



CD4 and CXCR5 in Patients with Diabetic Nephropathy

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: Diabetes is a metabolic condition characterized by hyperglycemia caused by defects in insulin secretion, insulin activity, or both. Diabetic nephropathy (DN) is one of the most common complications of diabetes mellitus especially type 2 diabetes. Blood CXCR5+CD4+ T cells represent three subsets: T follicular helper 1 (Tfh1), Tfh2 and Tfh17 cells. Increasing the numbers of circulating Tfh cells and also stimulation of Tfh cells have been linked with autoimmune diseases and may have a role in pathogenesis of diabetic nephropathy.

Aim of the Work: Was to investigate CD4 and CXCR5 in diabetic nephropathy patients and assess their role in the pathogenesis of diabetic nephropathy.

Subject and Methods: This study was conducted on 20 diabetic patients without nephropathy (Group I), 20 diabetic nephropathy patients (Group II) and 20 apparently healthy control subjects (Group III). Complete history taking and full clinical examination were done. Assessments of CD4 and CXCR5 were done by flowcytometry (FACS caliber, BD, CA, USA).

Results: The present study revealed a statistically significant difference between diabetic patients (Group I), diabetic nephropathy patients (Group II) and healthy control (Group III) regarding CD4+CXCR5+T follicular helper cells level. CD4+CXCR5+T follicular helper cells levels were

significantly higher in diabetic nephropathy patients than in diabetic patients without nephropathy and control subjects. CD4+CXCR5+T follicular helper cells level positively correlated significantly with fasting blood glucose, postprandial blood glucose, hemoglobin A1c, creatinine, urea and urinary protein level, and negatively correlated with estimated GFR in diabetic nephropathy patients.

Conclusions: This study showed that CD4+CXCR5+T follicular helper cells level might be a useful marker for prediction and diagnosis of diabetic nephropathy patients.

Keywords: CD4; CXCR5; diabetic nephropathy.

1. INTRODUCTION

Diabetes is a metabolic condition characterized by hyperglycemia caused by defects in insulin secretion, insulin activity, or both [1]. WHO categorized diabetes into two main types, known as insulin-dependent diabetes mellitus (IDDM, type 1 diabetes) and non-insulin-dependent diabetes mellitus (NIDDM, type 2 diabetes) both have hyperglycemia in common. Diabetes leads to many acute and chronic complications including diabetic ketoacidosis (DKA), non-ketotic hyper-osmolar state, retinopathy, neuropathy, and nephropathy [2].

Diabetic nephropathy (DN) is one of the most common complications of diabetes mellitus especially type 2 diabetes and is also a common cause of end stage renal disease and renal failure [3]. DN risk factors include hyperglycemia, high blood pressure levels, genetic predisposition, elevated serum lipids, smoking habits, and the dietary protein quantity and origin. Some factors such as cytokines, chemokines, growth factors, adhesion molecules, nuclear factors as well as immune cells such as monocytes, lymphocytes and macrophages result in DN pathogenesis [4]. T follicular helper (T_{fh}) cells are of great importance for expression of CXCR5, PD-1, SAP (SH2D1A), IL-21, and ICOS, among other molecules. TFH cells are important for the development of germ centers. Once germ centers are set up, TFH cells are required to support them and to regulate the differentiation of germ center B cells into plasma cells and memory B cells [5]. Increasing the amount of T_{fh} cells circulating and also the stimulation of T_{fh} cells were related to autoimmune diseases [6]. Hence the aim of the present work is to assess the role of CD4 and CXCR5 T follicular helper cells in pathogenesis of diabetic nephropathy.

2. SUBJECT AND METHODS

The present study was conducted from August 2017 till October 2019. This case control clinical

study was carried out on 60 subjects divided into three groups:

2.1 Diabetic Patients without Nephropathy (Group I)

Twenty subjects selected from outpatient clinic, Internal Medicine department, Tanta Faculty of Medicine. They were 12 males and 8 females detected of type 2 DM. Their age ranged from 40-72 years with a mean value of 57.65±9.02.

2.2 Diabetic Patients with Nephropathy (Group II)

They were 14 males and 6 females. Their age ranged from 45-75 years with a mean value of 57.10±8.93. The eligibility criteria included estimated glomerular filtration rate (eGFR) lower than 60 mL/min/1.73 m² or the ratio of urinary albumin/creatinine higher than 30 (mg alb./gm creat) for more than three months, and there were no other complications.

