



T Helper 1 Cytokines and Their Relationship with Beta Cell Function in Type 1 Diabetes

T Helper 1 Sitokinleri ve Tip 1 Diyabette Beta Hücre Fonksiyonu ile İlişkileri

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Abstract

Objective: In type 1 diabetes (T1D), T helper (Th) 1 cells affect β cell functions significantly. This study aims to explore the association between serum levels of Th1 cytokines [interferon-gamma (IFN- γ), interleukin (IL)-2 and tumor necrosis factor-alpha (TNF- α)] and β cell function in T1D. **Material and Methods:** The study included 110 patients with T1D (T1DPs) and 31 healthy controls. The β cell functions in T1DPs were assessed by calculating mixed-meal stimulated C-peptide levels. T1DPs were categorized into three groups depending on results of this test (1a-lowest, 1b, 1c-highest). Cytokine levels, IFN- γ /IL-2, and TNF- α /IL-2 ratios in T1DPs were compared with that in controls. Correlation analysis between cytokine levels and diabetes-related parameters was then carried out. **Results:** IFN- γ , TNF- α , IL-2 levels, and TNF- α /IL-2 of T1DPs were higher ($p=0.02$, $p=0.01$, $p=0.008$, and $p=0.01$, respectively) than that of controls. The highest IFN- γ /IL-2 and TNF- α /IL-2 ratios were observed in group 1b ($p=0.03$ and $p=0.04$, respectively) while the lowest IFN- γ /IL-2 and TNF- α /IL-2 ratios were observed in group 1a ($p=0.03$ and $p=0.04$, respectively). The TNF- α levels were found to be negatively correlated with fasting glucose levels ($r^2=-0.003$, $p=0.031$). However, after adjustment for age and gender, this correlation diminished ($r^2=-0.028$, $p=0.076$). **Conclusion:** IFN- γ , IL-2, and TNF- α may exhibit a triggering role in the pathogenesis of T1D. IFN- γ /IL-2 and TNF- α /IL-2 ratios possibly have more significant roles in the progression of β cell dysfunction than other cytokines (ClinicalTrials.gov number: NCT02389335).

Özet

Amaç: Tip 1 diyabette (T1D) T helper (Th) 1 hücrelerinin β hücre fonksiyonuna belirgin etkisi vardır. Bu çalışmada, T1D tanısı olan hastalarda serum Th1 sitokin [interferon-gama (IFN- γ), interlökin (IL)-2 ve tümör nekrozis faktör-alfa (TNF- α)] seviyeleri ile β hücre disfonksiyonu arasında bir ilişki saptanması amaçlandı. **Gereç ve Yöntemler:** Toplam 110 tip 1 diyabetli hasta (T1DH) ve 31 sağlıklı kontrol çalışmaya alındı. T1DH'lerin β hücre fonksiyonu, karışık öğün testi ile stimüle edilmiş C-peptid düzeyi ile ölçüldü. Hastalar, bu testin sonuçlarına göre üç gruba ayrıldı (1a- en düşük, 1b, 1c- en yüksek). Sitokin seviyeleri ve IFN- γ /IL-2 ile TNF- α /IL-2 oranları kontroller ile karşılaştırıldı. Sitokin seviyeleri ile diyabet ilişkili parametreler arasında korelasyon analizi yapıldı. **Bulgular:** T1DH'lerin IFN- γ , TNF- α , IL-2 seviyeleri ve TNF- α /IL-2 oranı kontrollerden istatistiksel olarak anlamlı şekilde yükseldi (sırasıyla, $p=0,02$, $p=0,01$, $p=0,008$, $p=0,01$). En yüksek IFN- γ /IL-2 ve TNF- α /IL-2 oranı grup 1b'de gözlemlendi (sırasıyla, $p=0,03$, $p=0,04$) ve en düşük IFN- γ /IL-2 ve TNF- α /IL-2 oranı grup 1a'da görüldü (sırasıyla, $p=0,03$, $p=0,04$). TNF- α seviyesi, açlık glukoz seviyesi ile negative korele olarak saptandı ($r^2=-0,003$, $p=0,031$). Ancak, yaş ve cinsiyete göre düzeltme yapıldığında bu korelasyon gözlenmedi ($r^2=-0,028$, $p=0,076$). **Sonuç:** IFN- γ , IL-2 ve TNF- α 'nın T1D patogenezinde daha büyük rolü olabilir. IFN- γ /IL-2 ve TNF- α /IL-2 oranlarının β hücre disfonksiyonunun ilerlemesindeki rolü de diğer sitokinlerden fazla olabilir (Bu çalışmanın klinik araştırma kayıt numarası NCT02389335).

