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Study of Regulatory T-cells in Type 1 Diabetes

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ABSTRACT

Background: T1D is a common autoimmune disease, globally, diabetes affects over four hundred million individuals, with Type 1 (insulin-dependent) diabetes (T1D) accounting for up to 10 percent of cases.
Aim of the work: to shed light on and evaluate the frequency of Treg cells in patients with type 1 diabetes.
Patients and methods: This study is a cross-sectional study that was conducted in the period 9/2019 to 3/2020 at diabetes and Internal medicine clinics of Al-Azhar and Ain Shams University Hospitals. 60 adult males and females aged between 19 - 40 years old who were diagnosed with type 1 DM (Group I) were offered participation in the study, later we subdivided them regarding the percentage of HBA1C to two groups:- (Ia) controlled DM, (Ib) uncontrolled DM.
Results: There is a considerable decrease of Tregs in patients with T1D in comparison with those in healthy subjects. There was a highly significant difference between patients with type 1 DM and healthy subjects regarding Tregs percentage, being higher in healthy subjects. There was a highly significant difference between patients with T1D and healthy subjects regarding FOXP3 percentage, being higher in healthy subjects while its level in diabetic subjects.
Conclusion: There is a considerable decrease of Tregs in patients with T1D in comparison with those in healthy subjects, there is no correlation between Treg percentage and the patients' age, there is no relation between the frequency of Tregs and the control of blood sugar in type 1 diabetic patients.

Keywords: *Type 1 Diabetes; FOXP3; Regulatory T-cells.*

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INTRODUCTION

Type 1 diabetes (T1D) is one of the most common autoimmune diseases. Diabetes affects over four hundred million people worldwide,[1] with Type 1 (insulin-dependent) diabetic patients accounting for up to 10 percent of cases.[2] within the USA, type 1 diabetes occurs with an average rate of 15-30 new patients in 100,000 kids with an age up to 14 years annually,[3] consecutively, the estimated number of type 1 diabetes cases among Africans, Asia and South America is fewer than 15 cases per 100,000 children each year.[3]

The complex aetiology of type 1 diabetes is still not clearly defined; however, it sounds like the autoimmunity that leads to the destruction of beta cells that produce the insulin, this process occurs over the course of several years leading to clinical diabetes,[7] with a detectable decline in serum C-peptide level (a marker of pancreatic insulin production).[5,6]

Autoantibodies to islet cells are found in Type 1 DM patients and are widely understood to be a sign of beta cell destruction within the onset of the disease.[6,8]

Despite there is no specific pattern for these autoantibodies when they appears, the occurrence of more than one autoantibodies has a very high possibility of development of type 1 diabetes later on. Studies that investigate the response of islet cells autoantibody in persons who have genetic susceptibility will be beneficial in identifying the beginning of autoimmunity against islet cells by investigating the conversion from a seronegative to a seropositive and the development of the response of these autoantibody which could be a sign of the time of type 1 DM start.[23]

The introduction of these autoantigen to cytotoxic T cells (CD8 positive) is carried out by the main histocompatibility complexes class I and II (MHC I and II), which kills the antigen-presenting beta cells.[9, 10]

Different studies have identified autoreactive T-cells that can detect islet cell autoantigens which may have

a main effect in the immunopathogenesis of Type 1 DM.[11]

A variety of dysregulated events intrinsic and extrinsic to T lymphocytes leads to the breakdown of beta cell-specific self-tolerance. Several regulatory mechanisms maintain he peripheral tolerance to self antigens, including Tregs.[12]

Different studies had recently reported findings regarding the association between the frequency and the performance of regulatory T cells in Type 1 DM, but unfortunately their results represents a somewhat conflicting body of findings.[10]

