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Research Article

Regulatory T cells in Type 2 Diabetes Mellitus: Are they playmakers?

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Abstract

Background: There has been growing interest in a possible role for regulatory T (Treg) cells in type 2 diabetes mellitus (T2D).The present study aimed to assess the percentages of circulating Treg cells in patients with T2D, as compared to a group of healthy controls. **Materials and Methods:** This case-control study included 50 patients with T2D and 30 healthy controls. Body mass index (BMI) was assessed for all subjects. Investigations included urinary albumin/creatinine ratio (UACR), fasting (FPG) and postprandial plasma glucose (PPG) levels, and hemoglobin A1c (HbA1c). Measurement of the percentages of Treg cells by flow cytometry was performed for all patients and controls. **Results:** Percentages of circulating $CD4^+CD25^{high}$ $Foxp3^+$ Treg cells were significantly lower among T2D patients than among controls, and were lower among diabetics with nephropathy than among diabetics without nephropathy. Treg cell percentages did not differ significantly according to gender, presence of hypertension, dyslipidemia, ischemic heart disease or diabetic neuropathy. Among diabetic patients, there was a significant inverse correlation between Treg cell percentages and age, BMI, disease duration, HbA1c, UACR, but no significant correlation with FPG or PPG levels. **Conclusion:** We demonstrate a possible role for Treg cell reduction in T2D which is correlated with body mass index, disease duration, diabetes control, and diabetic nephropathy. Future large-scale studies are needed to evaluate the therapeutic effect of Treg cell induction in patients with T2D and its possible role as a new therapeutic modality among those patients.

Keywords: diabetes, flow cytometry, Foxp3, nephropathy, Treg.

Introduction

Diabetes mellitus (DM) is a disorder characterized by hyperglycemia and altered glucose, fat, and protein metabolism. Type 1 diabetes (T1D) results from β-cell destruction and leads to absolute insulin deficiency, whereas type 2 diabetes (T2D) results from a progressive insulin secretory defect on the background of insulin resistance [1].

$CD4^+$ T helper (TH) cells modulate immune responses, and are classified into TH_1, TH_2, TH_{17}, and regulatory T cell (Treg) subsets, based on their cytokine profiles and effector function [2]. Naturally occurring Tregs represent 1–2% of peripheral blood $CD4^+$ T cells, and develop in the thymus during normal T-cell maturation [3]. These cells are characterized by the expression of Foxp3, a DNA-binding forkhead-winged-helix transcriptional regulator which is necessary for the development and function of these cells. Mutations in this regulator have resulted in loss of Treg cell function and the development of autoimmune diabetes[4]. Treg cells possess the ability to suppress autoimmune responses and counteract pro-inflammatory cell populations, among which TH_1 and TH_{17} cells are most important [5].

Treg cells have shown a significant role in T1D as an autoimmune disease. The progression of T1D has been demonstrated to increase after Treg cell depletion in

non-obese diabetic mice [6]. Studies have demonstrated a decrease in numbers and function of Treg cells [7], as well as loss of Foxp3 expression, and hence loss of stability of Treg cells [8], in the inflamed pancreatic islets in T1D.

Obesity is recognized as a major contributor to insulin resistance and T2D, possibly through providing a state of chronic, low-grade inflammation in adipose tissue. Increasing adiposity is closely linked to macrophage entry into the abdominal depot, in addition to increased inflammatory cytokine concentrations. A possible role for Treg cells in adipose tissue inflammation has been demonstrated, and Treg cells were depleted in adipose tissue in an insulin-resistant model of obesity compared with adipose tissue from lean mice [9]. Furthermore, induction of Treg cells decreased adipose inflammation and improved insulin resistance in an ob/ob mouse model of T2D [10]. In contrast to T1D, the role of Treg cells in T2D is less clear, with very few studies in the literature investigating their relationship. Accordingly, we undertook the present study to assess the percentages of circulating Treg cells in patients with T2D, as compared to a group of healthy controls.

