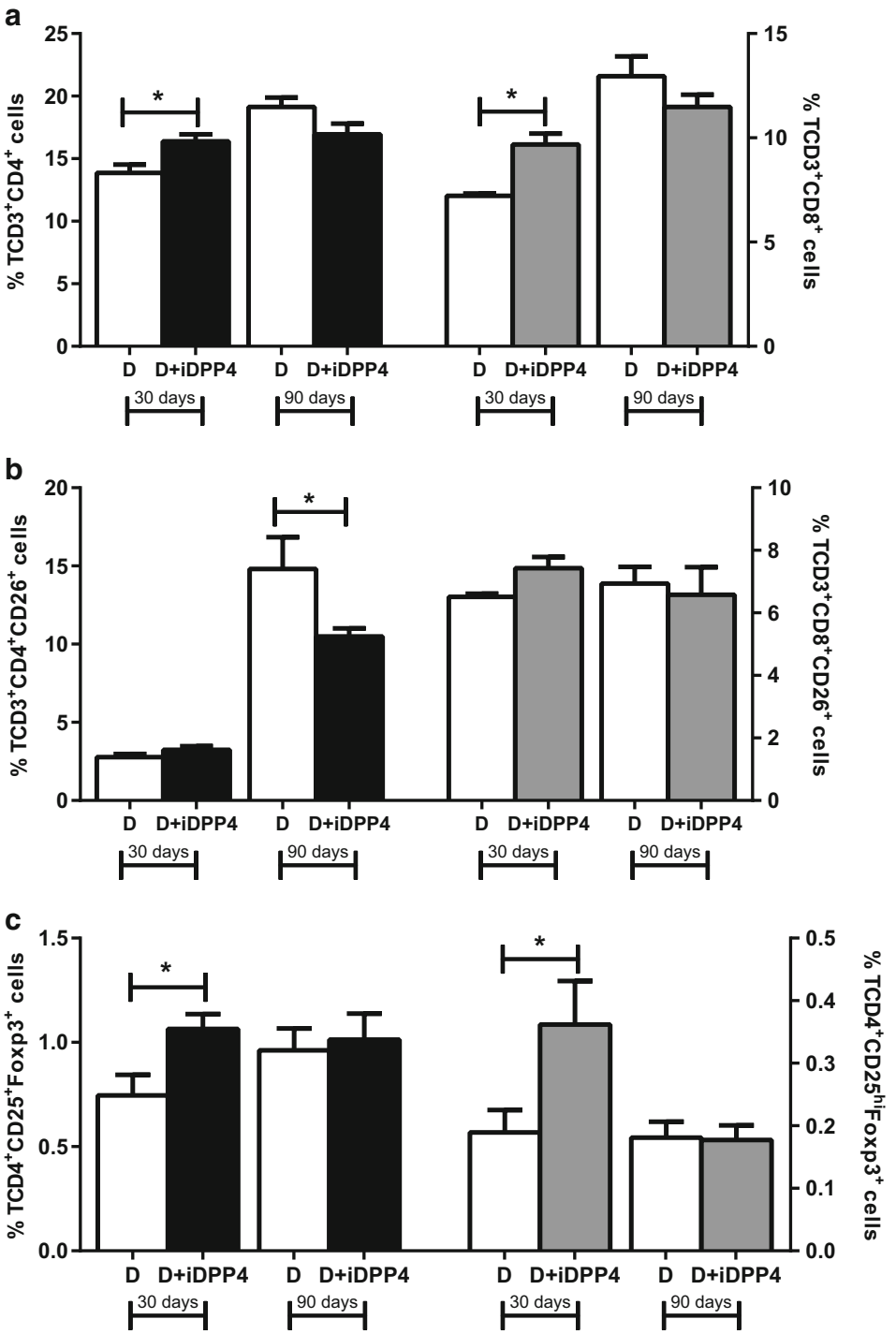

Fig. 3. DPP-4 inhibitor treatment decreases frequency of inflammatory $CD4^+$ T cells and increases frequencies of regulatory T cell subsets in the spleen of type 1 diabetic mice. $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells (**a**), $CD3^+CD4^+CD26^+$ and $CD3^+CD8^+CD26^+$ T cells (**b**), and $CD4^+CD25^+Foxp3^+$ and $CD4^+CD25^{hi}Foxp3^+$ regulatory T cells (**c**) were quantified in the spleen of diabetic group and diabetic group treated with iDPP-4 after 30 and 90 days of treatment. After treatment with iDPP-4, animals had their spleens collected and processed for immunophenotyping by flow cytometry. $*p < 0.05$ diabetic group compared to the diabetic group treated with iDPP-4 after 30 days in $CD3^+CD4^+$, $CD3^+CD8^+$ T cells and $CD4^+CD25^+Foxp3^+$, $CD4^+CD25^{hi}Foxp3^+$ regulatory T cells and after 90 days in $CD3^+CD4^+CD26^+$ T cells.