Tregs are cells that are involved in self-tolerance, and any dysfunction may lead to autoimmunity. Tregs are CD4-positive CD25-positive T lymphocytes that are formed in the thymus during childhood and have the potential to bind autoantigens via their T-cell receptor. Adaptive Treg cells, on the other hand, are produced in the peripheral blood by the conversion of CD4-positive CD25-positive naive T lymphocytes within a particular microenvironment.[13,14]

Sakaguchi was the first one to recognize that Tregs are important in occurrence of autoimmune diseases. When Sakaguchi and his colleagues transferred CD4-positive T lymphocyte after they have blocked the Interleukin-2 receptor alpha chain (also called CD25) using monoclonal antibodies that are specific to CD25 receptor, into BALB/c athymic nude mice that lead to occurrence of multiple autoimmune disorders spontaneously (like autoimmune thyroid diseases, autoimmune diabetes, autoimmune adrenal insufficiency, autoimmune ovarian dysfunction, and many other autoimmune diseases). They were able to prevent the occurrence of these autoimmune disorders that are developed in those mice when they transferred CD4-positive CD25-positive T lymphocytes into them.[22]

The introduction of forkhead winged-helix transcription factor Foxp3 (forkhead box p3) as a major regulator for Treg cells added a pivotal marker for such T lymphocyte subset. Foxp3 is primarily expressed in mice and humans at high levels in both natural and adaptive CD4+CD25 high Tregs.[15,16]

If there is any mutation in the genes specific for Foxp3 that are present on the chromosome X that will result in multiple autoimmune disorders like Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome, this syndrome will lead to death during childhood.[16]

PATIENTS AND METHODS

This study is a cross-sectional study that was conducted between September 2019 to March 2020, The work included 2 Groups: Control Group (I) (30 patients), Study Group (II) (60 patients) which subdivided according to HBA1C percentage into two subgroups:- (Ia) controlled DM, (Ib) uncontrolled DM. The patients and control were recruited from diabetes and Internal medicine clinics of Al-Azhar and Ain Shams University Hospitals.

After taking approval from the local ethics committee, all participants signed a written informed consents. The following procedures were done to all subjects in this study: detailed medical history and complete clinical examination. Laboratory investigations: Blood sampling was performed for measuring Fasting C-peptide, HbA1c percentage, Estimated GFR and Treg cells percentage using flowcytometry analysis.

To measure the percent of Tregs : We have withdrawn blood samples from patients and control. After that we have centrifuged blood samples to be able to isolate peripheral blood mononuclear cells (PBMC). Then we have resuspended cells that we have Isolated before in PBS to be able to count cells that are still viable. We have used these cells later for flowcytometry staining. After that we have incubated Antibody clones CD4-ECD for about twenty minutes in 200 µL of PBS with three percent fetal calf serum containing two million cells and then we have washed in PBS. Then we have resuspended the cells that we have washed in two ml of 1× Fix/Perm buffer, After that we have incubated the cells for about forty minutes in a dark room at a three degree Celsius temperature. Then we have washed the cells by three ml of 1× permeabilization buffer. After that we have incubated the cells again for about twenty minutes in a dark room at a three degree Celsius temperature with three percent rat blocking serum. At this step we have added FoxP3-PE antibody before we incubated the cells again for about forty minutes in a dark room at a three degree Celsius temperature. Then we have used the permeabilization buffer to wash the cells with. Then we have resuspended the cells in PBS with three percent fetal calf serum. After that we have to protect tubes from light to avoid cells damage and we have stored the cells at three degree Celsius temperature. For acquisitions we have prepared rainbow Calibration Particles, 8 peaks (3.0–3.4 mm) (Biolegend) by adding only one drop of beads into 300 mL of deionized water. after that we have performed the acquisitions on a Navios cytometer (Beckman Coulter), then we have used Kaluza 1.3 software (Beckman Coulter) to perform all data analyses and finally get the count of Tregs.