Materials and Methods

This case-control study was conducted at Ain Shams University Hospital, and included 50 patients with type 2 diabetes mellitus, who were recruited from the Diabetes outpatient clinic at Ain Shams University Hospital. Type 2 diabetes mellitus was diagnosed according to the American Diabetes Association criteria [11]. Exclusion criteria included type 1 diabetes mellitus, gestational diabetes mellitus, patients with associated major systemic diseases, and patients receiving oral steroids or immunosuppressives within 3 months before enrollment. In addition, 30 healthy non-diabetic individuals were recruited as a control group. An informed consent was taken from all study participants after explaining the aim and procedures of the study and ensuring the confidentiality of the data. The study was carried out after the approval of Ain Shams University Ethics Committee. All patients were subjected to full history taking and thorough clinical examination for evidence of any complications of diabetes. Body mass index (BMI) was assessed for all subjects and was categorized as normal weight (18.5 - 24.9 kg/m^2), overweight (25 - 29.9 kg/m^2) or obese (≥ 30 kg/m^2).

Laboratory investigations

Five mL of venous blood was drawn from each patient and control: 3 mL was placed in an EDTA Vacutainer (Becton Dickinson and Company, Franklin Lakes, New Jersy) tube for estimation of $CD4^+CD25^{high}$ $Foxp3^+$Treg cells by flow cytometry and for hemoglobin A1c (HbA1c) measurement and 2 mL was collected in plain Vacutainer for Fasting and 2h-post prandial serum glucose estimation. Random urine sample was collected for estimation of urinary albumin/creatinine ratio (UACR). They were measured in the clinical laboratory of Ain Shams University Hospitals:

- Fasting and 2h-post prandial serum glucose: were assayed by enzymatic procedure using Synchron CX9 autoanalyzer (Beckman Instruments Inc.California, USA).
- Urine albumin and creatinine were measured on Synchron CX9 autoanalyzer (Beckman Instruments Inc. California, USA). Then albumin creatinine ratio was calculated.
- HbA1c was measured by high performance liquid chromatography (HPLC) using Biorad D10 machine (Bio-Rad Laboratories, Inc., Hercules, CA).
- *$CD4^+CD25^{high}$ $Foxp3^+$Treg cell assay:*

Detection of the combined expression of $CD4^+$, $CD25^{high}$ and intracellular Foxp3 on peripheral blood lymphocytes was performed by using three colors EPICS XL flow cytometer (Coulter Electronics, Florida, USA). The test was done on lysed whole blood using the following monoclonal antibodies conjugated with different fluorescent dyes: Fluorescein isothiocyanate(FlTC)-conjugated anti-humanCD4, Phycoerythrin (PE)-conjugated anti-human CD25, Phycoerythrin-Cyanine 5 (PE-Cy5)-conjugated anti-human Foxp3, PE-Cy5isotype control, and PE isotype control, all supplied by (eBioscience, USA). Two plastic test tubes were used;one for the test and the other for the isotypic control. According to the manufacturer's instructions, 50µL of EDTA anticoagulated whole blood were added to each tube, which contained 1 mL lysing solution, followed by a wash with phosphate-buffered saline (PBS). Then the cell pellet was stained with combinations of the following antibodies (5 µL each): anti-CD25-PE, anti-CD4-FITC in the test tube; PE isotype control and anti-CD4-FITC in the control tube. Both tubes were then incubated in the dark at room temperature for

20 minutes, followed by a single wash with PBS. The cell pellet was re-suspended in 0.5 mL of freshly-prepared fixation/permeabilization working solution and incubated for 30 minutes at 4°C in the dark. This was followed by washing once with PBS, then washing once again with 1 mL of 1x permeabilization buffer. Ten µL of anti-Foxp3-PE-Cy5 and PE-Cy5 isotype control were added in the test tube and control tube, respectively, and incubated for 30 minutes at 4°C in the dark. This was followed by washing once with PBS, then re-suspension in 0.5 mL PBS for analysis. Lymphocytes were gated according to their forward and side scatter properties, and CD4 positive T cells were gated out of total lymphocytes. Tregs were identified by the combined expression of $CD4^{+}$, $CD25^{high}$ and Foxp3.

Data acquisition and analysis were performed on EPICS XL flowcytometry, using SYSTEM II version 3 software with a standard three-color filter configuration. Isotype-matched controls were used to set up the gating. Lymphocytes were identified according their size and complexity on the forward and side scatter plot (figure 1-A). $CD4^{+}$T cells were identified based on $CD4^{+}$expression (figure 1-B). PE isotype control was used to set gates separating $CD4^{+}CD25^{+}$ cells from $CD4^{+}CD25^{-}$ cells. $CD25^{high}$ cells were identified relative to the intensity of CD25 on $CD4^{-}$ cells, dividing it into two parts on the plot: the dim area ($CD4^{+}CD25^{low}$) (figure 1-D region F), and the bright one ($CD4^{+}CD25^{high}$) (figure 1-D region E). PE-Cy5 isotype control was used to set the gates for separating $Foxp3^{+}$ cells from $Foxp3^{-}$ cells.

