Frequency of Circulatory Regulatory Immune Cells in Iranian Patients with Type 1 Diabetes

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ABSTRACT

Type 1 diabetes (T1D) is the result of the autoimmune destruction of insulin-producing beta cells. Regulatory T cells (Tregs) and plasmacytoid dendritic cells (PDCs) act as mediators of peripheral tolerance. We investigated the possible alterations of such cells in peripheral blood of patients with T1D compared to normal individuals. This comparison may lead to a better understanding of the immunopathogenesis processes involved in T1D.

92 participants, including 49 patients with T1D and 43 healthy controls were studied. 3 mL of blood was taken from all participants. After isolating peripheral blood mononuclear cells (PBMCs), PDCs as well as 2 subtypes of Tregs, $CD4^+CD25^+FoxP3^+$ and $CD8^+CD28^-$ cells were counted by 3-colorflow cytometry. The association between such enumeration and T1D was studied by multivariate regression and discriminate function models.

The frequency of $CD4^+CD25^+FoxP3^+$Tregs (p=0.038) and PDCs (p=0.039) in the peripheral blood of diabetic patients was less than that in healthy subjects. Having compared some models consisting different cells as well as their combinations, we did not find any profound explanation of each subset or their combinations to identify T1D.

The decrease of $CD4^+CD25^+FoxP3^+$cells and PDCs in diabetic patients may suggest their role in the onset or development of the disease. Therefore, it is likely that their pharmacologic stimulation may direct immune responses towards tolerance and prevent the development or even the onset of diabetes in susceptible individuals.

Keywords: $CD4^+CD25^+FoxP3^+$regulatory T cells; $CD8^+CD28^-$regulatory T cells; Diabetes mellitus; Plasmacytoid dendritic cells

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INTRODUCTION

New researches have explained the possible effect of plasmacytoid dendritic cells (PDC), as a sub group of the dendritic cells, in different autoimmune diseases such as rheumatoid arthritis,[1] systemic lupus erythematosus,[2] Sjogren's disease,[3] and non-obsess diabetic (NOD) mice.[4] This effect has also been shown in type 1 diabetes (T1D) patients from both aspects of number[5] and function.[6] It is likely that such cells can modulate the inflammatory responses through the production of interferon type 1 which has anti-proliferative effects.[7] However, they stimulate tolerance in experimental models of asthma[8] and prevent the body's sensitivity to harmless allergens.[9] Also, PDC off-loading increases autoimmune properties of T and B cells to destruct collagen type 2 and cause rheumatoid arthritis.[10] These findings support the tolerogenic properties of such cells, actually through inhibition of $CD8^+$ T effecter responses,[11] activation of regulatory T cells (Tregs), and production of cytokines as well as 2, 3-indoleaminedioxygenase (IDO).[12] One study reported fewer number of blood PDCs in T1D patients compared to those in the control group. Such difference was not found in the number of myeloid DCs (mDC1 and mDC2).[13] Moreover, studies on animal models have shown that the loss of PDCs is linked to acceleration of pancreatic inflammation, the first step in T1D.[14]

Tregs are essential environmental tolerance inducers which are able to suppress cytokine production and proliferative properties of auto reactive immune cells[15] in different auto immunities. For example, lower Tregs numerically in multiple sclerosis (MS),[16] systemic lupus erythematosus (SLE),[17] Kawasaki,[18] inflammatory bowel disease[19] and rheumatoid arthritis;[20] lower Tregs functionally in T1D[21-23] and MS,[24] and lower Tregs both numerically and functionally in autoimmune hepatitis[25] have been shown. More prevalence of diabetes and other autoimmune diseases have also been reported in mice lacking $CD4^+CD25^+FoxP3^+$Tregs.[26] In diabetes, $CD4^+CD25^+FoxP3^+$Tregs prevent the initiation and progression of the disease through the induction of peripheral tolerance in such a way that their reduced number may anticipate early diabetes.[27-29] In few studies, which reveal the normal percentage of Tregs in T1D,[21-23] long term[21-23] and recent onset[22] diabetes showed no impact on such percent. However, that would be interesting to consider that 2 above studies[21,22] reported a reduced function of the Tregs in vitro. In NOD mice,

$CD8^+$Tregs inhibit the development of T1D and also reverse the established disease.[30]

Since current knowledge is confronting challenges regarding the frequency status of different subsets of such tolerogenic cells in T1D, we aimed to evaluate simultaneously the numerical status of both cell types in such patients.