Statistical analysis of the data was done using SPSS software package (V. 20, Echosoft Corp., USA). For quantitative measurements, data were expressed as Mean SD, and for classified data, both number and percentage were used. Specific tests were performed as follows: Student T-test was used to compare two independent groups of parametric numbers. With non-parameter data, Sperman's measuring test examines the relationships that exist between each variant of each group. In terms of divided data, the Chi-square test was used to investigate the relationship between all two objects or to compare two independent groups.

RESULTS

Results analysis of the of studied groups as regards laboratory data showed a high considerable difference in control group and patient group as regards HBA1C, Fasting C-peptide, FOXP3 and Treg while there is no considerable difference in control group and patient group as regards Creat, eGFR and CD4.(Table 1)

		Control group Group II	Patient group Group I	Test value	P-value	Sig.
		No. = 30	No. = 60			
HBA1C	Mean±SD	4.98 ± 0.46	7.43 ± 0.92	-13.757	0.000	HS
	Range	4 – 5.6	5.6 – 9.1			
F C-peptide	Mean±SD	2.05 ± 0.76	0.26 ± 0.11	18.076	0.000	HS
	Range	0.99 – 3.62	0.01 – 0.44			
Creat	Mean ± SD	0.81 ± 0.22	0.86 ± 0.22	-1.036	0.303	NS
	Range	0.52 – 1.43	0.52 – 1.4			
eGFR	Mean ± SD	124.35 ± 31.38	121.14 ± 30.44	0.466	0.642	NS
	Range	64.39 – 186	59.12 – 189.96			
CD4	Mean ± SD	49.46 ± 13.18	45.28 ± 13.31	1.408	0.163	NS
	Range	25.4 – 81.2	24.4 – 88.9			
FOXP3	Median(IQR)	16.75(14.3 - 18.6)	6.05(3.9 - 9.45)	-6.908	0.000	HS
	Range	7.3 – 36.7	0.4 – 16.9			
Treg	Median(IQR)	14.1(11.1 - 17.4)	4.8(2.45 - 7.55)	-6.604	< 0.001	HS
	Range	4.7 – 36.5	0.3 – 16			

Table 1: Descriptive analysis for all studied groups as regards laboratory data.

On comparing controlled, uncontrolled DM and control group regarding different parameters, there was a highly statistically significant difference between the three groups regarding HBA1C, Fasting C-peptide, CD4, FOXP3 and Treg while there was no statistically significant difference between the three groups regarding Creat and eGFR. (Table 2)

		Control group Group II	Controlled DM Group Ia	Un controlled DM Group Ib	Test value	P-value	Sig.
		No. = 30	No. = 21	No. = 39			
HBA1C	Mean ± SD	4.98 ± 0.46	6.39 ± 0.33	7.99 ± 0.57	323.508	0.000	HS
	Range	4 – 5.6	5.6 – 6.9	7.1 – 9.1			
F C-peptide	Mean ± SD	2.05 ± 0.76	0.26 ± 0.10	0.25 ± 0.12	161.527	0.000	HS
	Range	0.99 – 3.62	0.02 – 0.41	0.01 – 0.44			
Creat	Mean ± SD	0.81 ± 0.22	0.81 ± 0.19	0.89 ± 0.24	1.414	0.249	NS
	Range	0.52 – 1.43	0.52 – 1.3	0.53 – 1.4			
eGFR	Mean ± SD	124.35 ± 31.38	125.76 ± 24.75	118.66 ± 33.13	0.471	0.626	NS
	Range	64.39 – 186	80.13 – 175.63	59.12 – 189.96			
CD4	Mean ± SD	49.46 ± 13.18	40.13 ± 9.59	48.05 ± 14.29	3.579	0.032	S
	Range	25.4 – 81.2	24.4 – 61.4	28.5 – 88.9			
FOXP3	Median(IQR)	16.75(14.3 - 18.6)	5.5(3.9 - 7.4)	7.2(3.9 - 9.9)	49.550	0.000	HS
	Range	7.3 – 36.7	0.4 – 12.3	0.5 – 16.9			
Treg	Median(IQR)	14.1(11.1 - 17.4)	3.7(2.3 - 5.1)	6.6(2.5 - 9.4)	46.044	0.000	HS
	Range	4.7 – 36.5	0.3 – 12.2	0.4 – 16			

Table 2: Comparison between controlled, uncontrolled DM and control group regarding different parameters.