2.3 Control (Group III)

They were 12 males and 8 females. Their age ranged from 45-70 years with a mean value of 55.80±6.8.

Informed consent was taken from all patients and inclusion criteria included patients with type 2 diabetes mellitus without complications and patients with diabetes mellitus complicated with nephropathy.

Exclusion criteria are other causes of renal diseases, liver diseases, other chronic diseases, autoimmune diseases, other complications of diabetes mellitus and malignancy.

All individuals of the study were subjected to complete history taking, complete clinical examination, fundus examination to exclude retinopathy, exclusion of neuropathy by electroneurography.

Laboratory investigations included are complete blood count that was done on automated blood cell coulter (ERMA PCE-210N), fasting, post prandial blood glucose were done by enzymatic colorimetric method, hemoglobinA1c was done by Spectrophotometric method (Biosystems), cholesterol, triglycerides, uric acids, albumin, urea and creatinine were done by automated chemistry analyzer, sample oriented, random access (Beckman Coulter), complete urine analysis and microalbuminuria by turbidimetric method (Biosystems).

Specific investigations for measuring CD4 and CXCR5 were done by Flowcytometric Immunophenotyping.

2.4 Sampling

Five milliliters of peripheral blood were collected from subjects after their consent by the use of disposable sterile plastic syringe under complete aseptic technique. The sample was distributed as follow:

- Two ml of blood was delivered into a tube containing 50 µl of EDTA (10% conc.) tubes for complete blood count and HbA1c and flowcytometric analysis.
- Three ml of blood were taken into an empty plastic tube, left to clot and serum was separated for other investigations.

2.5 Methods

2.5.1 Flowcytometric Analysis

- 1) FACs caliber flowcytometry from Becton Dickinson was used.
- 2) Automated cell quest software was used for data acquisition and analysis.
- 3) The instrument setting was set by using calibrated beads provided by the manufacture.
- 4) Isotypic quality control was used.
- 5) 10,000 events were acquired.
- 6) Lymphocytes were gated according to their forward and side scatter light properties.

Follicular T helper cells were identified as CD4+, CXCR5+ cells.

2.6 Statistical Analysis of the Data

Data entry and statistical analysis were performed using SPSS (statistical package of social sciences) version 22. Data were

expressed in mean and standard deviation. Categorical data were expressed in numbers and percentage. The quantitative data were examined by Kolmogorov Smirnov test for normality of data. Independent sample t test (student t test), Chi-square, Fisher's exact test, Yates' corrected chi-square are computed for 2x2 tables, Analysis of variance (ANOVA) test was used for multivariate continuous normally distributed data, Linear Correlation Coefficient [r], ROC-curve (Receiver Operating Characteristic curve analysis) were used. Sensitivity, Specificity, PPV: Positive Predictive value, NPV: Negative Predictive value and accuracy were calculated.

3. RESULTS

This study was carried out on 60 subjects; 20 subjects with diabetes, 20 subjects with diabetic nephropathy and 20 healthy individuals served as a control group.

There was statistically significant difference between Diabetic patients group, diabetic nephropathy patients group and control group as regard to HB, lymphocytes, FBG, PPBG, HbA1c, triglycerides, uric acid, albumin, urea, creatinine, urinary proteins, microscopic hematuria, eGFR and CD4 CXCR5 TFH cells co-expression (p-value<0.001) with no statistically significant difference between studied groups as regard to age (p-value = 0.772), cholesterol (p-value = 0.005) and WBCs (p-value = 0.164) Table 1.

There was significant positive correlation between Tfh cells (CD4, CXCR5) and their co-expression and lymphocytes (P value =0.015), blood glucose levels (P value=0.001), creatinine (P value=0.001), urea (P value=0.001) and urinary protein level (P value=0.001). Also, significant negative correlation between Tfh cells (CD4, CXCR5) co-expression and eGFR (P value=0.007). While no correlation between Tfh cells (CD4, CXCR5) co-expression and age (P value=0.836), hemoglobin (P value=0.145), cholesterol (P value=0.672), uric acid (P value=0.450) and albumin (P value=0.687). Table 2.

