Role of alteration in Treg/Th17 cells’ balance in nephropathic patients with Type 2 diabetes mellitus

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Type of article: Original

Abstract

Introduction: In type 2 diabetes mellitus, the adaptive immune system drives systemic inflammation, promoting insulin resistance and related complications, such as diabetic nephropathy. Increased infiltration of activated T lymphocytes has been found in patients with diabetic nephropathy. T-cell influx and accumulation are the factors that aggravate diabetic nephropathy and link with glomerular filtration surface and albumin excretion. An appropriate balance between pro-inflammatory (T helper 17: Th17, and T helper 1: Th1) and anti-inflammatory (regulatory T cells: Tregs) subsets of T cells is critical to maintain homeostasis and avoid inflammatory disease. The aim of this study was to determine the balance between T helper 17 and regulatory T cells in type 2 diabetic patients who have diabetic nephropathy.

Methods: This case control study was conducted between December 2013 and June 2014 in Theodor Bilharz Research Institute in Egypt. Forty patients and 20 healthy volunteers were recruited in the study, and three groups were formed, i.e. two groups of cases with 20 patients in each group and one group of 20 controls) The groups were 1) 20 type 2 diabetic patients with nephropathy (group A); 2) 20 type 2 diabetic patients without nephropathy (group B); and 3) 20 healthy individuals (control group). Evaluation of T cells was done by standard 2-color flow cytometry.

Results: The study found higher mean of Th17 counts and Th17/Treg ratio among type 2 diabetic nephropathy patients compared to other groups; but a lower mean of Treg count was identified among type 2 diabetic nephropathy patients than in the other groups (p-value = 0.001).

Conclusion: The important role for regulatory T cells in the protection against nephropathy in type 2 diabetic patients was demonstrated, and also it was observed that T helper 17 cells were associated with renal affection.

Keywords: Type 2 diabetes mellitus, Treg/Th17 cells, diabetic nephropathy

1. Introduction

In type 2 diabetes mellitus (T2DM), the adaptive immune system drives systemic inflammation, promoting insulin resistance and related complications, such as diabetic nephropathy (DN) (1). Increased infiltration of activated T lymphocytes, in addition to amplified expression of inflammatory cytokines in the kidneys, has been found in patients with DN (2). Various studies have described T cells as a chief cause for DN in type 2 diabetic patients. T-cell influx and accumulation in the juxtaglomerular apparatus are the factors that aggravate diabetic nephropathy and link with the glomerular filtration surface and albumin excretion rate (3). T cells in T2DM patients are naturally distorted toward pro-inflammatory subsets that likely endorse chronic inflammation in T2DM through prominent cytokine production. However, regulatory T cells (Tregs) protect against inflammation, thus reducing insulin resistance, which points toward the fact that Tregs may recover T2DM (4). A suitable balance between pro-inflammatory (T helper 17: Th17, and T helper 1: Th1) and anti-inflammatory (Tregs) subsets of T cells is vital to maintain homeostasis and circumvent inflammatory disease (4). Determining the balance between Th17 and Treg cells may facilitate the sighting of new therapeutic strategies in the treatment of type 2 DN. The aim of this research was to evaluate the balance between T helper 17 and regulatory T cells in type 2 diabetic patients having diabetic...
nephropathy in comparison to type 2 diabetic patients without nephropathy and to a control group of healthy volunteers.

2. Material and Methods

2.1. Study design and setting
This case control study was carried out in the period between December 2013 and June 2014. Forty patients were recruited from those who came to the Nephrology Department of Theodor Bilharz Research Institute (outpatient clinic and inpatient ward) and 20 healthy volunteers. The participants were divided to three groups as follows: Group A (Diabetic nephropathy group): Twenty type 2 diabetic patients with nephropathy as evidenced by presence of microalbuminuria (30-300 mg/d) or macroalbuminuria (more than 300 mg/d) and/or decreased GFR (5); Group B (Diabetic group): Twenty type 2 diabetic patients without nephropathy as evidenced by absence of microalbuminuria or decreased GFR; Group C (Controls): Twenty healthy individuals volunteered from medical and paramedical staff of the same age and gender.

