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Increase in TGF-β and CD4+CD25+Regulatory T Cells in Iranian Patients With Type 2 Diabetes :A Case–Control Study

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Abstract

Background: Patients with type 2 diabetes mellitus (T2DM) often have imbalance in inflammatory cells, increased inflammatory cytokines, and defects in homeostasis. The present study aimed to evaluate the percentage of Treg cells, lymphocytes, T cells, and T helper cells and the expression of the TGF-β cytokine gene in T2DM patients.

Methods : This study was conducted on 50 patients with T2DM and 50 healthy controls according to the inclusion criteria. The percentage of Treg cells, T cells, and T helper cells was determined by flow cytometry. Also, the expression of the CD4, CD25, and FOXP3 markers of Treg cells was examined. The gene expression of TGF-β cytokine was evaluated by real-time polymerase chain reaction (PCR).

Results: The percentage of Treg cells was significantly lower in patients with T2DM than in healthy controls. The number of T helper cells and lymphocytes decreased in T2DM patients as compared to the healthy controls. Based on the results, the percentage of T cells was higher in T2DM patients than in healthy controls. The expression of CD25 and FOXP3 markers in Treg cells significantly decreased in T2DM patients compared to the healthy controls; however, this decrease was not significant for the CD4 marker. Conversely, the expression of the cytokine TGF-β increased in patients with T2DM compared to the healthy controls.

Conclusion: The expression of TGF-β and the percentage of CD4+CD25+regulatory T cells were impaired in patients with T2DM.

Keywords: Regulatory T cells, Transforming growth factor β (TGF-β), Type 2 diabetes mellitus (T2DM)

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Introduction

Type 2 diabetes mellitus (T2DM) is one of the most common chronic metabolic diseases associated with insulin resistance, glucose tolerance, hyperglycemia, and dysfunctions of pancreatic beta cells (1). According to the World Health Organization (WHO), it is estimated that 200–300 million people will develop T2DM by 2025 worldwide. Considering the increasing trend of T2DM prevalence, 5.2 million Iranians are predicted to develop this disease by 2025 (2). The complications of T2DM can be divided into two categories: macrovascular (arthrosclerosis and amputation) and microvascular (retinopathy, nephropathy, and neuropathy). Factors such as inflammatory conditions play a role in the occurrence of these complications (3).

T2DM is a heterogeneous disease in which various genetic, endocrine, and immunological factors are involved (4). T lymphocytes are involved in the development and progression of T2DM (5). Effector T cells promote inflammation and pathogenesis of T2DM by producing cytokines and inducing effector activity and inflammation. On the other hand, regulatory T cells (Treg cells) are involved in controlling inflammatory cells and their produced cytokines, regulating the response of other T cells, and suppressing their response to blood antigens. Overall, the presence of these cells is essential for the control and maintenance of homeostasis (6).

Evidence shows that Treg cells play an essential role in controlling autoreactive activity against pancreatic islet cells in humans and mice. Dysregulation of Treg cells causes autoimmune diseases, such as type 1 diabetes (T1D) (7). These cells reduce inflammation in T2DM

patients by suppressing the production of inflammatory cytokines (6). It is known that there is an imbalance in Th17 and Treg cells among patients with T2DM and obese people. It has also been shown that there is no homeostasis between T cells in these patients, which results in inflammation (8). In a study by Zeng et al on patients with T2DM, there was an imbalance among Th1, Th17, and Treg cells; the Treg/Th1 and Treg/Th17 ratios are lower in these patients (9).

Treg cells comprise 5%–20% of TCD4 + cells, including CD4 + and CD25 + (IL2R) (10). The CD4 + CD25 + cells, which suppress the proliferation and production of cytokines from both CD4 + and CD8 + cells, can be detected in the human peripheral blood. The interleukin-2 receptor alpha chain (CD25 or IL-2Ra) is a marker with high expression on the surface of Treg cells (11). The IL-2 signaling pathway induces FOXP3 + gene expression in murine and human CD4 + CD25 + T cells by activating the STAT5 transcription factor, both *in vitro* and *in vivo* (12). Moreover, Treg cells contain FOXP3 transcription factors. FOXP3, which may act as a repressor or activator of specific genes, is a key transcription factor for Treg cells (11).

