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Original Article

The Interaction Between Interleukin-29 and Toll-Like Receptor-4 in the Peripheral Blood Mononuclear Cells of Patients With Type 2 Diabetes

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Abstract

Background and aims: The Toll-like receptor-4 (TLR-4) signaling pathway and its interaction with cytokines are involved in insulin resistance (IR) progression, primarily through activating adipose tissue macrophages. Interleukin-29 (IL-29) has been suggested as a potential contributor to IR. Therefore, this study aimed to assess the relationship between TLR-4 and IL-29 expression alterations in the peripheral blood mononuclear cells of newly diagnosed type 2 diabetes (T2D) patients.

Methods: Overall, 60 participants were enrolled, comprising equal numbers of newly diagnosed T2D patients and healthy controls. Quantitative real-time polymerase chain reaction was employed to determine the messenger RNA expression levels of toll-like receptor-4 (TLR-4), interleukin (IL)-29, TIR-domain-containing adapter-inducing interferon-β (TRIF), and interferon regulatory factor 3 (IRF3). Moreover, serum IL-29 concentrations were quantified using an enzyme-linked immunosorbent assay. Data were analyzed through SPSS 16. In addition, an independent-sample t-test was used to compare the data between the two groups. Ultimately, the relationship between variables was assessed using Pearson's correlation coefficient.

Results: Overall, IL-29 serum levels were significantly increased in the T2D group compared to the control group ($P<0.001$). Additionally, the gene expression levels of *IL-29*, *TLR4*, *TRIF*, and *IRF3* were markedly upregulated in T2D patients relative to healthy individuals ($P<0.001$).

Conclusion: These findings revealed that IL-29 overexpression is linked to the activation of the TLR-4 pathway, which may contribute to the pathogenesis of IR in T2D.

Keywords: Type 2 diabetes, Insulin resistance, Interleukin-29, Toll-like receptor-4

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Introduction

Type 2 diabetes (T2D) is the most common type of diabetes. The global prevalence of this chronic disease has been rapidly increasing during the last few years. Overall, 415 million patients with T2D were identified in 2015 and are likely to reach 625 million in 2045. Hyperglycemia, insulin resistance (IR), dyslipidemia, and hypertension are the most critical indicators of metabolic syndrome, which can increase the risk of developing T2D (1, 2).

Moreover, chronic low-grade inflammation has emerged as a central mechanism in the pathogenesis of T2D (3, 4). Inflammatory responses, particularly those involving immune cells (e.g., macrophages and monocytes), contribute to IR and impaired glucose metabolism (5, 6). Research has shown that macrophage activation in metabolic tissues leads to elevated production of inflammatory cytokines in patients with T2D (7). Interleukin (IL)-29, a member of the interferon family, has recently been implicated in these processes. It contributes to inflammation and enhances monocyte chemoattractant protein-1(MCP-1) expression in hypertrophic adipocytes (8, 9). According to a previous study, IL-29 promotes IR by reducing glucose uptake in individuals suffering from Simpson-Golabi-Bemel syndrome. However, the exact role of IL-29 in metabolic disorders remained unclear (8).

The activation of peripheral blood mononuclear cells (PBMCs) is the first step in the onset of many inflammatory diseases, including IR, T2D, and the like (10, 11). For example, the production of pro-inflammatory cytokines and chemokines has increased in the monocytes of patients with T2D (12). Emerging evidence indicates that the expression of toll-like receptors (TLRs) in PBMCs plays an essential role in regulating pro-inflammatory mediators (13, 14). For instance, TLR-2 and TLR-4 are highly expressed in PBMCs and play a key role in mediating inflammatory responses linked to IR (15). In addition, TLR-4 signal transduction through MyD88 adapter-like/toll-IL-1 receptor-domain-containing adapter protein and toll-interleukin-1 (IL-1) receptor-domain-containing adapter-inducing interferon-β (TRIF)/TRIF related

adaptor molecule axis could promote IR (16).

Interferon regulatory factor 3 (IRF3), which lies downstream of TLR-4, induces IR in adipocytes through the induction of tissue inflammation (17). A recent study revealed that IL-29 can increase TLR-4 expression by producing IL-6, IL-8, and tumor necrosis factor-alpha along the nuclear factor kappa B messenger pathway (18). For example, previous studies confirmed an increased level of IL-29 in PBMCs, serum, adipose tissue, and synovial fluid of patients with rheumatoid arthritis (18, 19). As a result, IL-29 can increase inflammation through the TLR-4 signaling pathway (20). Hence, the current study aims to investigate IL-29 expression in PBMCs and serum of patients with T2D and healthy subjects and evaluate its association with the TLR-4/TRIF/IRF3 axis.