2.2. Ethical consideration
The study protocol and its ethics were approved by the Theodor Bilharz Research Institute. In addition, written informed consent was provided by all participants after the details, benefits, and risks of the study were explained to them.

2.3. General methods
All patients were subjected to the complete history and physical examination and a complete laboratory investigation, including blood sugar estimation, including fasting blood sugar (FBS), 2-h postprandial (2hPP) blood glucose level, and glycated hemoglobin. The kidney function test included serum creatinine, serum urea, creatinine clearance, and serum electrolytes, including serum sodium (Na+) and serum potassium (K+). We also collected urine over a 24-hour period and analyzed the urine for microalbuminuria, macroalbuminuria, and creatinine clearance. We also conducted liver function tests and analyzed the lipid profile, including serum total cholesterol, triglycerides, high density lipoprotein (HDL), and low density lipoprotein (LDL). Complete blood count, abdomino-pelvic ultrasound, 12 lead electrocardiography, and echocardiography also were performed for all participants.

2.4. Flow cytometry
Evaluation of Tregs and Th17 cells was done by standard 2-color flow cytometry based on Ryba and Myśliwska (6) for Tregs and on Chalan et al. (7) for Th17 cells. In this step, we used the following monoclonal antibodies: Phycoerythrin (PE)-labeled anti-CD161, Phycoerythrin-Cyanine 5 (PC5)-labeled anti-CD4, Fluorescein isothiocyanate (FITC)-labeled anti-CD25, and Isotypic control antibodies (Beckman Coulter, USA). Fluorochrome-labeled monoclonal antibody was incubated with washed cells expressing its antigenic determinants. Then unbound conjugated antibody was washed from the cells. The cells were stained fluorescently with the intensity of staining directly proportional to the density of expression of the antigenic determinants. Cell surface expression of cell surface markers was determined by flow cytometric analysis. In the procedure of flow cytometry, first, 1-100 µl of whole blood were diluted 1:1 with phosphate-buffered saline (PBS) (Sigma Chemicals, St Louis, MO, USA). Then, a volume of 25 µl of the mixture was added to 5 µl of fluorochrome-labeled monoclonal antibody (anti-CD4 and anti-CD25 human antibodies for Tregs and anti-CD4 and anti-CD161 human antibodies for Th17 cells). For each set, appropriate isotopic control was done. The samples were mixed well and then left protected from light in the dark in the refrigerator (2-8 oC). In the next steps, we added 1 ml of lysis buffer, and Vortex was done for 10 seconds; then, the material was incubated at room temperature in the dark for 30 min.

2.5. Data analysis
Data were analyzed using EPICS-XL PROFILE II Coulter flow cytometer (Coulter, USA). A minimum of 10000 events were collected. Data were expressed as the percentage of positive events. Results were expressed as means ± standard deviation of the means (SD) or number (%). Comparison between normally distributed parameters in the three studied groups was performed using one way AVOVA followed by “least significant difference test” if significant results was recorded. Comparison between not normally distributed parameters in the three studied groups was performed using Kruskal Wallis ANOVA test followed by Mann Whitney test if significant results were recorded. Comparison between parameters in the two studied groups was performed using either unpaired t test or Mann Whitney test, whenever it was appropriate. Comparison between categorical data was performed using the chi-squared test. Correlation between different parameters was performed using Spearman rank correlation.
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coefficient. Receiver-operating characteristic (ROC) curve was used to calculate the diagnostic indices of Th17, Treg and Th17/Treg ratio. The data were considered significant if the p-value was \( \leq 0.05 \) and highly significant if p-value < 0.01. Statistical analysis was performed using the SPSS computer program (version 12 for Windows).