Treg cells produce transforming growth factor-beta (TGF-β), interleukin-10 (IL-10), and interleukin-4 (IL-4) (13, 14). IL-10 is a multifunctional cytokine produced by Treg cells (15). The IL-10 cytokine suppresses the proliferation of T lymphocytes and their cytokine secretion. Therefore, this cytokine plays a key role in inflammatory responses related to insulin resistance (16). The TGF-β cytokine maintains the expression of FOXP3 transcription factor in CD4 + CD25 + Treg cells and enhances their immunosuppressive function (17, 18).

Generally, Treg cells play an important role in many diseases; therefore, their reduction contributes to the pathogenesis of many diseases. Treg cells play an important role in T1D, since the immunotherapy or active transfer of these cells causes the CD25 + mean fluorescence intensity (MFI) of CD4 + CD25 + cells to rise and remain high for up to seven days (19). In this regard, Crispin et al found that the percentage of CD4 + CD25 + T cells decreased in untreated systemic lupus erythematosus (SLE) patients (20).

The present study aimed to evaluate Treg cells in patients with T2DM and to compare them with healthy non-diabetic controls. In this study, the expression of Treg cell markers (CD4, CD25, and FOXP3) or the MFI of the markers was evaluated in patients with T2DM and compared with a healthy control group. Also, in this study, the percentage of lymphocyte cells, T cells, and T helper (TH) cells and TGF-β expression were examined and compared between T2DM patients and healthy controls.

Materials and Methods

T2DM patients and healthy controls

Peripheral blood samples (5 mL) were taken from 50 patients with T2DM and 50 healthy non-diabetic individuals (healthy controls) in Arak, Markazi Province, located in the central western Iran. The healthy control group was matched with the patient group in terms of age, sex, and body mass index (BMI). The healthy control group was also evaluated for fasting blood sugar (FBS) levels and clinical signs. On the other hand, patients with T2DM were selected according to standard indicators of diabetes. The clinical characteristics of the participants are summarized in Table 1.

Inclusion criteria

The inclusion criteria were as follows: (1) mean age of 25–55 years in both sexes; (2) FBS ≥ 126 mg/dL; (3) BMI > 25 kg/m^2; and (4) use of oral hypoglycemic drugs, including monotherapy with metformin or glyburide (no use of insulin).

Exclusion criteria

The exclusion criteria were as follows: (a) T1D; (b) kidney failure (serum creatinine > 2 mg/dL) or liver failure (liver enzymes more than twice the normal range); (c) acute or chronic inflammatory diseases; and (d) malignancies.

Flow cytometry analysis

Monoclonal antibodies and flow cytometry reagents

To analyze Treg cells, they need to be stained with appropriate CD4, CD25, and FOXP3 monoclonal antibodies. For this purpose, peridinin chlorophyll protein (PerCP)-conjugated anti-CD4, phosphatidylethanolamine (PE)-conjugated anti-CD25, and fluorescein isothiocyanate (FITC)-conjugated anti-Foxp3 (BD Biosciences, USA) were used. All antibodies were obtained from BD Biosciences (Bedford, MA, USA).

To examine the blood samples by flow cytometry, a fresh blood sample (50 μL) was poured into two microtubes. Monoclonal anti-CD4 and anti-CD25 antibodies were

Table 1. Comparison of demographic and clinical data in T2DM and healthy controls

Variable	Healthy controls (Mean ± SE)	Type 2 diabetes (T2DM) patients (Mean ± SE)	*P* value
Age (y)	55 ± 1.61	55.42 ± 1.29	1
Sex (male/female)	17/19	30/42	-
BMI (kg/m^2)	28.09 ± 0.94	28.10 ± 0.69	1
FBG (mg/dL)	97.53 ± 7.46	137.72 ± 13.99	0.049
HbA1c (%)	NA	6.95 ± 0.18	-
ALT (U/L)	20 ± 1.73	24.79 ± 1.86	0.881
Creatinine (mg/dL)	1.03 ± 0.02	0.99 ± 0.01	0.887

