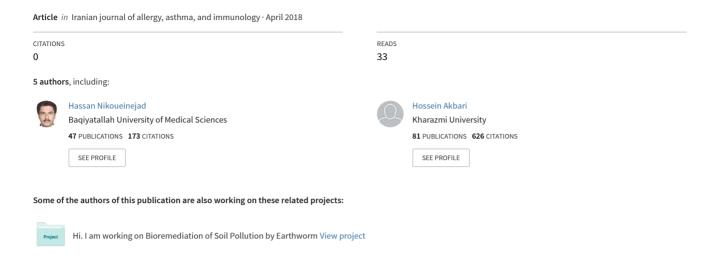# Association of Regulatory T Cells with Diabetes Type-1 and Its Renal and Vascular Complications Based on the Expression of Forkhead Box Protein P3 (FoxP3), Helios and Neurophilin-1

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Association of Regulatory T Cells with Diabetes Type-1 and Its Renal and Vascular Complications Based on the Expression of Forkhead Box Protein P3 (FoxP3), Helios and Neurophilin-1

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ABSTRACT

In recent years, it has been recognized that regulatory T cells (Tregs) play a critical role in maintaining immune tolerance. Moreover, the expression of two markers named Helios and neurophilin-1 (NRP-1) has been highlighted in such cells. Helios, an intracellular transcription marker, largely differentiates twomost operative sub group of Tregs, namely naturally occurring (nTreg) and induced (iTreg) Tregs, and NRP-1 is reckoned as a membranous activity marker of Tregs. We aimed to count peripheral mononuclear cells expressing such markers in a group of type 1 diabetes patients to elucidate the possible role of Tregs in the pathogenesis of such disease and its complications.

Blood samples from 61 adult patients with type 1 diabetes and 61 sex and age-matched healthy controls were tested to count two types of Tregs, namely naturally occurring and inducible types, according to the expression of cell surface markers of CD4/CD25/CD47–FITC/PE/APC and intracellular markers of FoxP3/Helios–PE-CY5/eFlour450 by flow cytometry, respectively.We also investigated the relation between expression of such markers with HbA1c, urine albumin/creatinine ratio (UACR), and common carotid intima thickness (CIMT).

The circulatory frequency of both Helios+ and Helios- T-cells were significantly decreased in patients compared to those in healthy controls ($p<0.001$). There was also a significant decrease in circulatory frequency of Helios+ NRP-1+ and Helios- NRP-1+ cells in the patients compared to controls ($p=0.029$).

According to expression of Helios and NRP-1 markers, the number and function of both Tregs were decreased in diabetic patients. Moreover, the neurophilin expression was inversely associated with complications of type 1 diabetes.

Keywords: Forkhead box protein P3 (FoxP3); Helios; Neuropilin-1; Regulatory T cells; Type 1 diabetes

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INTRODUCTION

Type 1 diabetes (T1D) is a T-cell–mediated disease caused, at least partly, by loss of immunological tolerance to self-antigens.[1] One mechanism involved in maintenance of tolerance to such disease includes the presence of a specialized subset of regulatory T-cells (Tregs) expressing the transcription factor of Forkhead Box Protein P3 (FoxP3) within the CD4$^+$CD25$^+$T-cell population.[2] Tregs are generally classified into two categories, naturally occurring Tregs (nTregs) and inducible/adaptive Tregs (iTregs).[3] nTregs primarily emerge from the thymus and have the CD4+CD25+FoxP3+Helios+ phenotype,[4-7] whereas iTregs are generated in the periphery from naive T cells after antigen exposure[8] and have the CD4+CD25+FoxP3+Helios-phenotype.[4,5,7,9] CD4+CD25+FoxP3+ Tregs which arise both in the thymus and in the periphery as a consequence of exposure to antigens[8] are immune response modulators of crucial importance in many physiological and pathological situations, which are characterized by their ability to inhibit antigen specific T lymphocyte activation.[10] Even though the molecular and cellular mechanisms underlying their suppressive activity are under intense investigation, they are not yet totally elucidated.