3. Results

The results of this study showed that Type 2 diabetic patients and healthy individuals groups were comparable as regards the mean age with no statistical significant difference, with higher mean age among type 2 diabetic nephropathy patients compared to other groups. The differences were statistically significant (Table 1). There was a higher incidence of hypertension and ischemic heart disease among type 2 diabetic nephropathy patients compared to other groups. A higher incidence of hypertension among type 2 diabetic patients compared to healthy individuals was noted. The differences were statistically significant (Table 1). There was a higher mean duration of hypertension among type 2 diabetic nephropathy patients compared to other groups and a statistically significant higher mean duration of diabetes among type 2 diabetic nephropathy patients compared to type 2 diabetic patients (Table 1). There was a higher incidence of albuminuria among type 2 diabetic nephropathy patients compared to other groups. The differences were statistically highly significant (Table 2). The groups are comparable as regards mean total cholesterol, LDL, HDL, and TAG with no statistical significant difference among type 2 diabetic nephropathy, type 2 diabetic patients, and healthy individuals. Furthermore, there was a higher incidence of statin use among type 2 diabetic nephropathy patients than the other groups. The differences were statistically significant (Table 2). There was higher mean Th17 and Th17/Treg ratio among type 2 diabetic nephropathy (Group A) than for the other groups (Table 2). Also, there was lower mean Treg among type 2 diabetic nephropathy patients than in the other groups (Table 2). The mean level of Th17 and Th17/Treg ratio among type 2 diabetic patients (Group B) were higher than it was for healthy individuals. The differences were statistically significant (Table 2). Type 2 diabetic patients and healthy individuals were comparable as regards mean Treg with no statistical significant difference (Table 2).

| Table 1. Demographic features of participants in different studied groups |
|---------------------------|-----------------|---------------------------|---------------------------|
| Variables                 | Control (n= 20) | T2DM (n= 20)  | T2DM-N (n= 20)  | p-value    |
| Age                       | 55.55 ± 2.24    | 55.00 ± 3.24    | 58.90 ± 3.31 \( \text{ab} \) | 0.001      |
| Gender (F/M)              | 7/13 (35%/65%)  | 14/6 (70%/30%)  | 10/10 (50%/50%) | 0.085      |
| DM (yes)                  | 0 (0%)          | 20 (100%)       | 20 (100%)       | ---        |
| HTN (yes)                 | 10 (50%)        | 9 (45%)         | 17 (85%)        | 0.019      |
| Ischemic heart disease (IHD) (yes) | 0         | 2         | 10         | 0.001      |
| Duration of DM (yrs.)     | ---             | 3.65 ± 1.79     | 9.95 ± 3.94     | 0.001      |
| Duration of HTN (yrs.)    | 4.20 ± 2.28     | 4.88 ± 3.83     | 8.53 ± 4.90     | 0.090      |
| Data are expressed as mean ± SD or number (%), \( \text{a} < 0.01 \) relative to control; \( \text{b} \) p < 0.01 relative to T2DM |