In Fig. 1 the dot blot shows the co-expression of CD4 and CXCR5 on T follicular helper cells in a diabetic subject without nephropathy. The co-expression in this patient was about 1.3% and lymphocyte count was about 4 (10⁹/L) so the absolute count of CD4 and CXCR5 co-expression is about 0.052 (10⁹/L).

Table 1. Showed comparison between control group and diabetic patients' with and without nephropathy groups in different studied parameters

	Diabetic patient without nephropathy group	Diabetic patient with nephropathy group	Control group	P
Age	40 –72	45-75	45 - 70	0.772
HB (gm/dl)	9.9 – 15	7.1 – 11.2	10 -14.1	0.001
WBCs (10 ⁹ / L)	4.9 – 9.1	4.7 – 8.6	4.5 – 9	0.164
Lymphocytes (10 ⁹ /L)	0.96 - 4.24	2.13 - 4.24	0.25 - 1.59	0.001
FBG (mg/dL)	145-250	140 – 266	75 - 98	0.001
PPG (mg/dL)	200 -390	200 – 370	78 - 128	0.001
HbA1c (%)	6.3 - 8.5	6.5 – 9	4 – 6	0.001
Cholesterol (mg/dL)	150 - 195	159 – 300	155 - 195	0.005
Triglycerides (mg/dL)	55 – 256	99 – 290	52 - 150	0.001
S. Uric acid (mg/dl)	2.7 – 6.3	2.7 – 6.3	2.5 - 6	0.001
S. Albumin (gm/dl)	2.6 – 4.5	1.6 – 4.6	4.2 – 4.8	0.001
Urea (mg/dL)	19 - 41	45 – 113	18 - 42	0.001
Creatinine (mg/dl)	0.5 – 1.5	1.8 – 4.7	0.5 – 1.4	0.001
Urinary proteins (gm/24h)	0 - 2	5.2 – 11.6	0 – 0.8	0.001
Microscopic Hematuria (RBCs/HPF)	1 - 10	10 – 40	0 – 2.42	0.001
eGFR (ml/min/1.73m ²)	78 - 96	10 – 59	91 – 112.3	0.001
CD4 CXCR5 TFH cells Co-expression %	0.3 – 1.3	0.3 – 4.3	0.2 – 0.7	0.003

Table 2. Correlation between Tfh cells (CD4, CXCR5) co-expression serum level and different studied parameters in diabetic nephropathy patients (group II)

G II	TFH cells CD4 CXCR5 Co-expression %	
	R	P
Age	0.050	0.836
HB	0.338	0.145
WBCs	0.011	0.964
Lymphocytes	0.534	0.015*
FBG	0.758	0.001*
PP	0.719	0.001*
HBA1c	0.719	0.001*
S. Triglycerides	- 0.056	0.816
S. Cholesterol	- 0.101	0.672
S. Uric acid	0.179	0.450
S. Albumin	0.096	0.687
Creatinine	0.771	0.001*
Urea	0.788	0.001*
Urinary proteins	0.715	0.001*
Microscopic Hematuria	- 0.155	0.514
eGFR	- 0.584	0.007*

In Fig. 2 the dot blot shows the co-expression of CD4 and CXCR5 on T follicular helper cells in a diabetic subject nephropathy. The co-expression in this patient was about 4.3% and lymphocyte

count was about 4.24 (10⁹/L) so the absolute count of CD4 and CXCR5 co expression is about 0.18 (10⁹/L).

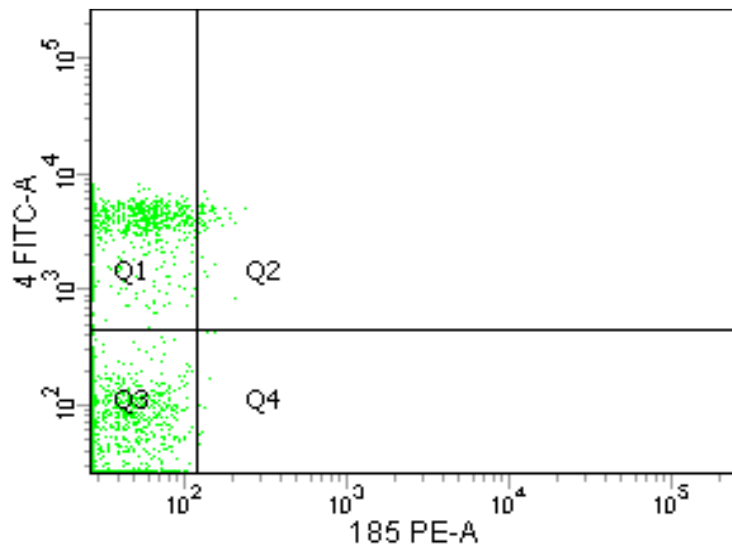


Fig. 1. Dot blot show co-expression of CD4+ (FITC) and CXCR5+ (185PE) on T follicular helper cells in diabetic patient without nephropathy