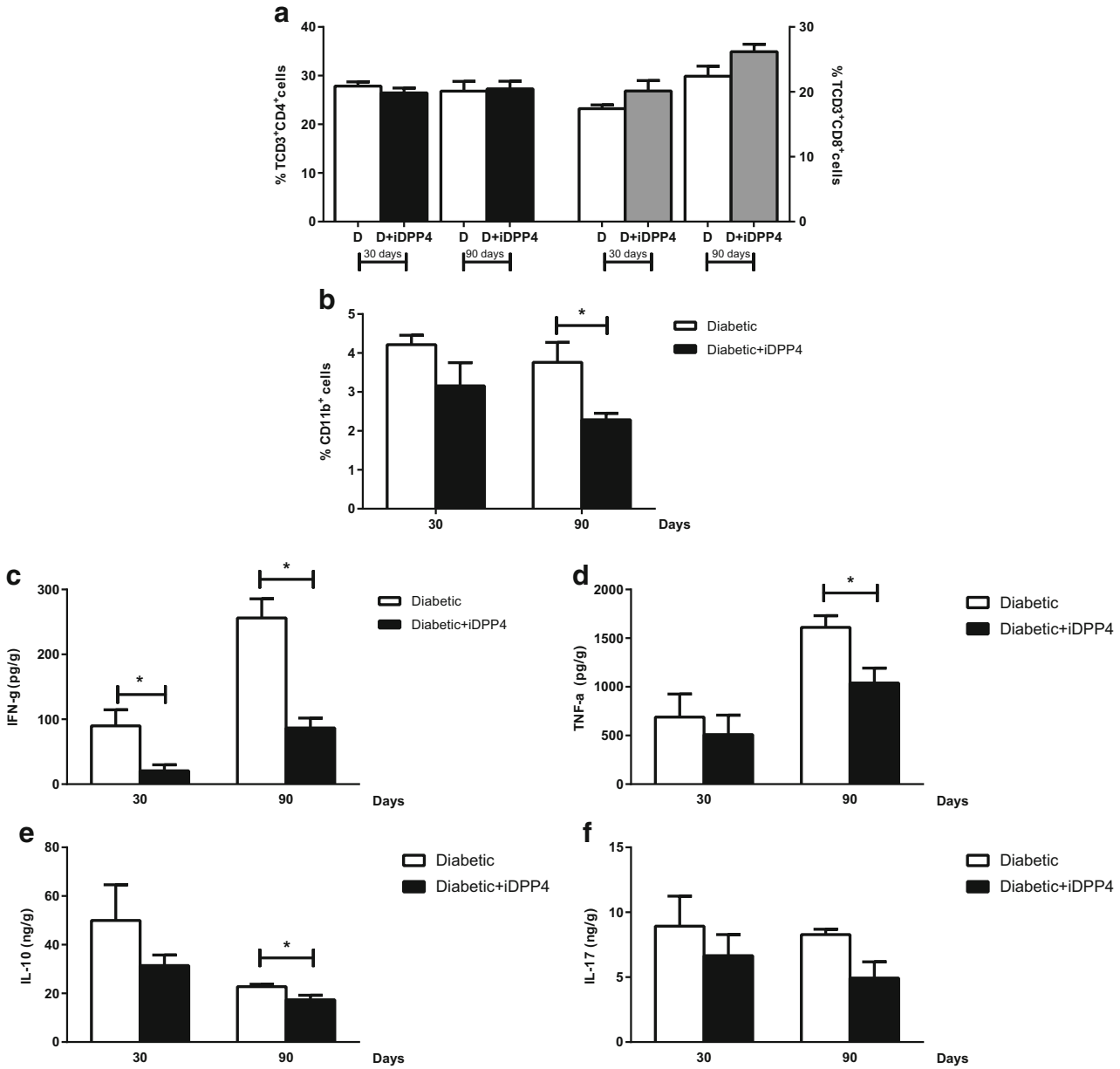

Fig. 4. DPP-4 inhibitor decreased macrophage numbers and inflammatory cytokine levels in the pancreatic microenvironment of type 1 diabetic mice. $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells (**a**) and $CD11b^+$ macrophages (**b**) in of diabetic group and diabetic group treated with iDPP-4 after 30 and 90 days of treatment. After the treatment with the iDDP-4, the animals had pancreatic lymph nodes collected and processed for immunophenotyping by flow cytometry. IFN-γ (**c**), TNF-α (**d**), IL-17 (**e**), and IL-10 (**f**) were measured in of diabetic group and diabetic group treated with iDPP-4 after 30 and 90 days of treatment. After the treatment with iDDP-4, the animals had their pancreas collected and processed in the presence of protease inhibitor. Cytokines were quantified by ELISA and its concentration represented by the ratio of pictogram of cytokine per gram of pancreatic tissue. *$p < 0.05$ diabetic group compared to the diabetic group treated with iDPP-4 in the same period.

Regarding inflammatory cytokines, IFN-γ concentration in pancreatic homogenate was decreased in treated diabetic mice after 30 and 90 days of treatment (Fig. 4c), suggesting a possible anti-inflammatory role of iDPP-4 in pancreatic microenvironment of diabetic mice.

After 90 days of treatment with iDPP-4, a significant decrease of TNF-α (Fig. 4d) and IL-10 in pancreatic homogenate was observed (Fig. 4e, Supplemental Table 1). The concentration of IL-17 showed a tendency to decrease in pancreas of treated diabetic mice, although not statistically significant (Fig. 4f).

DISCUSSION

Current knowledge indicates that pancreatic beta cells can be renewed even under prolonged autoimmune state and metabolic stress in chronic type 1 diabetes (T1D) patients [47, 48]. These data encourage the development of alternatives therapies that promotes maintenance and regeneration of residual pancreatic beta cells in T1D setting.

