

T Helper 22 Pathway Evaluation in Type 1 Diabetes and Its Complications

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ABSTRACT

A subset of CD4+ T cells named T helper (Th)22 cells play some pathogenic roles in some autoimmune disorders such as type 1 diabetes (T1D). We aimed to study the correlation between the circulatory number of these cells and serum levels of its related cytokines with T1D as well as diabetic complications including metabolic control, atherosclerosis, and nephropathy.

Fortynine patients with T1D and 43 healthy controls underwent the assessment of circulatory number of Th22 cells (by flow cytometry), serum level of Th22 related cytokines including Interleukin-22 (IL-22), Interleukin-10 (IL-10), Transforming growth factor- β (TGF- β), Tumor necrosis factor- α (TNF- α) (by ELISA) and carotid intima-media thickness (cIMT) measurement (by doppler ultrasonography). In addition, fasting blood and urine samples were taken to measure levels of hemoglobin A1C, lipid profile, cell blood count (CBC), serum and urine creatinine and urine protein in all participants.

Th22 frequency and serum levels of IL-22 and TNF- α in patients were significantly higher than those in controls ($p < 0.001$). Serum levels of IL-10 and TGF- β in healthy individuals were higher than those in patients ($p < 0.001$). None of the Th22 related markers had a significant correlation with diabetic complications. There was only a significant effect of IL-22 on HbA1C variations.

Th22 pathway has a significant correlation with T1D but not with its complications of cIMT and Urine Albumin/Creatinine Ratio (UACR). We report that Th22 pathway is not a good prognostic as well as diagnostic marker of early macrovascular complications in T1D.

Keywords: Diabetic nephropathy; Diabetes type 1; Intima media thickness; Interleukin-10; Interleukin-22; T helper 22; Transforming growth factor- β ; Tumor necrosis factor- α

INTRODUCTION

Type 1 diabetes (T1D) is perceived as an

autoimmune disease that causes the elimination of insulin-producing beta cells, in the pancreatic islets.¹ The exact cause of the autoimmune attack in this

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debilitating illness remains unknown but T-cells appear to play a pivotal role in the destruction process of the insulin-producing beta cells and the potential contributions of T helper (Th1/Th2) subsets in the disease process seem to be substantial.² These cells can participate in disease process through different ways. However, aberrations of their cytokines appear to play a pivotal role in the pathogenesis of T1D. Recently, there has been an increase in researches involving Th22 effector subpopulations of T lymphocytes.

Interleukin-22 (IL-22), as signature cytokine of Th22 cells, belongs to the Interleukin 10 (IL-10) cytokine family and has some roles in different autoimmune conditions.³ IL-10 by itself plays regulatory as well as tolerogenic roles in T1D. In this regard, early systemic treatment with exogenous murine IL-10 can inhibit T1D in non-obese diabetes (NOD) mice.⁴ Confirming such regulatory role, IL-10 and the other regulatory and tolerogenic cytokine, Transforming growth factor- β (TGF- β), have been reported to be associated with benign insulinitis; whereas the inflammatory cytokine of tumor necrosis factor- α (TNF- α) is associated with destructive form of insulinitis.⁵ The uncertain status of the production of both of IL-10 (as an anti-inflammatory cytokine) and of IL-22 (as an inflammatory cytokine) from Th22 cells is essential to be cleared in the inflammatory autoimmune state of diabetes. Also considering the both decreased production and function of regulatory T cells (as main producer of TGF- β) in autoimmune conditions⁶ such as T1D,⁷ the investigators have hypothesized that there is a decreased production of IL-10⁸ and TGF- β in accordance with Th22 pathway function in T1D.

Diabetic complications progress due to inflammation which is originated from multiple pathways and mechanisms.⁹ The relationship between metabolic control and development of chronic adverse complications of T1D has always been a big concern over the years; and nowadays the main indicator of long-term glycemic control in T1D and its complications is the glycosylated hemoglobin (HbA1c).¹⁰

Coronary atherosclerotic heart disease (CHD) is one of the major concerns in T1D. The systemic chronic inflammation triggered by immune system activity has been postulated to bridge the increased risk of cardiovascular disease and T1D. Th22 cells might play an independent role in CHD and represent a novel proxy for cardiovascular risks in diabetes.¹¹ Increased

common intima media thickness (cIMT), measured by ultrasound, is an early and strong predictor of atherosclerotic changes leading to future vascular events.¹² Considering diabetes as the main risk factor of atherosclerosis,¹³ a few investigators have indicated that children,¹⁴ adolescents and adults¹¹ with T1D have significantly higher cIMT than that the healthy people.