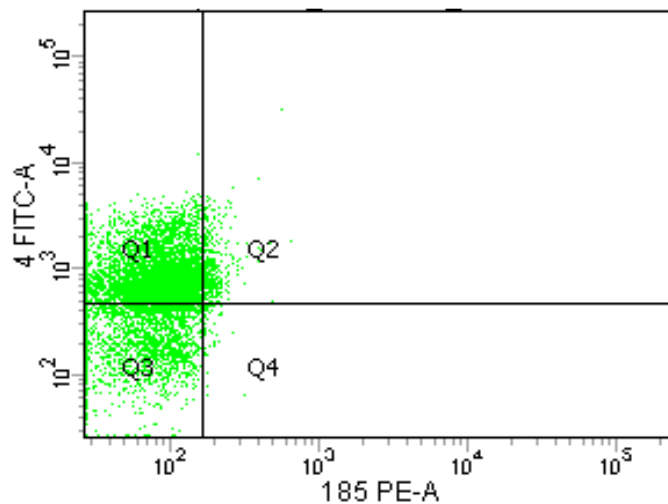


Fig. 2. Dot blot show co-expression of CD4+ (FITC) and CXCR5+ (185PE) on T follicular helper cells in diabetic patient with nephropathy

4. DISCUSSION

In the present study there was no significant difference in age between the three studied groups, and this was in agreement with Hassan GH. et al. [7] which found that there was no significant difference in age between T2DM without nephropathy and control group. On the other hand, Chang-Chiang et al. [8] reported significant increase in age in T2DM with nephropathy in comparison with control group.

Considering sex, there was no statistically significant difference between the three studied

groups, and this was in agreement with Garcia et al. [9], who proved that diabetes prevalence is similar in male and female.

As regard hemoglobin, it was significantly lower in diabetic patients with nephropathy group (II) than in diabetic patients without nephropathy group (I) and control group (III). This was in agreement with Lakshmi P., [10] who proved that there is significant association between patients with anemia and diabetic nephropathy as the number of patients having diabetic nephropathy in his study was greater in the anemic group than in the non-anemic group.

As regard lymphocytes, there was significant increase in diabetic nephropathy patients' group (II) than in diabetic patients without nephropathy group (I) and control group (III). This was in disagreement with Chung F. et al. [11], who explained that the peripheral lymphocyte count was inversely related to the severity of diabetic nephropathy in their study.

In this study there were statistically significant differences in fasting blood glucose, post-prandial blood glucose and HBA1c levels among the three studied groups. The three parameters were elevated in diabetic and diabetic nephropathy patients than in control group. This is in accordance with Qinghua Z. et al. [12] who proved that blood glucose levels and HBA1c were elevated in diabetes and diabetic nephropathy patients.

As regard serum triglycerides, in our study there was significant difference between the diseased groups and the control group. Triglycerides was higher in diabetic nephropathy than in diabetic patient without nephropathy. Also, it was higher in diabetic patient without nephropathy than in healthy controls. This is in agreement with Chang-Chiang et al. [8] who explained the increase in serum triglycerides in diabetic nephropathy group by dysregulated lipid metabolism results in diabetic nephropathy development in patient with type 2 diabetes mellitus. Additionally, Zhang et al. [13] were in agreement with our study and reported that serum triglycerides were significantly higher in patients with T2DM without nephropathy than in the control group.

As regard serum total cholesterol, the results of the present study revealed there was no significant difference between the diseased groups and control group. This is in agreement with Chang-Chiang et al. [8] reported that there was no statistically significant difference in serum total cholesterol between T2DM with nephropathy and control group. They suggested that, this result may be related duration of T2DM and medications received by diabetic patients.