In 2006, FDA (Food and Drug Administration, USA) has approved several new drugs for treatment of type 2 diabetes (T2D) patients, especially the analogs and mimetics of GLP-1 and DPP-4 inhibitors. DPP-4 inhibitors comprehend a class of oral glucose-lowering agents that act to block GLP-1 degradation by DPP-4 activity, and thereby enhancing insulin secretion in a glucose-dependent manner [4, 49, 50]. Incretins, such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP), are gut hormones that stimulate pancreatic β cell proliferation. Since their approval for clinical use, these drugs have shown satisfactory therapeutic efficacy in T2D. Long-term treatment of T2D mice with DPP-4 has improved insulin sensitivity and decreased blood glucose levels [51, 52]. Furthermore, there are some reports describing anti-inflammatory effects by the decrease of TNF-alpha level [reviewed by 53] and suppression of inflammatory molecules [54] in patients with T2D followed by DPP-4 inhibitor treatment.

Once incretin hormones also have protective effects on pancreatic beta cells by promoting cell survival and proliferation [55, 56], increasing their concentrations through administration of DPP-4 inhibitor could also confer therapeutic benefit for T1D patients. Thereby, in the last years, DPP-4 inhibitors have also been tested in T1D experimental models [35, 57, 58] and patients [31, 59].

In this scenario, there are few reported studies evaluating the immune changes after treatment of T1D with DPP-4 inhibitors and their correlation with therapeutic efficacy. Thus, here we have evaluated the therapeutic efficacy of DPP-4 inhibitor MK0431 (sitagliptin) in the streptozotocin-induced T1D experimental model and the immune mechanisms involved. We demonstrated that long-term treatment of T1D mice with sitagliptin ameliorates metabolic control (by increasing GLP-1 levels, reducing progression of hyperglycemia and increasing insulin secretion) and immunomodulates the pancreatic microenvironment, as observed by Cho and Pospisilik and colleagues in pancreas after DPP-4 inhibitor treatment by islet neogenesis [60, 61].