| Table 2. Laboratory data of participants in different studied groups |
|---------------------------|-----------------|---------------------------|---------------------------|
| Variables                 | Control (n= 20) | T2DM (n= 20)  | T2DM-N (n= 20)  | p-value    |
| Creatinine                | 0.75 ± 0.13     | 0.78 ± 0.19    | 3.30 ± 1.01 \( \text{ab} \) | 0.001      |
| Creatinine clearance      | ---             | 101.90 ± 7.06   | 40.25 ± 10.56    | 0.001      |
| FBS                       | 83.15 ± 9.62    | 154.60 ± 32.87 \( \text{a} \) | 148.00 ± 41.22 \( \text{a} \) | 0.001      |
| PP                        | 117.70 ± 12.98  | 219.50 ± 52.87 \( \text{a} \) | 231.95 ± 72.16 \( \text{a} \) | 0.001      |
| HbA1c%                    | ---             | 7.91 ± 1.34     | 8.84 ± 2.13      | 0.103      |
| Albuminuria (yes)         | 0 (0%)          | 0 (0%)         | 17 (85%)         | 0.001      |
| Proteinuria               | ---             | 0.03 ± 0.04     | 0.77 ± 0.31      | 0.001      |
| S. cholesterol (mg/dl)    | 183.30 ± 37.51  | 204.60 ± 46.98  | 207.20 ± 56.21   | 0.153      |
| LDL (mg/dl)               | 95.75 ± 89.57   | 82.05 ± 20.95   | 101.60 ± 41.94   | 0.310      |
| HDL (mg/dl)               | 44.45 ± 7.42    | 46.70 ± 7.31    | 45.15 ± 7.53     | 0.590      |
| TG (mg/dl)                | 183.15 ± 110.21 | 234.50 ± 108.21 | 223.15 ± 99.99   | 0.133      |
| Statins                   | 3 (15%)         | 9 (45%)        | 10 (50%)         | 0.022      |
| Th17 (%)                  | 3.07 ± 0.28     | 5.92 ± 0.42 \( \text{a} \) | 7.15 ± 0.51 \( \text{ab} \) | 0.001      |
| Treg (%)                  | 0.34 ± 0.05     | 0.33 ± 0.06     | 0.17 ± 0.03 \( \text{ab} \) | 0.001      |
| Th17/Treg ratio           | 9.33 ± 1.84     | 18.26 ± 3.74 \( \text{a} \) | 42.98 ± 6.87 \( \text{ab} \) | 0.001      |
| Data are expressed as mean ± SD or number (%), \( \text{a} < 0.01 \) relative to control; \( \text{b} \) p < 0.01 relative to T2DM |
There was a statistically significant strong positive correlation between serum creatinine and DM duration from one side and Th17 and Th17/Treg ratio level on the other side in the diabetic patients. While, it was strongly negative with Treg cells (Table 3). There was a statistically-significant, strong negative correlation between creatinine clearance from one side and Th17 and Th17/Treg ratio level on the other side in diabetic patients, while it was strongly positive with Treg cells (Table 3). There were higher mean Th17 and Th17/Treg ratio levels among the diabetic patients with hypertension than among the healthy individuals with hypertension. The differences were statistically significant (Table 4). The groups were comparable as regard the mean Treg with no statistical significant difference among type 2 diabetic nephropathy, type 2 diabetic patients, and healthy individuals with hypertension. The diagnostic indices of Th17, Treg, and Th17/Treg ratio using ROC curve are presented in Table 5.

### Table 3. Correlation between Th17, Treg, and Th17/Treg ratio and different studied parameters in all type 2 diabetic patients (n = 40)

| Variables       | Th17 r | p-value | T.reg r | p-value | Th17/Treg ratio r | p-value |
|-----------------|--------|---------|---------|---------|-------------------|---------|
| Cr              | 0.781  | 0.001   | -0.726  | 0.001   | 0.733             | 0.001   |
| Cr. clearance   | -0.697 | 0.001   | 0.747   | 0.001   | -0.741            | 0.001   |
| Duration of DM  | 0.703  | 0.001   | -0.691  | 0.001   | 0.711             | 0.001   |
| Duration of HTN | 0.303  | 0.141   | -0.361  | 0.076   | 0.301             | 0.144   |
| Cholesterol     | -0.044 | 0.787   | -0.046  | 0.778   | 0.017             | 0.918   |
| LDL             | 0.079  | 0.630   | -0.125  | 0.443   | 0.091             | 0.576   |
| HDL             | -0.023 | 0.887   | 0.108   | 0.505   | -0.071            | 0.662   |
| TG              | -0.055 | 0.738   | 0.086   | 0.600   | -0.119            | 0.465   |

### Table 4. Comparison between mean values of Th17, Treg and Th17/Treg ratio in all type 2 diabetic patients (n = 40) classified according to hypertension disease

| Variables       | Not Hypertensive (n = 14) | Hypertensive (n = 26) | p-value |
|-----------------|--------------------------|-----------------------|---------|
| Th17            | 6.19 ± 0.73              | 6.72 ± 0.75           | 0.038*  |
| Treg            | 0.29 ± 0.07              | 0.23 ± 0.10           | 0.056   |
| Th17/Treg ratio | 23.47 ± 9.10             | 34.47 ± 14.27         | 0.005** |