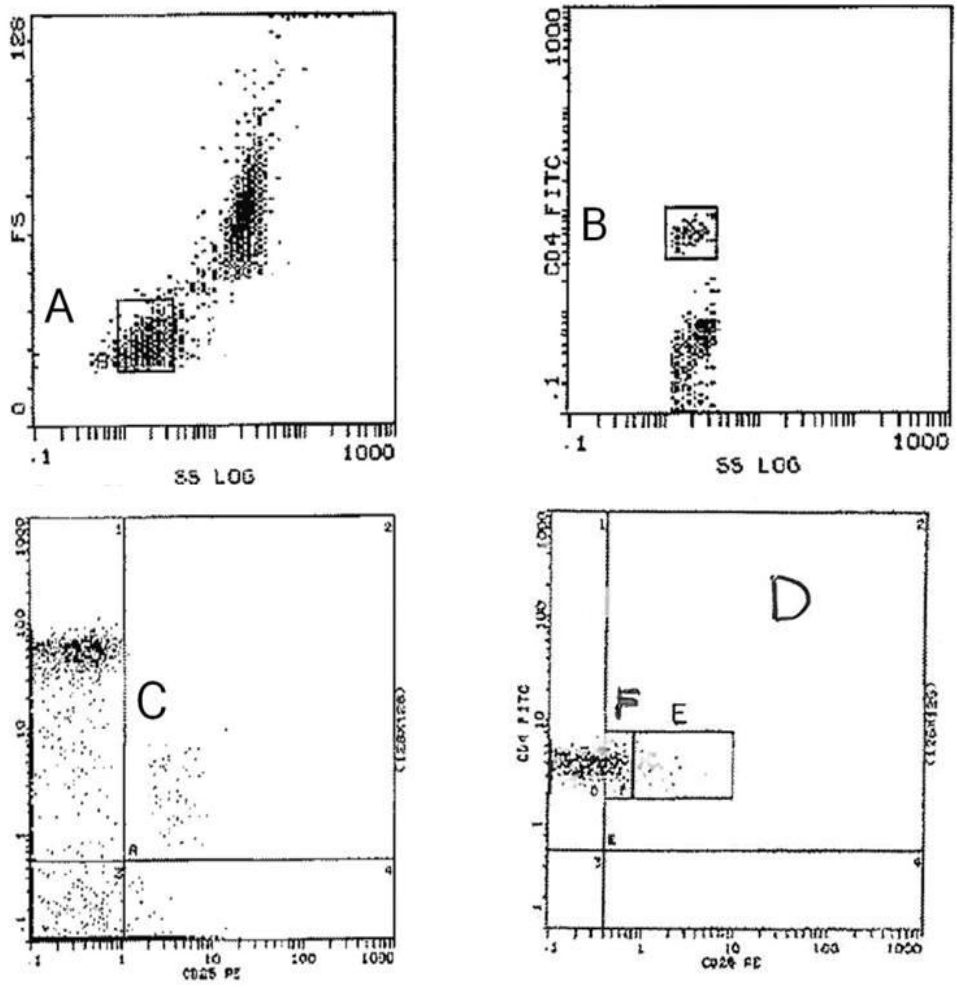

Figure 1: Representative dot plots of flow cytometry and the gating strategy used (A to D). (A) shows forward and side scatter to gate lymphocytes. (B) shows that $CD4^{+}$ cells were acquired after gating the lymphocyte population by forward and side scatter properties. (C)and(D) show the gating approach for $CD25^{+}$ cells and for discrimination between $CD25^{high}$ and $CD25^{low}$ on $CD4^{+}$cells. The gates for the $CD25^{high}$ and $CD25^{low}$ populations were set by comparing the CD25 expression levels of $CD4^{-}$ cells.

Outcome measures

The primary outcome measure of the study was the difference in percentages of regulatory T cells ($CD4^{+}CD25^{high}$ $Foxp3^{+}$) between patients with type 2 diabetes mellitus and healthy controls. Secondary outcome measures were possible relations between regulatory T cell percentages and body mass index, disease duration and control of diabetes, and presence of diabetic complications.