MATERIALS AND METHODS

Study Subjects

A total of 49 people suffering from T1D for at least 5 years with no yet-known diabetic complications from Kashan Diabetes Center in Iran and 43 sex, age and body mass index-(BMI)-matched healthy controls were enrolled in our case-control study. Control subjects with no family history of diabetes were recruited from local Blood Donation Organization. The diagnosis of T1D was based on the American Diabetes Association's criteria. Exclusion criteria were liver, kidney, rheumatoid, endocrine, cardiovascular, and metabolic diseases; familial cardiovascular diseases; cancer; and a history of using antihypertensive or lipid-lowering medications as well as smoking.

The protocol was approved by the local Committee of Ethics (No. 93128) and was in accordance with the Helsinki Declaration. Written informed consent was obtained from all participants older than 16 years and in the case of younger ones from their parents/guardians.

Sampling Protocol and Measurements

Blood samples were drawn at 08:00-10:00 in the morning after 12 hours overnight fasting. An immunoturbidometric method of Bayer Diagnostics Europe Ltd (Ireland) protocol measured HbA1c levels. Height and weight were measured using a wall-mounted stadiometer to the nearest 0.1 cm and an electric digital scale to the nearest 0.1 kg, respectively.

Flow Cytometric Analysis
Treg Analysis

Fresh peripheral blood mononuclear cells (PBMCs) were separated from 3 mL of anti-coagulated blood by Ficoll-Hypaque (Lymphodex, InnoTrain, Germany) density gradient centrifugation. Natural $CD4^+CD25^+FoxP3^+$Tregs were detected in one tube by staining with a cocktail of anti-human surface CD4-FITC/CD25-PE, and intracellular FoxP3-PE-Cy5

according to the manufacturer's instructions (eBioscience, USA). Inducible CD8$^+$CD28$^-$Tregs were detected in another tube by staining with antihuman CD3-PE, CD8a-PE-Cy5, and CD28-FITC. All antibodies and their isotype-matched controls were purchased from eBioscience, USA. The percentages of CD4$^+$CD25$^+$FoxP3$^+$ and CD8$^+$CD28$^-$ cells were analyzed by a 3-color flow-cytometry using BD FACScalibur flow-cytometry (BD, USA). Lymphocytes were gated on the basis of light scattering properties and CD3$^+$ characteristics and at least 20,000 events were obtained for each patient sample.

PDC Analysis

Fresh whole blood DCs were stained by a human specific PBMC PDC enumeration kit according to the manufacturer instructions (Imgenex, San Diego, CA). Human lineage (CD3, CD14, CD16, CD19, CD56)-FITC negative and HLA-DR-PerCP-Cy5.5 positive cells expressing human CD123-Alexa Fluor 647 were counted as PDCs using a Mouse IgG1 isotype control Alexa Fluor 647. Finally, cells were analyzed by flow-cytometry using BD FACScalibur flow-cytometry (BD, USA) and at least 100,000 events were obtained for each patient sample.