Data are expressed as mean ± SD

### Table 5. Diagnostic indices of Th17, Treg, and Th17/Treg ratio using ROC curve

| Variables       | Th17 | Treg | Th17/Treg ratio |
|-----------------|------|------|-----------------|
| Area under the roc | 1.00 | 1.00 | 1.00            |
| Cut off         | > 6.43 | ≤ 0.21 | > 23            |
| Sensitivity     | 100  | 100  | 100             |
| Specificity     | 100  | 100  | 100             |
| Positive predictive value | 100  | 100  | 100             |
| Negative predictive value | 100  | 100  | 100             |

### 4. Discussion

It is well understood that T2DM is not an immune disease, but, at this time, we could consider that there is evidence that the combination of immunologic and inflammatory mechanisms play a pivotal role in its presentation, development, and progression (8). Several studies have shown that kidney inflammation is crucial in promoting the development and progression of DN. Inflammation may be a key factor that is activated by the metabolic, biochemical, and hemodynamic derangements known to exist in the diabetic kidney (9). Diabetic nephropathy is a leading cause of chronic kidney disease, resulting in end-stage renal disease, which has become a major problem for people worldwide. Diabetic nephropathy takes place in nearly 33% of patients with type 1 DM and in approximately 25% of patients with type 2 diabetes (8). Now, we know that activation of the immune system and chronic inflammation are both involved in pathogenesis of DM and, as a result, DN (8). It was revealed that T cells in type 2 DM patients are skewed toward a pro-inflammatory phenotype, requiring monocytes for maintenance and promoting chronic inflammation through elevated cytokine (10). Treg cells are potent suppressors of autoimmunity in the
periphery, and they can dampen immune effector cell responses in the β-islets. Treg cells were identified to be specified to suppress Th 1, Th 2 or Th17 responses (1). So, Treg cells may participate in dampening the inflammation in the diabetic kidney, given the worsening of renal injury (9). Th17 cells produce Il 17, which is a potential inflammatory cytokine that contributes to several autoimmune and inflammatory diseases, including T2DM. Recent studies have shown that Il 17 is a potent inducer of T2DM (11). In our study, there was a higher incidence of hypertension and higher mean duration of hypertension among type 2 diabetic nephropathy patients (85%) compared to other groups with statistically significant difference (p-value 0.019). Also, there was a higher incidence of hypertension among type 2 diabetic patients (45%) compared to healthy individuals with high statistically significant difference. The findings of Viswanathan et al. (12), who conducted an observational study over 11 years on 152 type 2 diabetic patients in India using proteinuria as an indicator of renal affection, was consistent with our findings. It was observed that there was a higher incidence of hypertension among T2DN patients (69%) in comparison to T2DM without nephropathy (43%) with statistically significant difference (p-value <0.05). Recently, Zhang et al. (2014) investigated 93 type 2 diabetic patients using albuminuria as an indicator of renal affection and showed that the incidence of hypertension was higher in T2DN patients (82%) in comparison to control subjects. Our study also showed that there is a higher incidence of ischemic heart disease among type 2 diabetic nephropathy patients compared to other groups with very high statistically significant difference (p-value <0.001). Also, consistent with our study, Viswanathan et al. (12) investigated 139 type 2 diabetic patients in South India using proteinuria and creatinine clearance as an indicator of nephropathy; the results showed increased incidence of ischemic heart disease among T2DN patient (61%) in comparison to T2DM without nephropathy (24%). We observed in our study that there was as a higher mean duration of diabetes among type 2 diabetic nephropathy patients compared to type 2 diabetic patients with statistically significant difference. In other words; incidence of nephropathy was higher among diabetic patients with longer duration of diabetes with very high statistically significant differences (p-value < 0.001). The same observation was made by Viswanathan et al. (12), who showed higher mean duration of diabetes among T2DN patients compared to T2DM patients without nephropathy with statistically significant difference (p-value < 0.05).