BMI, Body mass index; FBG, Fasting blood glucose; HbA1c, Hemoglobin A1c; ALT, alanine aminotransferase.

then added to one of the microtubes and incubated for 30 minutes in the dark at room temperature. The lysis solution (RL) was then added, and the mixture was incubated at room temperature for ten minutes. After centrifuging, discarding the supernatant, and washing with a phosphate-buffered saline (PBS) solution, a fixation buffer was added, and the mixture was incubated in the refrigerator for 30 minutes. After rinsing with PBS buffer, the monoclonal anti-FOXP3 antibody was added. Following 45 minutes of incubation in the dark, the samples were added to a FACSCalibur™ flow cytometer (BD Biosciences) to count the Treg cells and the MFI was determined using FlowJo software (Tree Star, Ashland, OR, USA) (21,22).

Real time-polymerase chain reaction (RT-PCR) assay

Total RNA extraction and cDNA synthesis were performed, according to Yekta Tajhiz Azma cDNA synthesis kit (Yekta Tajhiz Azma, Iran).

Initially, the primers for TGF-β and GAPDH (as housekeeping gene) were designed using AlleleID 6.0 (Premier Biosoft Intl., USA). Next, quantitative PCR (qPCR) was carried out using SYBR® Green qPCR Master Mix (Yekta Tajhiz Azma, Iran); all reactions were performed in triplicate on a LightCycler system (Roche Diagnostics, Germany). Primer-dimer and non-specific bands were excluded, based on the melting curve assay. To measure the relative gene expression in each sample, the Pfaffl method was used, and the ratios were considered as the final results for statistical analyses.

Statistical analysis

Values were mainly expressed as the mean ± standard deviation (SD). Data were analyzed using SPSS 16.0 statistical software (Inc, Chicago, IL, USA). The independent samples *t*-test was used for data analyses. A *P* value < 0.05 was considered to be statistically significant.

Results

There were no significant differences between T2DM and healthy controls with regard to age, sex or BMI ratios (Table 1). The median duration of diabetes in the patients participating in the study was 4 years.

The analysis of Treg cells in the peripheral blood of T2DM patients and the healthy controls showed that the percentage of CD4 + CD25 + FOXP3 + Treg cells in T2DM patients was significantly lower as compared to the healthy control group (Figure 1A). Based on this comparison, the percentage of T cells in T2DM patients was significantly higher compared to the healthy control group (Figure 1B).

The results showed that the percentage of lymphocytes was lower in T2DM patients than in the healthy controls (Figure 1C). Based on this comparison (Figure 1D), it was found that the percentage of TH cells in T2DM patients

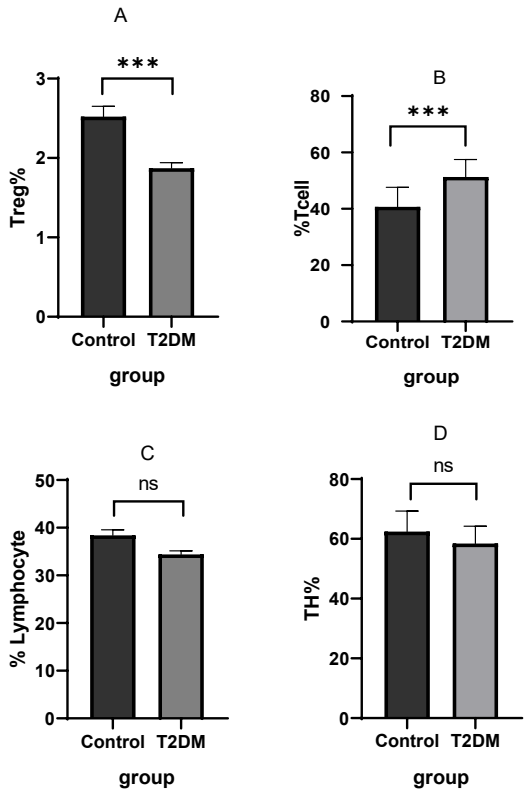

Figure 1. Comparison of the percentage of Treg cells (A), T cells (B), lymphocytes (C) and TH cells (D) in T2DM patients and healthy controls. NS: not significant (with $P > 0.05$); *** $P < 0.001$

was lower than the healthy control group; this difference was not statistically significant as compared to the healthy control group.