Statistical Analysis

The normality of the data was checked by Kolmogorov-Smirnov test. The results were expressed as mean ± SD. The groups were compared by independent t-test and Mann-Whitney U test based on their normality. Pearson's coefficient was determined for correlating between the 3 subset of regulatory cells

according to the study groups. Constructing discriminant function model for quantitative variables with normal distribution, we explained the expression status of different considered cells through canonical correlation and wilk's lambda assessment as criteria of goodness-of-fit model. Also, we used multiple logistic regression models to evaluate the effect of PDCs, CD4$^+$CD25$^+$FoxP3$^+$ and CD8$^+$CD28$^-$ cells on T1D. Finally, we compared the results of both models with corrected classification. All analyses were performed by SPSS (version 16; Statistical Package for the Social Sciences, SPSS Inc., Chicago, Illinois, USA).

RESULTS

Demographic, laboratory and clinical characteristics of the study groups as well as their CD4$^+$CD25$^+$FOXP3$^+$Treg, CD8$^+$CD28-Treg and PDC frequencies are shown in Table 1. There were no significant changes in the frequency of CD8$^+$CD28$^-$ Tregs between 2 groups ($p=0.12$). However, the frequency of CD4$^+$CD25$^+$FOXP3$^+$Tregs ($p=0.038$) as well as PDCs ($p=0.039$) were reduced significantly in T1D patients.

Looking for a possible relationship between the frequency of each cell with that of other cells, we found a significant association between the frequency of CD4$^+$CD25$^+$FOXP3$^+$ and CD8$^+$CD28$^-$Tregs ($p=0.024$) (Table 2, Figure 1).

Evaluating the effect of both subsets of Treg as well as PDC frequencies to explain the existence of T1D, we constructed both models of multiple regression (Table 3) and discriminant function (Table 4).

Table 1. Demographic and laboratory characteristics of the type 1 diabetes patients and control group

Variants	Healthy controls (n = 43)	T1D (n = 49)	p value
Age (years)	10.95±3.83	12.20±3.86	0.12
Gender (male/female)	21/22	22/27	0.7
Duration of diabetes (months)	-	73.22±5.9	-
BMI (kg/m2)	21.02±4.25	21.23±4.6	0.826
HbA1c (%)	4.73±0.73	7.34±1.24	<0.001
eGFR (mL/min/1.73m2)	87.1±34.4	90.6±27.8	0.64
CD4$^+$CD25$^+$FOXP3$^+$Tregs	2.18±0.73	1.87±0.68	0.038
CD8$^+$CD28$^-$Tregs	0.87±0.4	0.75±0.28	0.12
PDCs	0.38±0.27	0.26±0.28	0.039

T1D: Type 1 diabetes; BMI: Body mass index; PDCs: Plasmacytoid dendritic cells

Table 2. Linear correlation coefficient between cell types of CD4$^+$CD25$^+$FOXP3$^+$Tregs, CD8$^+$CD28$^-$Tregs, and PDCs in both groups of type 1 diabetic patients and healthy controls

	Cell type	CD4$^+$CD25$^+$FOXP3$^+$Tregs	CD8$^+$CD28$^-$Tregs	PDCs
Diabetics	CD4$^+$CD25$^+$FOXP3$^+$Tregs	1	0.322, P=0.024	-0.11 (P=0.45)
	CD8$^+$CD28$^-$Tregs	0.322 (P=0.024)	1	-0.085 (P=0.58)
	PDCs	-0.11 (P=0.45)	-0.085 (P=0.58)	1
Controls	CD4$^+$CD25$^+$FOXP3$^+$Tregs	1	0.067 (P=0.67)	0.218 (P=0.16)
	CD8$^+$CD28$^-$Tregs	0.067 (P=0.67)	1	-0.112 (P=0.47)
	PDCs	0.218 (P=0.16)	-0.112 (P=0.47)	1