Upon conducting correlation study, we found that Treg are positively correlated with FOXP3 in all studied groups (all patients, Controlled and Uncontrolled DM), CD4 is positively correlated with FOXP3 in all patients, while eGFR is negatively correlated with FOXP3 in all patients. (Table 3), Also CD4 is positively correlated with Treg in all patients and uncontrolled DM group, while eGFR is negatively correlated with Treg in all patients.(Table 4)

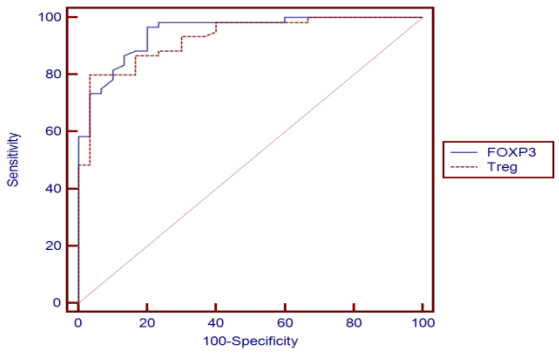
	FOXP3					
	All patients		Controlled DM		Uncontrolled DM	
	r	P-value	r	P-value	r	P-value
Treg	**0.944****	**0.000**	**0.871****	**0.000**	**0.957****	**0.000**
Age	-0.041	0.758	-0.284	0.212	0.052	0.752
Weight	-0.160	0.223	-0.051	0.827	-0.221	0.176
HBA1C	0.138	0.292	-0.175	0.447	-0.091	0.582
F C-peptide	-0.213	0.102	-0.269	0.238	-0.233	0.154
Creat	0.192	0.141	0.073	0.753	0.175	0.286
eGFR	**-0.311***	**0.016**	-0.042	0.856	-0.299	0.064
CD4	**0.256***	**0.048**	0.094	0.684	0.267	0.100

Table 3: Correlation between FOXP3 and numerical variables in different groups.

	Treg					
	All patients		Controlled DM		Uncontrolled DM	
	r	P-value	r	P-value	r	P-value
FOXP3	**0.944****	**0.000**	**0.871****	**0.000**	**0.957****	**0.000**
Age	-0.030	0.820	-0.396	0.076	0.100	0.543
Weight	-0.107	0.418	0.067	0.772	-0.169	0.303
HBA1C	0.178	0.174	-0.253	0.268	-0.002	0.989
F C-peptide	-0.217	0.096	-0.330	0.144	-0.220	0.179
Creat	0.207	0.112	0.142	0.540	0.196	0.231
eGFR	**-0.312***	**0.015**	-0.157	0.496	-0.305	0.059
CD4	**0.342****	**0.007**	0.188	0.416	**0.317***	**0.049**

Table 4: Correlation between Treg and numerical variables in different groups.

Our study showed ROC curve of FOXP3 had cut off 13.2% equal or below it diabetic patients have high probability of having type 1 DM with sensitivity 96.67 % and specificity 80%. ROC curve of Treg had cut off 8.3% equal or below it diabetic patients have high probability of having type 1 DM with sensitivity 80% and specificity 96.67%. (Figure 1)



Parameter	AUC	Cut of Point	Sensitivity	Specificity	PPV	NPV
FOXP3	0.948	≤13.2	96.67	80.00	90.6	92.3
Treg	0.929	≤8.3	80.00	96.67	98.0	70.7