STZ-induced diabetic mice that received the DPP4 inhibitor MK-0431 incorporated into diet showed significant increase serum GLP-1 levels after 30 days of treatment; however, blood glucose levels and the insulin/glucagon ratio was not altered [62]. In pre-diabetic NOD mice, treatment with another DPP4 inhibitor (MK-626) resulted in improved glucose tolerance, decreased insulitis score, and increased frequency of pancreatic lymph node regulatory T cells after 4 weeks [63]. Recently, promising effects of sitagliptin have been demonstrated in patients with T1D [2, 40, 41]. Exogenous GLP-1 administration in T1D patients reduced the levels of fasting blood glucose *via* decreased glucagon concentration [64]. However, in our study, no significant difference in serum levels of glucagon was observed in diabetic treated mice, but we observed a more pronounced improvement in glycemia and insulin level after 90 days, indicative of beta cell proliferation as observed by increasing the number of small pancreatic islets, as described in previous studies [35, 60]. Sitagliptin treatment decreased beta cell apoptosis and increased proliferation and regeneration [65] in diabetic mice. Other studies have observed an increase of small islet number, suggesting increased neogenesis [61, 66]; further investigations will be necessary to describe the mechanism involved in the increase of small pancreatic islets with type 1 diabetic animals after sitagliptin treatment.

Our study described an important role of homeostasis of incretin hormones during type 1 diabetes progression. Also, diabetic mice treated with DPP-4 inhibitor presented attenuated glycemia response to oral glucose tolerance test when compared with untreated diabetic animals at both periods after starting treatment. Despite that 30 days of treatment provided an increase of serum GLP-1 level, frequency of regulatory T in spleen, and a decrease of IFN-γ in pancreas, this scenario was not enough to control glycemia. Only after 90 days, DPP-4 treatment decreased glycemia and increased insulinemia. Also, after 90 days, sitagliptin treatment attenuated inflammatory pattern

through $CD11b^+$ and TNF-α decrease in pancreas microenvironment. Considering that after 30 days, DPP-4 inhibitor treatment showed its preliminary benefits, we believe that this treatment should be administrated as soon as T1D diagnosis is completed.

Besides the ability to promote metabolic/hormonal changes in diabetes models, DPP-4 inhibitors also have an important immunomodulatory capacity. It has been demonstrated that DPP4 inhibitors decrease T cell proliferation *in vitro* [67] and pro-inflammatory cytokine secretion *in vivo* [68]. After 30 days of sitagliptin treatment, the spleen of treated diabetic animals presented increased frequency of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells. This finding was also observed in peripheral lymph nodes of NOD mice treated with GLP-1 [45].

Hundred studies have demonstrated the importance of regulatory T cells (Tregs) in maintaining immune homeostasis and control of inflammatory and autoimmune responses, both in humans and in animal models [69]. In our work, a significant increase in the frequency of $CD4^+CD25^{hi}Foxp3^+$ regulatory T cells was observed in the spleen of iDPP-4-treated animals after 30 days. The treatment of NOD mice with iDPP-4 showed that treated animals that presented remission of the disease (less than 200 mg/dL glucose) had an increased number of Treg cells in the thymus [46] and pancreatic lymph nodes [36] when compared to animals NOD diabetic untreated. In contrast, GLP-1 administration was associated with significant decreased numbers of Tregs in the thymus, and no change was observed in other lymphoid organs [35, 45].

In this way, the balance of regulatory T cells after iDPP-4 treatment still remains controversial, and the present study showed this profile through the increase of Treg cells after 30 days that was not observed after 90 days with iDPP-4 treatment. We believe that type 1 diabetes state presented an intensive pro-inflammatory environment which does not allow the maintenance of high frequencies of Tregs for long periods due to iDPP-4 treatment.