PDCs: Plasmacytoid dendritic cells
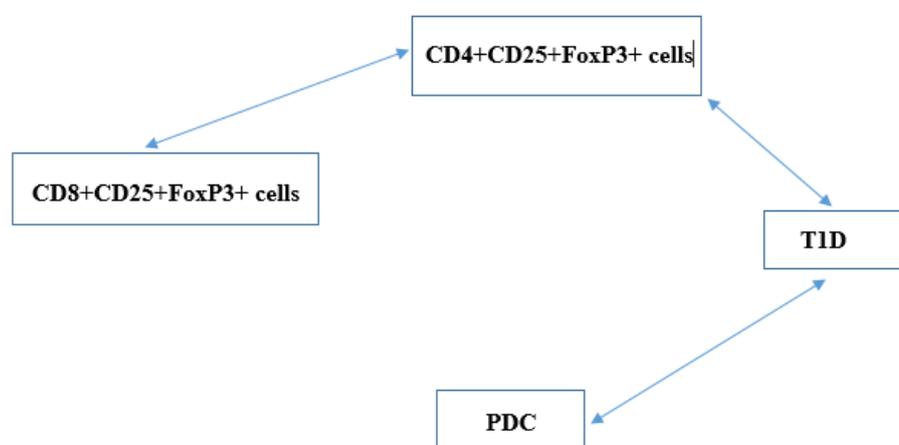
Figure 1. Proposed diagram based on the results obtained by analysis of the association between CD4$^+$CD25$^+$FOXP3$^+$Tregs, CD8$^+$CD28$^-$Tregs, plasmacytoid dendritic cells (PDC) and type 1 diabetes

As shown in Table 3, only the number of PDCs could demonstrate the phenomenon of T1D (p=0.05). Notably, among 3 kinds of such cells, the odds ratio was less for PDC numbers than that of other cells (OR=0.202). It means that each unit increase in the number of PDCs decreases the probability of T1D as 20%. Logistic regression model could only explain 13.8% of the changes in T1D (R square=0.138). The corrected classification in this model was only 59.8%. It means that the power of diagnosis of T1D using such measurements is 59.8%.

Table 4 shows 4 models of discriminant function describing the effect of both cell type frequency and all together on T1D. According to model 1, the frequency of CD4$^+$CD25$^+$FOXP3$^+$Tregs explained only the 4.7% of variance changes with a corrected classification of 62%. Such value got decreased to 59.8% in models 2 and 3 showing the effect of CD8$^+$CD28$^-$Tregs and PDCs, respectively. The corrected classification in model 4 which simultaneously contains 3 variables reached again to 62%. This means that CD8$^+$CD28$^-$Tregsand PDCs could not increase the explanation power of CD4$^+$CD25$^+$FOXP3$^+$Tregs in T1D.

Considering both models simultaneously, we observed that logistic regression and discriminant function model in the presence of different cells could explain T1D at 59.8% and 62% cases, respectively. False positive cases in both models were equal to 53.5% and false negative cases were equal to 28.6% and 30.6% in logistic regression and discriminant function models, respectively.

Table 3. Parameters of logistic regression model based on the frequencies of CD4⁺CD25⁺FOXP3⁺Tregs, CD8⁺CD28⁻Tregs, and PDCs in type 1 diabetes

Different cells	B	S.E.	Sig.	Exp(B)	95.0% CI for EXP(B)	
					Lower	Upper
CD4⁺CD25⁺FOXP3⁺Tregs	-0.544	0.329	0.098	0.58	0.304	1.106
CD8⁺CD28⁻Tregs	-0.894	0.661	0.176	0.409	0.112	1.495
PDCs	-1.602	0.818	0.05	0.202	0.041	1.001

PDC: Plasmacytoid dendritic cells

Table 4. Parameters of discriminant function model* based on the frequencies of CD4⁺CD25⁺FOXP3⁺Tregs, CD8⁺CD28⁻Tregs, and PDCs in type 1 diabetes

Models	variables	Parameters	Total variance explained	Corrected classification
1	CD4⁺CD25⁺FOXP3⁺Tregs constant	0.234 -0.205	0.047	62%
2	CD8⁺CD28⁻ constant	2.882 -2.331	0.028	59.8%
3	PDC constant	0.232 -0.203	0.046	59.8%
4	CD4⁺CD25⁺FOXP3⁺Tregs CD8⁺CD28⁻ PDC constant	0.761 1.289 2.299 -3.321	0.102	62%

PDC: Plasmacytoid dendritic cells

*Considering that there was no profound explanation of each lonely subset of regulatory cells evincing T1D, we used discriminant function models in which a combination of such cells is used to analyze their explanation power on T1D.