Diabetic nephropathy (DN) is the other diabetic complication which may cause end-stage renal disease.¹⁵ DN is characterized by basement membrane thickening, expansion of the mesangial cells, reduced filtration, albuminuria, and ultimately renal failure.¹⁶ Albuminuria is a well-established independent predictor of diabetic nephropathy as well as cardiovascular morbidity and mortality in both type 1 and type 2 diabetic patients.¹⁷

In present study, we aimed to identify the circulatory frequency of Th22 cells and the serum level of its related cytokines including IL-22, IL-10, TGF- β , and TNF- α in type 1 diabetic patients. We also evaluated the potential of such biomarkers as diagnostic and prognostic tools of T1D complications.

MATERIALS AND METHODS

Patients and Sampling Protocol

A total of 49 people suffering from T1D for at least 5 years with no yet-known diabetic complications and 43 sex, age and 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 (N. 92143) 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.

Blood and urine samples were drawn at 08:00-10:00 in the morning after 12 hours overnight fasting. CBC and serum levels of HbA1c, creatinine, and lipids profile 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. 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.

Ultrasonography

Having left all participants in supine position for at least 10 min in a quiet room at 22°C, a radiologist unaware from the subject's clinical details performed their ultrasonography according to standardized scanning protocol for the right and left common carotid arteries to determine the cIMT 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.

Flow Cytometric and ELISA Analysis

Fresh peripheral blood mononuclear cells (PBMCs) were separated from 2 ml of anti-coagulated blood by Ficoll-Hypaque (Lymphodex, innoTRAI, Germany) density gradient centrifugation. At least one million PBMCs were stimulated in a duplicated form in the presence of PMA (50 ng/mL, Sigma) and ionomycin (1 µg/mL, Sigma) in a RPMI 1640 media (Invitrogen) at a 37 °C incubator with 5% CO₂ for 2 hours. Then the cells were cultured for another 4 hours in the presence of Brefeldin (0.5 µg/mL, Sigma). Negative control was produced via culture only in the RPMI 1640 media. Stimulated cells firstly were stained by antiCD4-APC (Allophycocyanin) (eBioscience, USA) and after fixation/permeabilization (eBioscience, USA) were stained by antiIL-22-PE (eBioscience, USA) according to the manufacturer's instructions. The percentages of CD4⁺IL-22⁺ 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 at least 100,000 events were obtained for each patient sample. Serum levels of IL-

22, IL-10, TGF-β, and TNF-α cytokines were measured through sandwich Elisa method according to manufacturer's instructions (eBioscience, USA).

Statistical Analysis

The results were expressed as mean±SD. The normality of the quantitative variables was confirmed by Kolmogorov-Smirnov test. The groups were compared by chi square test for gender of participants and independent t-tests for quantitative variables. Pearson's coefficient was determined for correlating between Th22-related immunologic factors as independent variables and diabetic complication findings as dependent variables. Also, we used multiple linear regression models to evaluate the effect of such markers on complications of T1D. Adjusted R square was used as a criterion of goodness of fit test and $p > 0.2$ was considered as an exclusion criterion from the model. All analyses were made by the SPSS package (Version 16.0. Chicago, SPSS Inc.).

RESULTS

Basic and clinical characteristics of the study groups are shown in Table 1. Th22 frequency (1.6 ± 1.05 vs 0.7 ± 0.5) and serum levels of IL-22 (33.57 ± 16.23 vs 20.04 ± 10.37) and TNF-α (6.41 ± 4.28 vs 3.21 ± 2.26) were significantly higher in patients than those in controls ($p < 0.001$). IL-10 (7.83 ± 4.67 vs 4.15 ± 2.57) and TGF-β (5.43 ± 2.9 vs 2.01 ± 1.37) levels were higher in healthy individuals than those in patients ($p < 0.001$).

T1D patients showed a significantly greater HbA1c (7.34 ± 1.24 vs 4.73 ± 0.73), cIMT (0.457 ± 0.009 vs 0.392 ± 0.006) and UACR (10.8 ± 8.57 vs 3.01 ± 2.64) than those in the control group ($p < 0.001$). We found a strong positive correlation between the Th22 frequency and serum levels of IL-22 in the patients ($r = 0.894$, $p < 0.001$). Such correlation was not found in control group ($r = 0.186$, $p = 0.231$).

Bivariate correlation showed no significant relationship between different Th22 related biomarkers and cIMT, UACR and HbA1c as indicators of diabetic complications including diabetic atherosclerosis, diabetic nephropathy, and poor metabolic control, respectively, in both control and patient groups (Table 2).