CD304, or Neuropilin-1 (NRP-1), a transmembrane C-type lectin on plasmacytoid dendritic cells,[11] found originally as a semaphoring-III receptor in neurological synapses.[12,13]Expressing on human immune cells, NRP-1 plays a crucial role in both regulation[14] and activation of immune responses through promotion of dendritic cell–CD4+ T-cell clustering via homotypic interaction.[13]Nowadays, NRP-1 is usually referred to as a marker of human Tregs.[15]

A few studies have been carried out on Treg's function in animals with type 1 diabetes.[6,16,17] In our research, considering Helios as a differentiating marker of nTreg/iTreg and NRP-1 as a specified marker of Tregs' (and not the auto-reactive T-cells) activity, we quantified the number of both Helios and NRP-1 expressing CD4+CD25+FoxP3+Tregs in peripheral blood of type 1 diabetic patients. We also investigated the relation between expression of such markers with HbA1c (as indicator of metabolic control of type of diabetes), urine albumin/creatinine

ration (as indicator of diabetic nephropathy), and common carotid intima thickness (as indicator of diabetic vasculopathy).

MATERIALS AND METHODS

Patients

This study was conducted on 122 cases, 61 of which were adult patients with type 1 diabetes and 61 sex and age-matched healthy controls. Type 1 diabetic patients diagnosed for more than a year who were non-smoker, prescribed with no antihypertensive and lipid-profile altering drugs, and no history of cardiac events were included. Existence of hepatic, renal, articular, metabolic, endocrine and cardiovascular diseases, cancers, infections, auto-immunity, pregnancyand a history of using antihypertensive or lipid-lowering medicationswere our exclusion criteria. The protocol was approved by the local Committee of Ethics (No. 93101) and was in accordance with the Helsinki Declaration. Written informed consent was obtained from all participants.

Sampling Protocol and Measurements

Blood and urine samples were drawn at 08:00-10:00 in the morning after 12 hours overnight fasting. CBC, HbA1c, creatinine, lipids (total cholesterol, LDL cholesterol, HDL cholesterol, VLDL cholesterol, and triglycerides) and urinary concentration of albumin and creatinine were evaluated using standard laboratory methods. An automatic cell counter Sysmex K4500 (Japan) counted blood cells and an immunoturbidometric method of Bayer Diagnostics Europe Ltd (Ireland) protocol measured HbA1c levels. Lipid profile was assessed using end-point enzymatic methods through Beckman Instruments, Fullerton, California's protocol. Urine albumin and urine/serum creatinine were measured by enzymatic immunoassay and Jaffe´ methods, respectively. In order to define albuminuria through albumin/creatinine ratio measurement, 3 urine samples were obtained.

Ultrasonography

Having left all participants in supine position for at least 10 min in a quiet room at 22ºC. An unaware reader of the subject's clinical details performed ultrasonography according to standardized scanning protocol for the right and left common

carotid arteries using a medison v20 equipped with a linear 11 MHZ transducer (Medison/Samsung Medicine System GmbH, South Korea). On each common carotid artery, a 2cm segment proximal to the bulb region on far wall of the carotid was scanned by at least 100 points. All images were taken at end-diastole and then stored digitally for subsequent off-line analysis. Computer software analyzed the IMT distance automatically via arithmetically calculating a mean of the thickenings of the 2 above-mentioned segments.

Mononuclear Cell Isolation

Peripheral blood mononuclear cells were isolated from 4mL of fresh blood sample with EDTA anticoagulant agent on ficoll-hypaque (Biosera, South Korea). Having added fixation/permeabilization solution to each tube, we stained each test tube for cell surface markers of CD4/CD25/CD304–FITC/PE/APC and intracellular markers of FoxP3/Helios–PE-CY5/eFlour450 anti-human monoclonal antibodies according to manufacturer's instructions (e Bioscience,-USA). The other tube of each participant was intended as isotype control. In summary, the following steps were taken:

For each sample and its control tube, 100 microliters equivalent to 1million cells were added to each flow cytometry tube. 20 µL of the staining cocktail containing 1 µcg CD4 and CD304 and 0.125 µcg CD25 were added to sample tubes. For each isotype control tube, 20 µL (microgram) of mouse PE IgG1 and 1 µg of mouse APC IgG1 were added. Cells were washed in staining buffer and were incubated in 1 mL fixation/permeabilization buffer at 4°C for 60 minutes. After being washed twice with 2mL of 1x dilution of permeabilization buffer, 10 µL of anti-human Helios/FoxP3 to the sample tube and 2.5 µL of rat IgG2a-PE-CY isotype control were added to control tubes. The tubes were incubated at 4°C for 30 minutes. Having washed the cells, we enumerated at least 50000 events using a 4-coloured (BD-USA) FACScalibur. For such purpose after gating of CD4+CD25+FoxP3+Treg lymphocytes, the cells expressing both markers Helios and CD304 were counted.