Keywords: Type 1 diabetes; IFN- γ ; IL-2; TNF- α ; T helper-1 cell

Anahtar kelimeler: Tip 1 diyabet; IFN- γ ; IL-2; TNF- α ; T helper 1 hücresi

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Introduction

In type 1 diabetes (T1D), pancreatitis destroys β cells. Cytokines of T helper (Th) 1 cell play a prominent role in the inflammation of the pancreas and impaired beta (β) cell function (1,2). The molecular alterations that occur in T cells before insulinitis are poorly understood. Also, the mechanism of breakdown of T-cell tolerance in T1D has not been clarified. It may result from an increase in effector function and/or loss of regulatory function. A few studies point to the loss of regulatory T cell (Treg) number or function, which can trigger the breakdown in tolerance in T cells. Hughson A. et al. observed a stable increase in effector function and a transient decrease in Treg suppression in type 1 diabetes patients (T1DPs) (3). Other mechanisms associated with the progression of this disease include increased levels of inflammatory cytokines, increased insulin resistance, and glucotoxicity resulting in reduced β cell function (4,5).

Cytokines secreted from Th1 cells, exert pro-inflammatory or anti-inflammatory effects at the onset of T1D (6). This study aims to investigate the association between serum levels of Th1 cytokines [interferon-gamma (IFN- γ), interleukin (IL)-2 and tumor necrosis factor-alpha (TNF- α)] and β cell function in T1D.

Material and Methods

The study was conducted at Istanbul Medeniyet University Goztepe Training and Research Hospital between 2013 and 2014. Patients were recruited from an endocrinology clinic.

Subjects

The study involved 110 T1DPs and 31 healthy subjects (control group; CG). The ethical committee of Istanbul Medeniyet University approved the study protocol and was conducted following the Declaration of Helsinki (24.01.2013, 30/I). Informed consent was obtained from all the participants before their inclusion in the study, which was registered with an approved clinical trial registry. The clinical trial registration number of this study is NCT02389335.

The presence of inflammatory diseases, the disease likely to interfere with glucose metabolism, malignancies, hemoglobinopathies,

recent trauma or antibiotic treatment, use of drugs affecting β cell function and insulin sensitivity, and the use of drugs with suppressive effects on inflammation formed the exclusion criteria for the study. Besides, fasting glucose intolerance and impaired glucose tolerance were the exclusion criteria for the CG.

T1D was diagnosed as per the criteria given by the American Diabetes Association (7). All patients were administered basal-bolus insulin treatment. The β cell function of the patients was evaluated by assessing mixed-meal stimulated C-peptide levels. T1DPs were categorized into three groups according to the C-peptide levels after Mixed-Meal Tolerance Test (MMTT)s: patients with undetectable ≤ 0.03 nmol/l (0.1 ng/mL) (group 1a, n=35) C-peptide levels; C-peptide levels between 0.03-0.26 nmol/l (0.1-0.8 ng/mL) i.e. minimal (group 1b, n=30); and sustained ≥ 0.26 nmol/L (0.8 ng/mL) (group 1c, n=45) (in normal range) C-peptide levels.

Laboratory Procedures

After at least 12 h of fasting and abstinence from smoking, and having used the long-acting insulin the previous day and but not prandial morning insulin, the levels of IFN- γ , IL-2, and TNF- α , venous glucose, C-peptide, HbA1c were assessed. After fasting samples were collected, a mixed meal comprising 33 g of carbohydrate, 15 g of protein, and 6 g of fat (240 kcal totals) was administered in less than 15 min for MMTT. As per the Diabetes Control and Complications Trial protocol (8), venous glucose and C-peptide levels were measured at the 90th minute.

Glucose was measured by the hexokinase method; high-performance liquid chromatography (Tosoh G7 and 2.2, Tokyo, Japan) was used to measure HbA1c level, and direct electrochemiluminescence immunoassay (Immulite 2000, Siemens, Germany) was employed to measure C-peptide levels. A C-peptide level ≤ 0.03 nmol/L (a common historical limit of detection) was considered as undetectable (9), that between 0.26-2.58 nmol/l as normal range and between 0.033-0.26 nmol/l as detectable levels (10,11). Limit of blank (LoB) and limit of detection (LoD) values for C-peptide 0.02 nmol/L and 0.03 nmol/L (0.08 ng/mL), respectively.