Statistical analysis

Analysis of data was performed using the SPSS program, version 15. Data were expressed as mean ± standard deviation (SD) for parametric data, and as median and interquartile range (IQR) for non-parametric data, respectively. Student t-test and Mann-Whitney U test were used to compare the means of two groups containing parametric and non-parametric data, respectively. Chi-square test was used to compare categorical data. To assess the strength of the relationship between Treg cell percentages and the other studied variables, Spearman's correlation coefficient test was performed. Linear regression analysis was performed to identify factors independently associated with Treg percentages among diabetic patients. A p-value of less than 0.05 was considered significant.

Results

The present study included 50 patients with type 2 diabetes and 30 healthy individuals as a control group. Characteristics of the study groups are displayed in table 1. Age, gender, and body mass index were comparable between both groups. Fasting and postprandial plasma glucose levels were significantly higher among patients with T2D than among healthy controls.

Table 1. Characteristics of the study groups

	Type 2 diabetes n= 50	**Controls n= 30**	***p***
Age (y)	55.06 ± 8.13	54.37 ± 7.47	**0.705**
Sex, n (%) **Male** **Female**	 22 (44) 28 (56)	 16 (53.3) 14 (46.7)	 **0.418**
BMI (kg/m^2)	32.85 ± 5.43	32.31 ± 5.06	**0.660**
FPG (mg/dL)	178.30 ± 64.82	87.60 ± 7.29	< **0.001**
2hPP (mg/dL)	249.86 ± 89.43	111.50 ± 9.81	< **0.001**

Values presented as mean ± standard deviation except sex, as number (%)
BMI, body mass index; FPG, fasting plasma glucose; 2hPP, 2-hour plasma glucose

Among patients, mean disease duration was 9.3 ± 7.8 years. Hypertension was present among 13 (26%) patients, and dyslipidemia and ischemic heart disease were present among 6 (12%) patients. Forty (80%) patients had uncontrolled diabetes. Mean hemoglobin A1c among patients was 8.6 ± 1.1. Fifteen (30%) patients had diabetic neuropathy, whereas 10 (20%) patients had diabetic nephropathy. Urinary protein/creatinine ratio ranged from 0.06 to 0.46 and had a mean of 0.19 ± 0.09. Figure 2 shows that Treg cell percentages were significantly lower among T2D patients as compared to healthy controls.

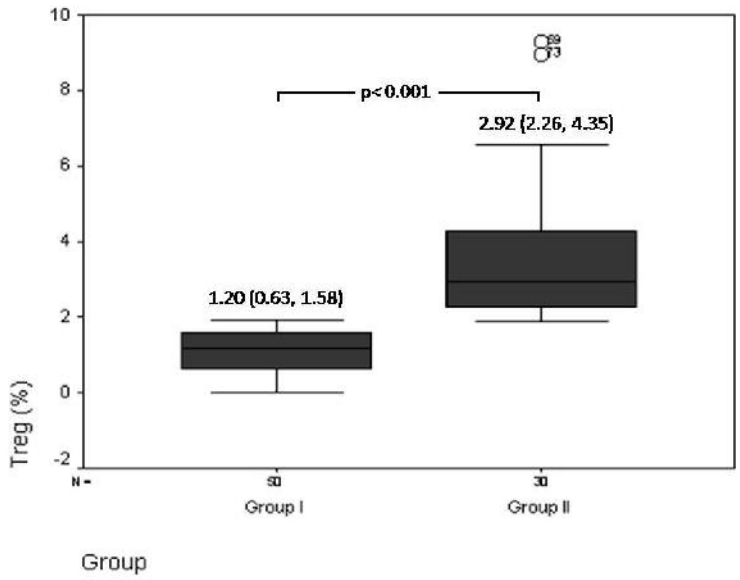

Figure 2: Comparison of Treg cell percentages between both study groups
Group I, patients with type 2 diabetes; group II, healthy controls
Treg, regulatory T cells

Percentages of Treg cells did not differ significantly according to gender, the presence of hypertension, dyslipidemia, ischemic heart disease, or diabetic neuropathy (table 2).