Modeling the effect of different markers on the prediction of diabetic complications through multiple linear regression analysis, we found that the diabetes itself is the most important factor affecting on HbA1c,

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UACR and cIMT changes. Other important factor affecting on HbA1c was IL-22 ($p=0.034$), while there was no linear relation between HbA1c and other inflammatory factors ($p>0.05$). Effective factors on cIMT were diabetes (group), age and sex ($p<0.05$) and there was not any linear relation between IL-22 and cIMT as well as UACR. Such modeling showed that

the only important factor affecting on UACR is diabetes (group) ($p<0.001$). It means that some other factors affect UACR except Th22 and IL-22. Such model also showed that both serum levels of IL-22 and diabetes are effective factors on the increase of HbA1C (Table 3).

Table 1. Basic and clinical characteristics of type1 diabetic patients and healthy control group

Variables	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/m ²)	21.02±4.25	21.23±4.6	0.826	
HbA1c (%)	4.73±0.73	7.34±1.24	<0.001	
Th22 frequency (%)	0.72±0.5	1.61±1.05	<0.001	
IL-22 (pg/mL)	20.04±10.37	33.57±16.23	<0.001	
TNF- α (pg/mL)	3.21±2.26	6.41±4.28	<0.001	
IL-10 (pg/mL)	7.83±4.67	4.15±2.57	<0.001	
TGF- β (pg/mL)	5.43±2.9	2.01±1.37	<0.001	
eGFR (mL/min/1.73m ²)	87.1±34.4	90.6±27.8	0.64	
UACR (mg/g Cr)	3.01±2.64	10.8±8.57	<0.001	
Urine protein (mg/dl)	2.01±2.17	1.69±1.52	0.538	
Urine creatinine (mg/dl)	265.73±158.60	136.58±57.97	<0.001	
Mean Intimae				
Sex	Male	0.4±0.008	0.46±0.01	0.001
Media	Female	0.39±0.008	0.46±0.01	<0.001
Thickness	p value	0.43	0.97	-
(mm)	Age			
	≤12 years (n)	0.39±0.01	0.46±0.01	0.008
	>12 years (n)	0.38±0.006	0.45±0.01	0.001
	p value	0.48	0.53	-
Total		0.392±0.006	0.457±0.009	<0.001

T helper 22; Interleukin 22; Tumor Necrosis Factor- α ; Interleukin 10; Transforming Growth Factor- β

Table 2. Correlation of T helper22-related biomarkers and diabetic complications in 2 groups of type-1 diabetic patients and healthy controls

Group	Variable	Th22 r (p value)	IL-22 r (p value)	IL-10 r (p value)	TNF- α r (p value)	TGF- β r (p value)
Control	HbA1c (%)	0.056 (0.72)	-0.094(0.54)	0.039(0.80)	0.085(0.59)	0.236(0.12)
	Mean cIMT	-0.126(0.52)	0.217(0.26)	0.068(0.73)	-0.210(0.28)	-0.129(0.51)
	UACR	0.068(0.76)	0.101(0.65)	-0.105(0.64)	-0.066(0.77)	-0.192(0.39)
Patients	HbA1c (%)	0.091(0.53)	0.254(0.07)	-0.184(0.20)	0.119(0.41)	-0.091(0.53)
	Mean cIMT	0.020(0.91)	0.116(0.54)	-0.243(0.19)	0.079(0.68)	-0.132(0.48)
	UACR	0.059(0.68)	0.060(0.68)	-0.095(0.51)	0.164(0.26)	-0.030(0.84)

T helper 22; Interleukin 22; Tumor Necrosis Factor- α ; Interleukin 10; Transforming Growth Factor- β

Independent variables: Th22, IL22, IL10, TNF- α , TGF- β

Dependent variables: HbA1c (%), Mean cIMT, UACR

r= correlation coefficient

Table 3. Linear multiple regression analysis for evaluating the effect of T helper22-related biomarkers on diabetic complications

Dependent Variables	Explanatory variables	B	SE	Wald Statistic	95% CI	p value	Adjusted R ²
HbA1c	Constant	3.759	0.449	8.371	(-2.87 , 4.64)	<0.001	0.633
	Group*(Diabetes)	2.619	0.269	9.745	(2.09,3.15)	<0.001	
	Sex(male)	0.350	0.215	1.627	(-0.07,0.77)	0.107	
	IL-22*	0.019	0.009	2.160	(0.001,0.036)	0.034	
	TGF	0.077	0.056	1.357	(-0.328,0.187)	0.178	
cIMT	Constant	0.358	0.023	15.495	(0.313,0.403)	<0.001	0.285
	Group*(Diabetes)	0.031	0.015	2.118	(0.001,0.06)	0.039	
	Age*	0.003	0.001	2.816	(0.001,0.005)	0.007	
	Sex(male)*	0.042	0.013	3.111	(0.016,0.068)	0.003	
	IL-22	0.001	0.0006	1.551	(-0.000,0.002)	0.127	
UACR	Constant	3.002	1.556	1.929	(0.048,6.05)	0.058	0.189
	Group*(Diabetes)	7.805	1.873	4.167	(4.13,11.4)	<0.001	