Statistical Analysis

The comparison of different markers in two groups of diabetic and non-diabetic participants was performed using Chi-square test. Regards to their distribution, we quantitated the variables using t-test or Mann-Whitney test. The relationship between the variables was determined by conducting Pearson test. Considering the different confounding factors, we analysed the data using logistic regression model. All analyses were made by the SPSS package (Version 16.0. Chicago, SPSS Inc., USA).

RESULTS

The participants' demographic and clinical data are shown in Table1.

The circulatory frequency of CD4+CD25+FoxP3+Helios+ ($p<0.001$), CD4+CD25+FoxP3+Helios- ($p<0.001$), and CD4+CD25+FoxP3+Helios+NRP-1+ Tregs were significantly lower than those in controls ($p=0.029$).

Eliminating the confounding effects of factors including age, BMI, haemoglobin, blood lipid status and creatinine by Pearson Correlation Coefficient, we revealed a non-significant negative correlation between CD4+CD25+FoxP3+Helios+ Tregs with UACR (r=-0.199) and HbA1C (r=-0.203). There was no linear correlation between CD4+CD25+FoxP3+Helios+ Tregs and CIMT (r=0.001). Moreover, there was similar correlation between CD4+CD25+FoxP3+Helios- Tregs and UACR (r=-0.151) but not with CIMT and HbA1C. Non-significant, univariate, inverse correlations were observed between CD4+CD25+FoxP3+Helios+NRP-1+ cells with CIMT (r=−0.149) and HbA1C (r=−0.151) (Table 2).

Using multivariate regression analysis to investigate the effect of different basic and clinical factors on diabetes complications, we showed significant effects of CD4+CD25+FoxP3+Helios+ Tregs on UACR (P=0.034) in the presence of diastolic blood pressure, HDL and HbA1C; CD4+CD25+FoxP3+Helios+NRP-1+ Tregs on CIMT ($p=0.05$); CD4+CD25+FoxP3+Helios+ Tregs ($p=0.012$) and CD4+CD25+FoxP3+Helios- Tregs ($p=0.033$) on HbA1c in the presence of TG ($p=0.011$) and Hct ($p=0.002$).(data not shown)

Table 1. Distribution of basic and clinical characteristic of type 1 diabetic patients and healthy individuals

Parameters	Healthy	Patients	*p* value
Sex (male/female)	30/31	28/33	0.717
Age (years)	13.21±40.8	12.93±4.09	0.707
Duration of diabetes (months)	-	78.1±59.6	-
BMI (kg/m2)	21.28±3.99	21.34±4.46	0.937
Systolic BP (mmHg)	114.44±8.91	112.62±9.95	0.29
Diastolic BP (mmHg)	73.05±9.66	71.85±8.53	0.47
Creatinine (mg/dl)	0.97±0.17	0.92±0.19	0.16
Total cholesterol (mg/dl)	162.26±24.03	166.74±24.23	0.42
Triglyceride (mg/dl)	76.28±24.6	75.34±27.54	0.84
HDL(mg/dl)	38.44±6.18	40±7.4	0.21
VLDL(mg/dl)	16.6±6.77	14.95±5.82	0.15
LDL(mg/dl)	105.6±18.26	111.48±17.84	0.076
HbA1c (%)	4.73±0.71	6.81±1.55	<0.001
RBC (Mil/µL)	4.99±425	5.13±1.28	0.43
Hemoglobin (g/dL)	14.07±2.12	13.81±1.25	0.41
Hematocrit (%)	43.19±3.68	41.66±3.27	0.017
CIMT[*]	0.388±0.034	0.447±0.066	<0.001
UACR[**]	3.93±2.87	10.58±10.23	<0.001
CD4+CD25+FoxP3+Helios+	1.33±0.91	0.68±0.63	<0.001
CD4+CD25+FoxP3+Helios-	1.2±0.79	0.73±0.6	<0.001
CD4+CD25+FoxP3+Helios+NRP-1+	0.087±0.11	0.053±0.042	0.029

CIMT: carotidintima media thickness; UACR: urinealbumin/creatinine ratio

Table 2.Pearson correlation coefficient between different Tregs and complications of type 1 diabetes