On the other hand, Hassan Ghasemi et al. [7] who suggested that there was significant difference in cholesterol serum level between diseased groups and control group, these results were based on the association of T2DM with an atherogenic lipid profile and the risk of cardiovascular disorders.

Cyster et al. [14] described a mechanism for cholesterol in mediating the correct migration of Tfh cells in the lymph node between the T cell zone and B cell follicle leading to upregulation of T follicular helper cells. This area needs more investigations in order to know the exact relation between cholesterol, DN and Tfh cells level.

As regard serum creatinine and blood urea, there was significant increase in diabetic nephropathy patients as compared with control subjects and T2DM patients without nephropathy, this was in agreement with Keith C. et al. [15] whose study revealed that increasing albuminuria carry higher risks of declining kidney function and associated outcomes. There was no significant difference between healthy subjects and T2DM patients without nephropathy in our study. This is in disagreement with Bamanikar et al. [16] who reported statistically significant increase in creatinine level in T2DM without nephropathy group compared to control group.

As regard urinary proteins, there was significant increase in diabetic nephropathy patients as compared with control group and T2DM without nephropathy group. This is in agreement with Mysore K., et al. [17] who reported that the gold standard for detection and prediction of diabetic nephropathy risk is albuminuria.

As regard hematuria, there was significant increase in diabetic nephropathy patients as compared with control group and T2DM without nephropathy group. This is in agreement with Hugo Y, et al. [18] whose study reported that 15% of patients with DN exhibited moderate hematuria and 47% showed mild and moderate hematuria.

As regard estimated GFR, in our study there were significant decline in diabetic and diabetic nephropathy patient groups than in healthy control group and this was in agreement with Megumi O. et al. [19] whose study revealed that eGFR declines were strongly associated with subsequent risk of ESRD in Japanese type 2 diabetic patients, in addition to 30% and 40% declines in eGFR, a 20% decline in eGFR over 2 years could be considered as an endpoint of ESRD in DKD.

As regard CD4+CXCR5+, there was significant increase in diabetic patients as compared with control group and this is in agreement with Weiwei Sh, et al. [20] who showed that proportion of circulating Tfh in the peripheral

CD4+T cells was significantly increased in T2DM patients than in controls. CD4+CXCR5+Tfh cells also show significant increase in diabetic nephropathy patients' group when compared with control group and diabetic patients without nephropathy and this is in agreement with Zhang N. et al. [13] who demonstrated increased circulating CD4+CXCR5+PD-1+ Tfh cell counts in DN patients which were reduced post-treatment.

In diabetic nephropathy patients there was positive correlation between CD4+CXCR5+ Tfh cells and lymphocytes, FBG, PPBG, HBA1c, urea, creatinine, urinary proteins and negative correlation with eGFR and this was in agreement with Zhang N. et al. [13] who reported the positive correlation between Tfh cells and blood glucose levels, urinary proteins and negative correlation between Tfh cells and eGFR.

Zihan Z. and Feng Z. [21] explained that CD4+ T helper cell tolerance of glucose has been represented to be an important factor in the pathogenesis of diabetes and so it is also highly relevant to DN. Also explained that inflammatory cytokines produced by T cells play an important role in pathogenesis of DN. They also suggested the role of common forms of oxidative stress in activation of T helper cells in pathogenesis of DN.

Moon J. et al. [22] showed a marked increase in CD4+ T cells in the renal interstitium of type 2 diabetic patients the aberrant infiltration and the activation of T cells in interstitium of the kidney, which may be the underlying immune-pathological mechanisms of diabetic kidney injury.

5. CONCLUSION

In conclusion this study revealed the increased expression of T follicular helper cells in diabetic nephropathy patients. Also revealed the significant positive correlation between CD4+CXCR5+ Tfh cells and kidney function tests (urea and creatinine), urinary proteins and negative correlation between CD4+ CXCR5+ Tfh cells and eGFR. Therefore, our finding suggests the probable role of Tfh cells in the pathogenesis of diabetic nephropathy. Future studies with more investigations and large sample size of DN patients will allow to detect the real roles of Tfh cells in the pathogenesis of DN.

6. RECOMMENDATION

The need for further studies on a larger number of patients for more comprehensive statistical analysis and future follow up studies may be needed to clarify the role of using CD4 and CXCR5 in the pathogenesis of DN.

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the authors.

CONSENT

As per international standard or university standard, Participants' written consent has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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