Interleukin 22; Transforming Growth Factor- β

unstandardized coefficient; it explains both potency and kind (direct or inverse) of its relation on dependent variable. It is the mathematical weightings of the explanatory variables in the equation

SE: standard Error of B; it is an estimated precision of the coefficients

95% CI: 95% confidence intervals for the coefficients

Wald statistic: the estimate of the coefficient divided by its standard error

Adjusted R²: the adjusted R-squared; it compares the explanatory power of regression models that contain different numbers of predictors.

* their effects on diabetic complications is statistically meaningful.

DISCUSSION

Type 1 diabetes (T1D) is perceived as an autoimmune disease resulting from the immune-mediated destruction of pancreatic β cells.¹⁸ Both the immune regulation and immune response are involved in the pathogenesis of T1D, in which infiltrated different immune cells take part in the damage of β cells.¹⁹

Discovering different subsets of CD4⁺ T cells,²⁰ investigators have identified a novel subset of T helper (Th) cells, Th22, which is induced by TNF- α as well as TGF- β cytokines and plays an emerging role in many inflammatory and autoimmune disorders²¹ such as T1D.²² Th22 subset is identified by secretion of various cytokines such as tumor necrosis factor- α (TNF- α), IL-10, and the most important one which is IL-22.³ Th22 plays dual roles in immune system. From one side it is protective against several infectious diseases, and from the other side it might be pathogenic. Both characteristics are invented from its inherent pro-inflammatory features; it would further elevated when

IL-22 is produced together with other pro-inflammatory cytokines, in particular IL-17.²³

We found that T1D patients have higher frequencies of Th22 lymphocytes and higher plasma IL-22 levels compared to healthy individuals. Along with ours, little studies²² showed a significantly elevated of such cells indicating their contribution in the pathogenesis of T1D itself and its complications. Interestingly, IL-22 shows dual effects in different pathologies.²⁴ For example, IL-22 could potentially induce regeneration of β cells and prevent their apoptosis²⁵ to preserve insulin levels in T1D. Therefore, its elevation may act as a protective/compensatory factor to relieve the disease progression. Such role has been explained in some other inflammatory states in which IL-22 repairs the tissue via induction of epithelial cell proliferation and survival. In this way, IL-22 prevents tissue destruction in several mouse models of hepatitis,²⁶ colitis,^{27,28} and graft versus host disease.²⁹ In contrast, IL-22 induces inflammatory molecules and in this way its increased concentration has been shown in a variety of human diseases including psoriasis,³⁰ rheumatoid arthritis,³¹

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inflammatory bowel diseases, influenza and human immunodeficiency virus, which collectively afflict millions of people worldwide.³²

Considering such paradoxical effects, we should introduce Th22 pathway having detrimental or beneficial effects on human islet cells. The fate of such different potentials may be from the duration of the disease in which the autoimmune attack of Th22 pathway may be weakened after a longer duration.²²

High glucose increases the risk of late, potentially life-threatening complications of T1D. Analyzing CD4+IL-22+Th22 subsets, we and others³³ found that T1D patients with higher levels of IL-22 (as the main cytokine of Th22) had greater HbA1c values. In this regard, our findings highlight the role of IL-22 immunity in the pathogenesis of human T1D and its metabolic complications.

We also found that elevated systemic IL-10 and TGF- β were limited to healthy individuals suggesting a potential therapeutic role for these cytokines in the autoimmune disease of T1D.

To our knowledge, this is the first study demonstrating the changes of Th22 subset and its related cytokines in T1D and its complications. The limitation of our study was lack of monitoring such changes longitudinally. This limitation allowed just a cross-sectional analysis of only limited robustness. Secondly, functional assays which provide further information on the possible immune-regulatory mechanism of Th22 pathway were not performed. As further studies emerge to address current limitations, improved therapies targeting such molecules may ultimately transit from treating the pathology to prevention.

Our data demonstrated that while the elevated circulatory Th22 frequencies are correlated with IL-22 serum levels in T1D patients, neither Th22 nor IL-22 have linear correlation with metabolic, cardiovascular, and nephropathic complications of T1D. We report that Th22-related biomarkers are not good prognostic as well as diagnostic indicators of T1D complications.

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