Our study indicated that there was a higher mean Th17 and Th17/Treg ratio among type 2 diabetic nephropathy patients (Group A) compared to other groups with very high statistically significant differences (p-value < 0.001). And, there was lower mean Treg among type 2 diabetic nephropathy patients compared to other groups with very high statistically significant differences (p-value < 0.001). Also, there was higher mean Th17 and Th17/Treg ratio among type 2 diabetic patients (Group B) compared to healthy individuals with very high statistically significant differences (p-value < 0.001). While, type 2 diabetic patients and healthy individuals were comparable as regards mean Treg with no statistical significant difference. However, the mean level of Th17 cells was much higher and the mean level of Treg cells was a little bit lower in Group A in comparison to Group B with very high statistically significant differences (p-value < 0.001). Earlier, Bogdan et al. (4) investigated Th17 and Treg cells levels in type 2 diabetic patients in relation to healthy individuals detecting surface markers using flow cytometry. It showed that the proportion of Th17 cells in T2DM patients was increased in relation to control group. Unfortunately, it showed that the proportion of Treg cells in T2DM patients was decreased in relation to control group, which was inconsistent with the results in our study. Moreover, a recent study was conducted by Zhang et al. (13) including 93 type 2 diabetic patients with and without albuminuria detecting surface markers of Th1, Th2, Th17 and Treg using flow cytometry. The study revealed that the proportion of Th17 cells increased in T2DM patients with or without nephropathy, but the proportion of Treg cells was markedly decreased, resulting in elevated Th17/Treg ratio. Moreover, the alteration of Th17 and Treg proportions was more evident in T2DM patients with nephropathy with statistically significant differences (p-value < 0.005). A very high statistically-significant, strongly positive correlation between BUN and serum creatinine from one side and Th17 and Th17/Treg ratio levels on the other side in the diabetic patients. While, there was strong negative correlation with Treg cells (p-value < 0.001). Inversely, there was very high statistically-significant, strongly negative correlation between creatinine clearance from one side and Th17 and Th17/Treg ratio level on the other side in diabetic patients, while there was a strong positive correlation with Treg cells (p-value < 0.001). Also, our study revealed higher mean Th17 and Th17/Treg ratio levels among the diabetic patients with hypertension other than the healthy individuals with hypertension with very high, statistically-significant differences (p-value < 0.001). However, the groups were comparable as regard the mean Treg with no statistical significant difference among type 2 diabetic nephropathy, type 2 diabetic patients and healthy individuals with hypertension (p-value < 0.056). Receiver-operating characteristic curve analysis revealed that the cutoff value of Th17, Treg and Th17/Treg cells ratio at > 6.43%, ≤ 0.21 % and > 23%, respectively, could differentiate nephropathic from non-nephropathic diabetic patients with a sensitivity of 100% and a specificity of 100%. This was observed in our study with very high, statistically-significant differences (p < 0.0001). In other
words, measuring Th17 and Treg cells levels in type 2 diabetic patients can be used as an excellent screening test to detect renal involvement in those patients; so increased Th17 cells level or suppressed Treg cells level in patients with type 2 diabetic patients indicates renal involvement even before the occurrence of proteinuria or reduced GFR.

5. Conclusions
From our study, we can conclude the importance of regulatory T cells in the protection from nephropathy in type 2 diabetic patients, while T helper 17 cells are associated with renal affection. In view of relatively limited number of involved patients in the current study further evaluation of such findings in large cohorts of type 2 diabetic nephropathy to understand role of T helper 17 and regulatory T cells in development and progression of type 2 diabetes and diabetic nephropathy.

Acknowledgments:
Our sincere thanks to the Theodor Bilharz Research Institute for supporting this research.

Conflict of Interest:
There is no conflict of interest to be declared.

Authors’ contributions:
All authors contributed to this project and article equally. All authors read and approved the final manuscript.

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