To measure serum levels of IFN- γ , IL-2, and TNF- α after overnight fasting, antecubital

venous blood samples were collected in non-anticoagulated tubes. They were then centrifuged at 2,000*g for 10 min at 4°C and serum was aliquoted and stored at -80°C for further use. Serum TNF- α and IFN- γ levels were calculated by the ELISA method using reagents produced by Assaypro LLC (MO, USA). The intra-assay and inter-assay coefficient of variability (CV) of TNF- α was 4.8% and 7.1%, respectively. The intra-assay and inter-assay CVs of IFN- γ was 4.8% and 7.0%, respectively. The minimum detectable doses of TNF- α and IFN- γ tests were nearly 0.016 and 0.01 ng/mL, respectively. Serum IL-2 levels were measured by an ELISA reagent (eBioscience Inc., CA, USA). The intra-assay and inter-assay IL-2 were

7.0% and 5.0%, respectively. The minimum detectable dose of the test was 9.1 pg/mL.

Statistical Analysis

Statistical analysis was performed using the SPSS software version 16. The normality of variables was tested using visual (histogram) and analytic methods (Kolmogorov-Smirnov/Shapiro-Wilk's test). Kruskal-Wallis and Mann-Whitney U tests were used to compare groups. Pearson's and Spearman's correlations were used for testing the correlation between variables. Bonferroni correction was employed to analyze multiple comparisons. A *p*-value of less than 0.05 was identified as considered significant.

Table 1. Demographic and clinical characteristics between patients and controls.

	Patient Group (n=110)	Control Group (n=31)	p
Age (year)			0.13
Median	29	30	
Interquartile range	22-36	27-38	
Gender (Male/Female)	58/52	13/18	0.56
Duration of diabetes (month)		-	-
Median	44		
Interquartile range	18-108		
Fasting blood glucose (mg/dL)			<0.001
Median	175	86	
Interquartile range	122-251	84-92	
HbA1c (%)			<0.001
Median	8.4	5.2	
Interquartile range	7.1-10.1	5.1-5.4	
Fasting C peptide (nmol/L)			<0.001
Median	<0.001	1.35	
Interquartile range	0.01-0.78	1.05-1.66	
IFN- γ (pg/mL)			0.02
Median	31.7	21.7	
Interquartile range	10.3-66.9	10.1-30.8	
IL-2 (pg/mL)			0.008
Median	45.2	41.8	
Interquartile range	41.5-48.4	38.8-46.4	
TNF- α (pg/mL)			0.01
Median	90.5	30.0	
Interquartile range	29.8-101.3	26.7-90.3	
IFN- γ /IL-2 ratio			0.05
Median	0.66	0.51	
Interquartile range	0.26-1.40	0.26-0.64	
TNF- α /IL-2 ratio			0.01
Median	1.93	0.73	
Interquartile range	0.73-2.11	0.69-1.89	

HbA1c: Hemoglobin A1c; IFN: Interferon; IL: Interleukin; TNF: Tumor necrosis factor.

Results

Characteristics of T1DPs and controls have been shown in Table 1. Serum levels of IFN- γ , IL-2, and TNF- α were higher ($p=0.02$, $p=0.008$, and $p=0.01$, respectively) in T1DPs than in controls. No difference was observed between IFN- γ /IL-2 ratios of T1DPs and controls ($p=0.05$). However, TNF- α /IL-2 ratio of T1DPs was higher than that of controls ($p=0.01$). No correlation

was found either between stimulated C-peptide levels and IFN- γ ($r^2=0.022$; $p=0.349$), IL-2 ($r^2=0.005$; $p=0.667$) and TNF- α ($r^2=0.001$; $p=0.286$) levels nor between stimulated C-peptide levels and IFN- γ /IL-2 ($r^2=0.023$; $p=0.178$) and TNF- α /IL-2 ($r^2=0.001$; $p=0.217$) ratios in T1DPs.

Characteristics of group 1a, group 1b, and group 1c have been depicted in Table 2. No difference was found between serum levels of IFN- γ , IL-2, and TNF- α in group 1a, group

Table 2. Comparison of groups 1a, 1b, 1c in terms of demographic and biochemical characteristics.