DISCUSSION

Many studies regarding the tolerogenic role of some immune cells in the pathogenesis of T1D have been conducted in recent years. The present study aimed to evaluate the potential role of 2 groups of immune cells containing PDCs and CD4⁺CD25⁺FoxP3⁺ as well as CD8⁺CD28⁻Tregs from the point of their frequency in the peripheral blood of a group of patients with T1D. We showed a significant reduction of PDCs as well as CD4⁺CD25⁺FoxP3⁺Tregs in T1D patients compared to those in healthy subjects. Of course, the direction of such probable cause-and-effect relationship between the number of those cells and occurrence of the diabetes is not clear yet. In other words, it is not known whether the reduction of the Tregs and PDCs is a result

of diabetes or a cause of the disease. Nonetheless, PDCs and Tregs interact to each other through their surface molecules and in this way DCs can be affected by Tregs and be identified as tools in the establishment of peripheral tolerance and adjustment of Tregs activity.[31] Although there are not enough human studies, but animal studies on NOD mice confirm that a shortage of PDCs is associated with progression of insulitis[32] which could be controlled by CD4⁺CD25⁺FoxP3⁺Tregs.[33,34] Furthermore, one recent study has reported a reduced number of both myeloid and plasmacytoid DCs of the first-degree relatives of T1D patients.[35] Inconsistently, some studies have reported the increased number of PDCs in NOD mice.[36] These conflicting results may be due to a different microenvironment present in mice compared to human beings especially in terms of the effective cytokines.

Any numerical or functional disorders of Tregs can be applicable in the pathogenesis of T1D.[37] Some researchers have reported the reduced number,[27] like ours, as well as performance[21,22] of these cells in the peripheral blood of patients with T1D. Nevertheless, there is no difference between the number of Tregs in diabetics compared to healthy subjects in some other studies.[21,23] Such conflicting results may be due to some differences between the patient groups such as their different stages of the disease. For example, Putnam et al. took a comparison between chronic T1D patients and healthy subjects and found no difference in the number of $CD4^+CD25^+$ cells.[23] According to numerous reports on newly diagnosed diabetics, it may be concluded that there is a relationship between the duration of the disease and the extent of circulatory Treg reduction in such a way that the newer the diabetes, the less number of Tregs. Such concept could affect the therapeutic strategies including the usage of Tregs. It seems that the number of Tregs is not associated to the age of the patients.[28] In our study, both patients and control subjects were selected with consideration of the same in terms of possible confounding factors including the age.

To clarify the problems regarding the frequencies of different subsets of tolerogenic cells in T1D, we designed some models containing different cells as well as their combinations in such disease. Having compared those models, we did not find any profound explanation of each subset or their combination evincing T1D.

Our study had some advantages. Firstly, we considered a relatively proper sample size and associated standard deviations that yield a proper power to detect differences in subgroup analyses. Secondly, there were no essential factors affecting the comparability of the groups including diabetic complications and large differences in age, duration of diabetes, and length of treatment. Such uniformity of the patients is less seen in other studies.

The main limitation of our study was the lack of longitudinal data that could be taken through serial samples to monitor the changes of Tregs as well as PDCs. This limitation allowed just a cross-sectional analysis of such cell profile of only limited robustness.

The decrease of $CD4^+CD25^+FoxP3^+$ cells and PDCs in diabetic patients suggests their role in the onset or development of the disease. Therefore, it is likely that pharmacologic stimulation of these cells might direct immune responses towards tolerance and prevents the development or even the onset of diabetes in susceptible individuals.

ACKNOWLEDGEMENTS

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