Fig. 1: Roc curve of FOXP3 and Treg

DISCUSSION

T1D is a common autoimmune disease, globally, diabetes affects over four hundred million people,[1] with Type 1 (insulin-dependent) diabetes (T1D) accounting for up to 10 percent of cases.[2] within the USA, type 1 diabetes occurs with an average rate of 15-30 patients in 100,000 kids with an age up to 14 years annually,[3] consecutively, the estimated number of type 1 diabetes cases among Africans, Asia and South America is fewer than 15 cases per 100,000 children each year.[3]

The complex aetiology of type 1 diabetes is still not clearly defined; however, it sounds like the autoimmunity that leads to the destruction of beta cells that produce insulin over the course of several years leading to clinical diabetes,[7] with a detectable decline in serum C-peptide level (a marker of pancreatic insulin production).[5,6]

The damage of the beta cells in the pancreas which caused by cytotoxic lymphocytes results in insulin deficiency and hyperglycemia. Environmental factors trigger Type 1 DM in genetically susceptible individuals.[10,11]

T lymphocytes are involved in the breakdown of beta cell-specific self-tolerance via a variety of dysregulated events both intrinsic and extrinsic to T cells. Various regulatory mechanisms, including T regulatory cells, retain peripheral tolerance to self antigens.[12]

Tregs are part of self-tolerance and impairment of their function could lead to autoimmunity. They are CD4 positive CD25 positive T cells that are formed in the thymus during childhood and have the ability to bind autoantigens via their T-cell receptor. Adaptive Treg cells, on the other hand, are produced in the peripheral blood by the conversion of CD4+CD25 naive T cells in the presence of a specific microenvironment [13,14].

Many studies regarding the tolerogenic role of some immune cells in the pathogenesis of T1D have been conducted in recent years. Our study aimed to evaluate the potential role of Treg cells from the point of their percentage in a group of patients who have type 1 DM.

Our study showed there is a considerable decrease of Tregs in patients with T1D in comparison with those in healthy subjects

These results were consistent with the results of Zuzana et al. which showed in 2016 that there was a significant decrease in the frequency of CD25 positive FoxP3 positive in patients with long duration of type 1 DM in comparison to healthy subjects, also patients with long duration of type 1 DM have a great reduction in the presence of CD25 in CD25 positive FoxP3 positive Tregs and FoxP3 positive Helios positive Tregs regardless their age or the duration of type 1 DM. Type 1 diabetes relatives a similar decrease in the expression of CD25 especially those who are autoantibodies positive.[17] Also Tahereh et al. found in 2017 that there was a great reduction in the frequency of Tregs in subjects who have T1D in comparison to the healthy group.[18] Moreover, these results were in agreement with Szypowska et al. who found in 2012 that there was a lower frequency of regulatory T cells in kids with type 1 DM with an age less than 5 than in those with an age that is equal or more than 5. Moreover, kids with type 1 DM with an age less than 5 have a lower percentage of Tregs in comparison to the healthy group.[19]

On the other hand, Todd - Brusko et al., mentioned that there was no significant difference in the percentage of regulatory T cells in type 1 DM patients and the control group. [20] Also Shelley - Lindley et al. discovered that although the number of Tregs is normal in patients with a recent-onset T1D, there is a marked decrease in their ability to stop the proliferation of T cells in comparison to the healthy group. [21]

In our study we divided the patients group according to HbA1C in to 2 subgroups then we have found that there is no relation between the frequency of Tregs and blood sugar control in type 1 diabetic patients.

The decrease of Tregs in patients with type 1 diabetes may give us a idea regarding the occurrence of the disease. So, if we stimulated regulatory T cells pharmacologically this may lead to tolerance of the immune system and prevent the occurrence of Type 1 DM in subjects who have genetic susceptibly.

CONCLUSION

There is a considerable decrease of Tregs in patients with T1D in comparison with those in healthy subjects. There is no correlation between Treg percentage and the patients' age. There is no relation between the frequency of Tregs and the control of blood sugar in type 1 diabetic patients.

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