In this study, it was found that the expression (MFI) of FOXP3 marker was lower in T2DM patients as compared to the healthy controls (Figure 2A). The expression of CD25 marker (MFI) was lower in T2DM patients as compared to the healthy controls (Figure 2B). Also, the expression of CD4 marker (MFI) in T2DM patients was lower compared to the healthy controls; however, this difference was not significant compared to the healthy control group (Figure 2C).

Based on this comparison, the expression of TGF-β cytokine was higher in T2DM patients compared to the healthy controls (Figure 3).

Discussion

T2DM is a chronic inflammatory disease, caused by changes in the number and function of immune cells. Some studies suggest that T cells and inflammation are involved in T2DM (23). However, studies on the immunopathogenesis of T2DM are limited. Treg cells are a subset of TCD4 + cells with a regulatory activity. These cells are identified by the CD4, CD25, and FOXP3 markers, and their regulatory function is defined by these markers. In diseases, such as T1D, the ability to regulate CD4 + CD25 + T cells is lower in patients compared to

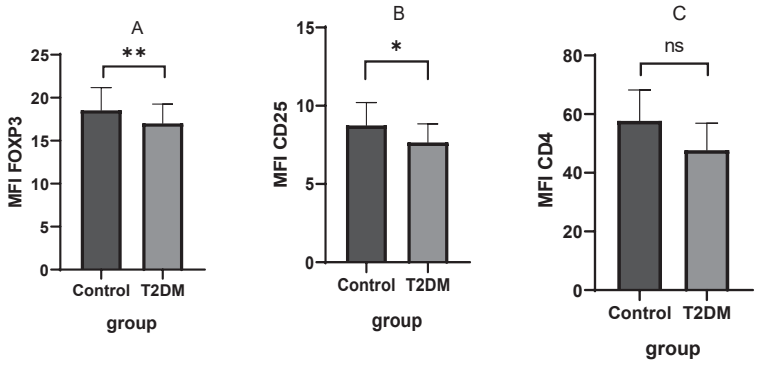

Figure 2. Calculated mean fluorescence intensity (MFI) of FoxP3 (A), CD25 (B) and CD4 (C) molecules in T2DM patients and healthy controls. NS: not significant ($P>0.05$); ** $P<0.01$; * $P<0.05$

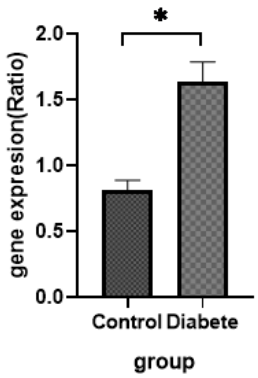

Figure 3. Comparison of TGF-β gene expression in T2DM patients and healthy controls. * $P<0.05$

healthy controls (24).

FOXP3, as a transcription factor, has been identified as a specific marker for Treg cells and is considered essential for the development and activity of these cells (24). Treg cells can inhibit inflammatory cells and improve insulin resistance through cell-cell contact or through the production of anti-inflammatory cytokines (IL10 and TGF-β) and secretion of immunosuppressants (25); therefore, in T2DM patients, a decrease in Treg cells can increase insulin resistance (5). Overall, Treg cells may reduce the number of immune cells, such as monocytes, macrophages, and dendritic cells, which are known to reduce the immune system response and improve diabetes (26).

The results of the present study showed that the percentage of Treg cells significantly decreased in T2DM patients as compared to the healthy controls. The reduction of Treg cells in T2DM patients is consistent with a number of studies (23). One of the reasons for the decrease in Treg cells in T2DM patients is the reduced Treg cell production in the thymus (27).

In another study by Qiao et al on patients with T2DM, the number of CD4+CD25+FOXP3+Treg cells was lower, without significant difference in the effector T cells (28). Moreover, in a study by Ilan et al, it was found that reduction of Treg cells by anti-CD25 monoclonal antibodies caused an increase in insulin resistance and FBS and impaired insulin sensitivity. Overall, complications of T2DM depend on the percentage of Treg cells and the serum levels of IL-10 (29). Decrease in Treg cells can contribute to the complications of diabetes (28).