	Group 1a (n=35)	Group 1b (n=30)	Group 1c (n=45)	p
Age (year)				0.19
Median	31	28	28	
Interquartile range	24-38	22-33	21-38	
Gender (Male/Female)	15/20	17/13	26/19	0.53
Duration of diabetes (month)				<0.001
Median	96	35	24	
Interquartile range	65-159	12-92	7-60	
Fasting blood glucose (mg/dL)				0.07
Median	182	207	154	
Interquartile range	118-293	144-282	122-216	
HbA1c (%)				0.72
Median	8.0	8.6	8.3	
Interquartile range	7.2-9.1	7.5-10.1	6.6-10.7	
Fasting C peptide (nmol/L)				<0.001
Median	0.01	0.23	0.86	
Interquartile range	0.01-0.01	0.15-0.26	0.48-1.26	
IFN- γ (pg/mL)				0.06
Median	27.5	45.1	31.4	
Interquartile range	10.1-46.6	27.4-128.4	10.1-54.7	
IL-2 (pg/mL)				0.35
Median	44.0	46.1	44.6	
Interquartile range	40.9-47.4	42.3-49.0	41.2-48.7	
TNF- α (pg/mL)				0.05
Median	33.2	95.8	89.6	
Interquartile range	28.1-95.8	88.5-108.5	30.5-100.6	
Age at diagnosis				0.12
Median	19	23	25	
Interquartile range	16-29	15-29	19-31	
90 th -minute C peptide (nmol/L)				<0.001
Median	0.01	0.64	2.26	
Interquartile range	0.01-0.01	0.27-0.71	1.69-4.14	
IFN- γ /IL-2 ratio				0.03
Median	0.57	0.93	0.63	
Interquartile range	0.25-0.99	0.57-2.65	0.25-1.12	
TNF- α /IL-2 ratio				0.04
Median	0.83	2.01	1.94	
Interquartile range	0.67-1.99	1.89-2.21	0.75-2.06	

HbA1c: Hemoglobin A1c; IFN: Interferon; IL: Interleukin; TNF: Tumor necrosis factor.

1b, and group 1c ($p>0.05$, $p>0.05$, $p>0.05$, respectively). However, group 1b expressed the highest IFN- γ /IL-2 and TNF- α /IL-2 ratio ($p=0.03$, $p=0.04$, respectively) while group 1a presented the lowest IFN- γ /IL-2 and TNF- α /IL-2 ratio ($p=0.03$, $p=0.04$, respectively) among T1DPs.

The TNF- α level was found to be negatively correlated with fasting glucose level ($r^2=-0.003$, $p=0.031$). However, this correlation diminished ($r^2=-0.028$, $p=0.076$) after adjustment for age and gender.

IFN- γ level was negatively correlated with the duration of diabetes ($r^2=-0.021$, $p=0.007$) and age at diagnosis ($r^2=-0.073$, $p=0.027$). After adjustment for age and gender, the correlation between IFN- γ and age at diagnosis and that between IFN- γ and duration of diabetes disappeared ($r^2=-0.003$, $p=0.453$; $r^2=-0.062$, $p=0.283$, respectively).

Discussion

The present study proved that serum levels of IFN- γ , IL-2, TNF- α , and TNF- α /IL-2 ratio were significantly higher in T1DPs than in healthy controls. Although IFN- γ /IL-2 ratio was lower in CG than in T1DPs, this difference was insignificant. IFN- γ /IL-2 and TNF- α /IL-2 ratios were higher in T1DPs with detectable and normal C-peptide levels than in T1DPs with undetectable C-peptide levels. These findings suggest the presence of higher levels of autoimmune inflammation in T1DPs with better β cell function than in T1DPs with undetectable C-peptide levels. Inflammation of β cells is the major pathology in T1D (11). β cell apoptosis is stimulated by cytokines such as IL-1 β , TNF- α , and IFN- γ (12). In contrast with the previous studies, the present study found higher IFN- γ and TNF- α levels, IFN- γ /IL-2, and TNF- α /IL-2 ratio in T1DPs with better β cell function than in T1DPs with undetectable β cell function. The process of dedifferentiation and/or differentiation of β cells may occur in T1D depending on the level of inflammation involved. It is, therefore, possible that IFN- γ and TNF- α may play protective roles for β cells against the phagocytosis and/or against the destruction by natural killers. However, it could also signify the ongoing inflammatory process in these patients, and high cytokine levels can

be attributed to increased inflammation in group 1b. Since only scarce beta cells remained in group 1c, the inflammation is thought to have diminished over time.