Table 2. Treg cell percentages according to the studied parameters in patients with type 2 diabetes

	Type 2 diabetes	*p*
Sex		
Male	1.28 (1.17, 2.52)	**0.953**
Female	1.07 (0.77, 2.33)	
Hypertension		
Present	0.99 (0.57, 1.24)	**0.174**
Absent	1.36 (0.66, 1.67)	
Dyslipidemia		
Present	1.07 (0.46, 1.69)	**0.858**
Absent	1.22 (0.65, 1.58)	
IHD		
Present	0.78 (0.59, 1.01)	**0.120**
Absent	1.28 (0.65, 1.64)	
Diabetic neuropathy		
Present	1.24 (0.58, 1.44)	**0.518**
Absent	1.11 (0.64, 1.68)	

Values presented as median (interquartile range)
IHD, ischemic heart disease

Treg cell percentages were significantly lower among diabetics with nephropathy than among those without nephropathy (figure 3), and correlated inversely with urinary protein/creatinine ratio (table 3). Treg cell percentages were significantly lower among patients with uncontrolled diabetes than among those with controlled diabetes [1.0 (0.57, 1.46) *vs.* 1.58 (1.50, 1.76), p= 0.003], and correlated inversely with hemoglobin A1c (table 3). As shown in table 3, Treg cell percentages among patients correlated

significantly with age, body mass index and disease duration, but showed no significant association with fasting or postprandial plasma glucose levels. Regression analysis was performed with T-reg cell percentages as the dependent variable, and age, BMI, HbA1c, and urine protein/creatinine ratio as independent variables. Only BMI was found to be significantly and independently associated with Treg cell percentages (coefficient for BMI -0.096, 95% CI: -0.105 to -0.086).

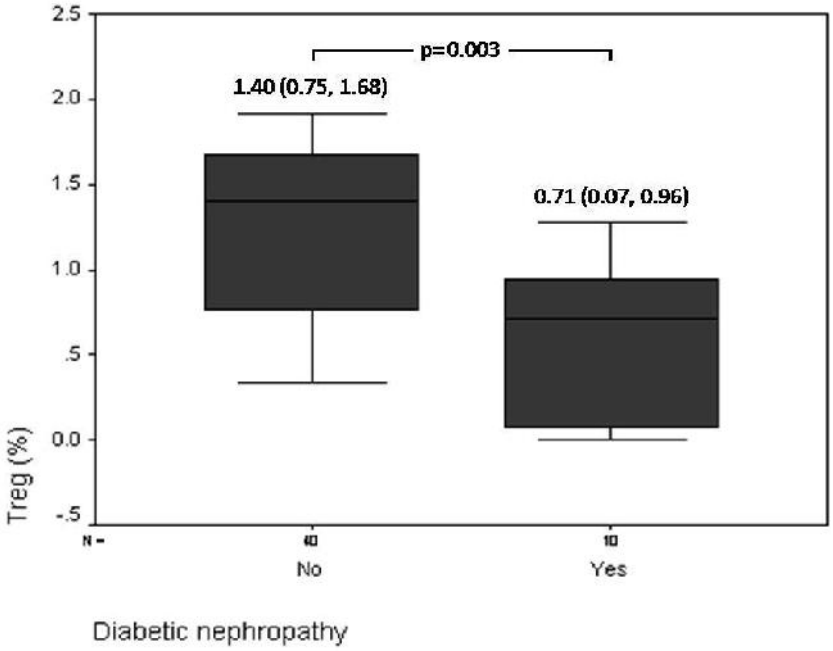

Figure 3: Comparison of Treg cell percentages between diabetic patients with nephropathy and those without nephropathy
Treg, regulatory T cells

Table 3. Correlations between Treg cell percentages and the studied parameters in patients with type 2 diabetes

Spearman's correlation	*r*	*p*
Age	-0.491	< **0.001**
BMI	-0.973	< **0.001**
Disease duration	-0.603	< **0.001**
FPG	-0.178	**0.217**
2hPP	-0.223	**0.119**
UPCR	-0.579	< **0.001**
HbA1c	-0.299	**0.035**

BMI, body mass index; FPG, fasting plasma glucose; HbA1c, hemoglobin A1c; UPCR, Urinary protein/creatinine ratio; 2hPP, 2-hour plasma glucose

Discussion

There has been growing interest in the possible relationship between percentages of Treg cells and T2D; however, studies in the literature are very scarce. In the present study, we demonstrated that percentages of Treg cells were significantly lower among patients with T2D than among healthy controls. Similarly, Jagannathan-Bogdan *et al*. also observed a statistically

significant reduction in the percentage of circulating Tregs in T2D patients compared with non-diabetic patients[12].A recent Chinese study also confirmed this finding [13].