DPP-4 or CD26 molecule, expressed in immune cell surfaces, has a role immune regulation [70, 71]. Inhibition of DPP-4 (CD26 molecule) in the cell surface not only suppressed T cell proliferation and production of pro-inflammatory cytokines but also stimulated the secretion of anti-inflammatory cytokines [72, 73]. In fact, inhibition of DPP-4 in NOD mice resulted in decreased insulitis [74, 75] and reversed established diabetes [46]. We found a decreased frequency of splenic $CD4^+CD26^+$ T cells after 90 days of sitagliptin treatment. As reported by others, CD26 expressing T cells was associated with diabetes parameters [76], but sitagliptin presented an important role decreasing $CD4^+$ T cell numbers in diabetic patients [77] and Th1 differentiation *in vitro* [78].

Increased levels of inflammatory cytokines are found in pancreas islets during the insulitis process in humans and animals with T1D [44]. The Th1 cytokines (IFN-γ, TNF-α, IL-2, and others) activate cell-mediated immunity, whereas the Th2 cytokines (IL-4, IL-10, and others) activate humoral immunity [79]. The unbalance production of these cytokines is directly related to pathogenesis of type 1 diabetes [80].

The role of Th17 cells in T1D pathogenesis has been studied in human [81] and animal models [82]. Insulitis process and incidence of T1D are directly related to IL-17 increase [83]. Although they appear to be the same cells, some studies have described different types of Th17 cells, polarized with transforming growth factor (TGF)-β + IL-6 or IL-23 + IL-6; the former polarization was characterized as a Th17 regulatory cell and the latter as a conventional Th17 cell [84]. This scenario could be the answer about controversial results found in development and progression of T1D [84], indicative of two different population of Th17 cells. Although *in vitro* treatment with DPP-4 inhibitor robustly decreases Th17 differentiation [78] and IL-17 production by $TCD8^+$ cells [35], our treatment after 30 and 90 days attenuated IL-17 production in pancreas.

We also observed decreased levels of IFN-γ and TNF-α in the pancreas of long-term iDPP-4-treated diabetic mice. Besides, our study revealed decreased percentage of $CD11b^+$ macrophages in the pancreatic lymph nodes of treated mice. Thus, inhibition of DPP-4 with sitagliptin was capable of immunomodulation in the inflammatory pancreatic environment of T1D mice.

According to our findings, sitagliptin administration in T2D mice decreased production of the chemokine MCP-1 (monocyte chemoattractant protein-1)/CCL-2 in the pancreatic tissue [68], in addition to reducing levels of inflammatory cytokines. MCP-1 is a potent chemoattractant for macrophages and can be produced by immune cells that comprise the inflammatory infiltrate in the pancreatic islets, contributing to exacerbation of inflammation. In the future, further evaluations should be performed with T1D models to confirm the mechanism responsible by $CD11b^+$ cell decrease and anti-inflammatory effects of sitagliptin.

CONCLUSION

Our study shows that long-term treatment with the DPP-4 inhibitor sitagliptin improved the metabolic control of type

1 diabetic mice by increasing insulin production and decreasing glucose levels, reflecting the increased number of small pancreatic islets, indicative of proliferation or neogenesis. In parallel, treated diabetic mice presented lower $CD4^+CD26^+$ T cells and $CD11b^+$ macrophage frequencies, decreased levels of inflammatory cytokines, and increased regulatory T cell frequency. Sitagliptin was able to immunomodulate the pancreatic microenvironment, by diminishing inflammation and increasing immunoregulation. Thereby, our findings suggest that administration of DPP-4 inhibitors is a feasible and effective therapeutic strategy for experimental and human T1D and could be relevant in attenuating inflammatory state and glycemia.

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AUTHOR'S CONTRIBUTION

MRD participated in the design of the study, performed experiments and sample collection, analyzed all results, and wrote the manuscript. CCO participated in the experiments, sample collection, and reviewed the manuscript. DTC provided infrastructure and administrative supports. JCV (*in memoriam*) participated in the conception and coordination of the study, provided financial support, and discussed the experimental plans and results. AMOL, KCRM, and CEBC supervised the work, discussed the experimental plans and results, and reviewed the manuscript. JNUY supervised the work, provided financial support, discussed the experimental plans and results, and reviewed the manuscript.

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COMPLIANCE WITH ETHICAL STANDARDS

Competing Interests. The authors declare that they have no competing interests.

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