The only Th 1 cytokine found in β cells is IFN- γ (13). Some studies reported that ablation or blockage of IFN- γ causes delayed or decreased incidence of T1D (14). IFN- γ has been found to increase the toxic effects of macrophages and T lymphocytes on β cell function (15-17).

Improvement in β cell function in T1D is considered to be a result of insulin sensitivity and reduced inflammatory/autoimmune process in islets (18). However, Kaas et al. established that stimulated C-peptide levels and IFN- γ levels did not have any link (19). The results of the present study showed no correlation between stimulated C-peptide and IFN- γ levels, which were consistent with those of Kaas's study.

Several studies have suggested the role of the upregulation of inflammatory factors and the downregulation of anti-inflammatory mechanisms (IL-10 and Tregs) in T1D pathogenesis (20). TNF- α has been confirmed to have an accelerator role in the development of T1D (21). Hotamisligil et al. identified that TNF- α downregulates the tyrosine kinase activity of the receptor (22). TNF- α could induce serine/threonine phosphorylation of the insulin receptor substrate, hinder normal phosphorylation of tyrosine, and diminish signal transduction of insulin, by increasing the activities of the NF- κ B transcriptional factor, protein kinase C, amino-terminal kinase, and inhibitor kinase. This finally results in insulin resistance, or TNF- α may result in the destruction of pancreatic beta cells and lead to the development of T1DM (23,24).

TNF- α interacts with beta cells via different mechanisms. Li et al. elucidated that Th17 cells may promote the development of T1D, and TNF- α could mediate diabetes in response to either Th17 cells or Th1 cells (25). A comprehensive interpretation of the levels of cytokines that could affect beta cells is important because studies prove that there is no single pathway to this.

The proportion of effector T cells was stable in T1D during pancreatitis; however, IL-2 treatment was determined to reduce IFN- γ levels, particularly in the pancreas (26). While TNF- α and IFN- γ reflect autoimmune

activity, IL-2 indicates regulatory activity, which in turn suppresses autoimmunity. The present study investigated the effect of IFN- γ /IL-2 and TNF- α /IL-2 balance on β cell function and found that T1DPs with higher autoimmune response have better β cell function compared to other T1DPs.

The sources of IFN- γ include activated T lymphocytes, natural killer (NK) cells, and sometimes β cells (6,27), while the sources of TNF- α are monocytes, macrophages, CD4⁺ and CD8⁺ T cells, B cells, lymphokine-activated killer (LAK) cells, NK cells, endothelial cells, non-hematopoietic tumor cell lines, mast cells, and neutrophils. Nevertheless, the only source of IL-2 is Th1 cells (28). T1DPs with better β cell function had higher IFN- γ /IL-2 and TNF- α /IL-2 ratios in the present study; this may reflect the effect of these cytokine sources in the process of autoimmune T1D.

This study is the first to explore the effects of balanced IFN- γ /IL-2 and TNF- α /IL-2 on β cell function. One limitation of this study is that autoantibody levels of T1DPs with high, moderate, and low β cell function could not be compared as the autoantibody levels of the patients were measured in different laboratories. However, all T1DPs had high levels of autoantibody against 65-kDa glutamic acid decarboxylase. Also, this cohort comprised only adult patients. A larger cohort with the inclusion of children may have presented different results.

Conclusion

IFN- γ , IL-2, and TNF- α may act as a triggering factor in the pathogenesis of T1D. IFN- γ /IL-2 and TNF- α /IL-2 ratio may be more significant than IFN- γ , IL-2, and TNF- α levels themselves in the progress of β cell dysfunction. Yet, it is not clear whether decreased β cell mass and function in T1D is the cause or the result of decreased Th1 cytokine levels.

Source of Finance

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Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: Gonca Tamer; Design: Gonca Tamer, Banu İşbilen Başok; Control/Supervision: Gonca Tamer, Burcu Doğan; Data Collection and/or Processing: Osman Köstek, Gonca Tamer; Analysis and/or Interpretation: Özge Telci Çaklılı, Osman Köstek; Literature Review: Özge Telci Çaklılı; Writing the Article: Özge Telci Çaklılı; Critical Review: Gonca Tamer; References and Fundings: Gonca Tamer; Materials: Gonca Tamer.

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