The link between Treg cells and obesity and insulin resistance involves macrophages. Macrophages accumulate in visceral adipose tissue, and their numbers increase with increasing obesity. This increase is accompanied by a phenotype skew in visceral adipose from an anti-inflammatory to a proinflammatory type, leading to a state of insulin resistance [14]. Treg cells are protective against insulin resistance possibly through reducing the influx of inflammatory macrophages to adipose tissue and reducing proinflammatory cytokine production by adipocytes [9]. Indeed, we observed that Treg cell percentages in our study were correlated negatively and strongly with BMI. In line with this finding, Feuerer *et al*. demonstrated that Treg cells were highly abundant in the abdominal fat of normal mice, but were markedly reduced at this site in insulin-resistant models of obesity. They observed a good correlation between insulin resistance and the fraction of Treg cells in abdominal fat. In addition, they observed a significant correlation between BMI and the drop in Treg cells in omental fat of obese humans [9]. Another American study confirmed these findings, and demonstrated that mice which were randomized to a high-fat diet intervention for 12 weeks showed a striking decrease in visceral adipose Tregs, which correlated with the severity of insulin resistance. In addition, visceral adipose tissue of morbidly obese humans showed a significantly reduced percentage of Treg cells, as well as decrease in Foxp3 expression, in comparison to lean controls [15]. A recent German study also reported that circulating Treg cells were reduced significantly in obese as compared to non-obese subjects and correlated inversely with body weight and body mass index [16].

Diabetic nephropathy is an extremely common complication of DM and a leading cause of end-stage renal disease. In the present study, 20% of patients with T2D had diabetic nephropathy. The percentages of regulatory Tcells were significantly lower among patients with diabetic nephropathy than among those without diabetic nephropathy. Furthermore, there was a significant inverse correlation between Treg cell percentages and urinary protein/creatinine ratio. We also observed a significant correlation between Treg cell percentages and disease duration, which is also possibly related to the presence of chronic inflammation and nephropathy among these patients. Similar findings have also been reported by a Chinese study which demonstrated that although the percentages of Treg cells were comparable between patients with T2D and healthy controls, Treg cell percentages were significantly lower among diabetics with nephropathy than among healthy controls, and were significantly lower among patients with macroalbuminuria than among those with microalbuminuria [17].An Austrian study showed that Treg cell depletion after treatment with a CD25-depletion antibody in db/db mice resulted in augmented insulin resistance and a more severe diabetic nephropathy. The urinary albumin/creatinine ratio was increased in Treg cell-depleted *versus* non-depleted db/db mice. In the same study, transfer of Treg cells improved insulin resistance and significantly decreased urinary albumin/creatinine ratio thus improving kidney function [5]. Another recent Chinese study also confirmed the significant negative correlation between Treg cell percentages and both disease duration and urinary protein/creatinine ratio [13].

Eighty percent of patients in the present study had uncontrolled DM. The percentages of regulatory T cells were significantly lower among patients with uncontrolled diabetes than among those with controlled diabetes. In addition, these cells correlated significantly and negatively with hemoglobin A1c, as a marker for diabetes control. However, no significant correlation was observed between the percentage of Treg cells and fasting plasma glucose or 2-hour plasma glucose levels. These findings have also been reported by a recent German study [16].

Reduced numbers of Treg cells have been observed among patients with acute coronary syndrome [18], and in atherosclerotic lesions in both humans [19] and experimental mice [20]. In addition, *in vivo* transfer of Treg cells attenuated experimental arteriosclerotic lesion formation [21]. In the present study, there was no significant difference in Treg cell percentages between diabetic patients with dyslipidemia and those without, or between diabetics with ischemic heart disease and those without; however, this is attributed to the small number of diabetics with dyslipidemia or ischemic heart disease in our study. Further larger clinical studies for investigating these associations are required.

In summary, we confirm the possible role of Treg cells in T2D, and demonstrate a significant reduction of Treg cell percentages among patients with T2D which correlated with body mass index, disease duration and

the degree of diabetic nephropathy. The reduction in percentages of Treg cells among patients with T2D may be interpreted as a causal contribution, as evidenced by the aggravation of insulin resistance after Treg cell depletion in adipose tissue [9], as well as the reduction in adipose inflammation and the improvement of insulin resistance in ob/ob mice models with T2D after induction of Treg cells [10]. Future large-scale studies are required to assess whether the induction of Treg cells among patients with T2D may reduce local adipose tissue inflammation, improve insulin resistance, and limit pathologic complications. Treg cell induction may represent a possible new therapeutic measure among these patients in the future.

Conflict of interest

The authors declare that no funding or grant was received for the study, and that they have no conflict of interest, financial or personal relationship related to the study.

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