The present results showed that TGF-β gene expression is higher in T2DM patients compared to healthy individuals. Generally, TGF-β cytokine is recognized as a multifunctional growth factor with inflammatory and anti-inflammatory effects, which may be secreted by many immune cells (30). Contrary to the results of our study, in the study by Yuan et al on newly diagnosed T2DM patients, serum TGF-β levels were lower. However, the difference in the results of various studies can be due to different study designs. The transcription factor Foxp3 plays an important role in the development and function of Treg cells (31). Elevation of TGF-β could be a compensatory mechanism for inducing FoxP3 expression and the subsequent production of the Treg phenotype of CD4 T4 cells in T2DM patients (32). Abbasi et al observed that TGF-β plays a role as a pro-inflammatory mediator by inducting Th17 cytokine production (33). Thus, TGF-β may play an important role in controlling immunity and inflammation in T2DM.

The percentage of T cells in T2DM patients was higher compared to the healthy controls. T cells play a vital role in inflammation and obesity in T2DM patients. Adipose-resident T cells increase inflammatory effector T cells and decrease the regulatory activity of Treg cells. Activation of T cells can increase the proliferation of these cells and lead to the production of inflammatory cytokines (34). In a study by Kraakman et al, it was shown that blockade of T cells reduced insulin resistance in mouse models of T2DM (35).

Moreover, the results of the current study showed that the expression of FOXP3 marker of Treg cells (MFI) was lower in T2DM patients as compared to the healthy controls. In a study by Aghili et al, the number of Treg cells in patients with T1D was evaluated. The number of these cells in patients with T1D was not significantly different from the controls, whereas the MFI of FOXP3

was significantly lower in these patients. It was also found that the decreased expression of FOXP3 might play a regulatory role in the Treg cells of diabetic patients and the pathogenesis of the disease (24). Decrease in FOXP3 expression could suppress and reduce the activity of Treg cells (36).

The results of the present study revealed that the expression of the CD25 marker (CD25 MFI) of Treg cells was lower in T2DM patients as compared to the healthy control group. In a study by Li et al, the suppressive activity of Treg cells was associated with the expression of the CD25 marker. Besides, the results of human studies have shown that the repressive function is reduced in Treg CD25-/^{low} cells (37). Moreover, previous studies have shown that CD4 + D25 + FOXP3 + Treg cells can contribute to the development and progression of T2DM (38).

Treg cells and their markers, such as CD25 and FOXP3, were lower in T2DM patients compared to the healthy controls while the percentage of T cells in T2DM patients was higher. Therefore, Treg cells play an important role in the development of T2DM patients by limiting the pro-inflammatory environment. Thus, we hypothesize that the enhancement of Treg cells may improve immune responses in T2DM patients.

However, the role of the immune system in the pathogenesis of T2DM requires further investigation. Future studies can help find the pathogenesis of T2DM, reduce its complications, and help the treatment of this disease.

Conclusion

This study showed a difference in the percentage of Treg cells and some markers in the immunopathogenesis of T2DM. However, further studies on the mechanisms involved in the immunopathogenesis of this disease can help design new treatment protocols.

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This study was extracted from the master's thesis of the first author.

Authors' Contribution

Conceptualization: Mehdi Salehi.
Data curation: Mehdi Salehi.
Formal Analysis: Ali Ganji.
Funding acquisition: Ali Ghazavi.
Investigation: Nazanin Shahnoruzi.
Methodology: Ghasem Mosayebi.
Project administration: Ali Ghazavi.
Resources: Mehdi Salehi.
Software: Ali Ganji.
Supervision: Ali Ghazavi.
Validation: Ali Ghazavi.
Visualization: Nazanin Shahnoruzi.
Writing – original draft: Nazanin Shahnoruzi.
Writing – review & editing: Ali Ghazavi.

Competing Interests

The authors declare that there is no conflict of interest.

Ethical Approval

The Ethics Committee of Arak University of Medical Sciences approved this study (Ethical Code: IR.ARAKMU.REC.1399.043; IRCT registration number: IRCT20141209020258N144; https://www.irct.ir/trial/48988) on May 3, 2020.

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