Diabetic complications Tregs	UACR	CIMT	HbA1C
CD4+CD25+FoxP3+Helios+	-0.199	0.001	-0.203
	(0.125)	(0.996)	(0.117)
CD4+CD25+FoxP3+Helios-	-0.151	-0.051	-0.042
	(0.246)	(0.701)	(0.747)
CD4+CD25+FoxP3+Helios+NRP-1+	-0.005	-0.149	-0.151
	(0.972)	(0.261)	(0.247)

UACR: urine albumin/creatinine ration; CIMT: carotid intima thickness; T1D: Type 1 diabetes

DISCUSSION

Multiple defects within the innate and adaptive immune systems are associated with the development of type 1 diabetes.[18] Collectively, these defects lead to an imbalance in immune regulation that facilitates the expansion of pathogenic autoreactive T- and B-cells, resulting in the eventual destruction of insulin-producing β-cells.[19,20] In recent years, appreciation has grown for the critical role Tregs on immune tolerance.[19] Increasing evidence in mouse models of type 1 diabetes (T1D), as well as other autoimmune disorders, suggests that there are defects in Treg-mediated suppression.[21] Immuno-therapies targeting T cells, and resetting the balance between T effectors and Tregs, have had some initial success in preserving beta cell function.[22] Two main subsets of CD4+Tregs, natural and inducible, which differ in terms of their development, specificity, mechanism of action and dependence on T-cell receptor and co-stimulatory signaling[8]are defined by expression of surface markers and production of specific cytokines that dictate their mode of action.[5]

Our study showed the decreased expression of the new markers of Helios (commonly reported as a marker of nTregs) and NRP-1 (as a marker of Treg activation) on circulatory Tregs of type 1 diabetic patients. Such decreases may affect type 1 diabetes immune pathogenesis. In line with our study, others have showed a decreased expression of Helios on Tregs of type 1 diabetic patients.[23]

Considering the nearly equal reduced number of both CD4+CD25+FOXP3+Helios+ and CD4+CD25+FoxP3+Helios- Tregs, we may conclude that there is no difference in expression level of Helios marker on different types of human Tregs in type 1 diabetes. In view of so-called marker as the identifier of naturally occurring versus inducible type of Tregs, decreased number of both types of Tregs may be a reason (or at least a predisposing factor) of type 1 diabetes. Also, considering NRP-1 as an activation marker of Tregs, we report a decrease of circulatory CD4+CD25+FOXP3+Helios+NRP-1+ frequencies among type 1 diabetics. Such concept may mean that the immunomodulatory effects of Tregs could prevent type 1 diabetes. In line with this idea, other studies have been stated that NRP-1+ cells show more roles in suppressing and controlling the immune system than

that of NRP-1- cells.[24]

Having omitted the possible confounding effects of age, BMI, haemoglobin, creatinine and serum lipid status on Treg frequencies, we showed that there was still a significant difference between the decreased numbers of Tregs with type 1 diabetes. It may be concluded more confidently now that the diabetes has an effect on the number and function of Treg cells.

Although non-significant, there was a relation between Treg frequencies and diabetic complications of poor metabolic control, nephropathy and vasculopathy. A reverse relation between number of CD4+CD25+FOXP3+Helios+NRP-1+cells with diabetic nephropathy and HbA1c levels, as an indicator of metabolic control of diabetes, was seen in our study. It may mean that there is less NRP-1-dependent activation onTregs in uncontrolled type 1 diabetes. Such matter also applies to vasculopathy complication of diabetes, as the number of NRP-1 expressing cells was inversely related to vasculopathy, although it was not significant.

In recent years, due to possibility of using Tregs in biologic treatment of common auto-immune diseases such as diabetes and multiple sclerosis and notable advantages of this method compared to conventional therapies, identifying the subgroups of Tregs and their specific cell markers has become an important matter of interest.[25] In line with this idea, our study proposes Treg therapy methods with such mentioned markers as a new promising therapeutic method.[3] There are also evidence for the imbalance of Th1/Treg cells (dramatically decrease of CD4+CD25+Tregs and improvement of Th1 cells) in T1D ,which may have significant impacts on the prevention of the disease and treatment of the patients.[26]

The main limitation of our study is 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.

According to expression of Helios and NRP-1 markers, the number and function of both naturally occurring and inducible Tregs were decreased in diabetic patients. Moreover, the NRP-1 expression was inversely associated with T1D complications. Therefore, pharmacologic stimulation of such markers may direct immune responses towards tolerance and prevent the development or even the onset of diabetes

in susceptible individuals.

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