Materials and Methods

Patients

Overall, 60 subjects (40–60 years old) referred to Hajar Hospital of Shahrekord (Iran), including 30 patients with T2D and 30 healthy individuals, were selected for investigation. Patients with T2D were included in the study according to American Diabetes Association criteria, with fasting blood sugar (FBS) ≥ 126 mg/dL, 2-hour blood sugar ≥ 200 mg/dL, or hemoglobin A1c (HbA1c) ≥ 6.5%. The control group had FBS below 100 mg/dL and no symptoms of diabetes. The height, weight, and body mass index (BMI) of all subjects were measured using a Kindle height and weight hand scale (model DT05L). In addition, the subjects' blood pressure (BP) was estimated using Richter's arm sphygmomanometer (Diplomat 1002 mercury model) under the same conditions and reported in mmHg. Furthermore, sera were separated for biochemical analysis and IL-29 level measurement. The exclusion criteria included chronic and acute inflammatory diseases, infectious diseases, autoimmune diseases, endocrine diseases, and pregnancy. Moreover, people treated with various drugs, including antihypertensive drugs (e.g., beta-blockers), anti-diabetic drugs, estrogens, thyroxine, diuretics, and hypolipidemic drugs, were excluded from the investigation. All subjects agreed to participate in this study by providing written informed consent.

Isolation of Peripheral Blood Mononuclear Cells

PBMCs were isolated by Ficoll gradient centrifugation (Sigma, USA). The process involved taking blood from individuals and diluting it in phosphate-buffered saline at a ratio of 1:1. The blood samples (800 g) were then centrifuged at 4°C for 40 minutes. Next, the middle layer containing PBMCs was removed and washed three times with phosphate-buffered saline. The resulting PBMCs were frozen at -70°C for further analysis.

Total Ribonucleic Acid Extraction, Complementary Deoxyribonucleic Acid Synthesis, and Quantitative Real-Time Polymerase Chain Reaction

The total RNAs of PBMCs were extracted using the RNX-Plus solution (Cinnagen, Iran). The extracted RNA was converted to cDNA using a cDNA synthesis kit according to the manufacturer's instructions (GeneAll Biotechnology, Korea). Additionally, qRT-PCR was performed using the SYBR Green master mix (Amplicone, Denmark) in the Rotor-Gene 3000 instrument (Corbett Research, Australia). Moreover, the specificity of primers was evaluated using Primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 1). In addition, the *ACTβ* reference gene was used to normalize gene expression as an endogenous internal control. Finally, the relative gene expression was assessed using the $2^{-\Delta\Delta Ct}$ method (21).

Serum Interleukin-29 Assay

The serum concentration of IL-29 was measured using enzyme-linked immunosorbent assay (ELISA) kits according to the protocol of the human IL-29 ELISA kit from CUSABIO (China) and the manufacturer's instructions. The sensitivity of this assay was 3.9 pg/mL.

Statistical Analysis

The obtained data were analyzed through SPSS software (version 16.0, Chicago, IL, USA) and GraphPad Prism software (version 7, San Diego, CA, USA). Regarding the normal distribution of data by checking the Kolmogorov-Smirnov test, the data from the two groups were compared using an independent-sample t-test. Further, the correlation between variables was calculated using Pearson's correlation coefficient (r). Data are expressed as means ± standard deviations (SD). $P < 0.05$ was considered statistically significant.

Results

Anthropometric and Biochemical Findings

Gender, age, mean body weight, height, BMI, systolic BP

Table 1. Specific Primer Sequences

mRNA Name	Accession Number NCBI	Forward Sequences	Reverse Sequences
ACTβ	>NM_031144.3	CCCGCGAGTACAACCTTCT	CGTCATCCATGGCGAACT
TRIF	>NM_001385678.1	GCACCAACTACCCAGTGGAG	TGGCGTCTGGTCTTTGACAG
IRF3	>NM_001197125.2	TCTTCCAGCAGACCATCTCC	TGCCTCACGTAGCTCATCAC
TLR-4	>NM_003266.4	AGTGTGTGTGTCCGCATGAT	CCACTTGGGGTCTAAGAACG
IL-29	>NM_172140.2	TGGGAACCTGTGTCTGAGAACG	AGGGCTCAGCGCATAAATAAGGTG

Note. mRNA: Messenger ribonucleic acid; NCBI: The National Center for Biotechnology Information; TLR: Toll-like receptor-4; TRIF: TIR-domain-containing adapter-inducing interferon-β; IL: Interleukin; ACTβ: Beta actin.

(SBP), and diastolic BP (DBP) underwent measurement (Table 2). There was no statistically significant difference in anthropometric data, except BMI ($P=0.026$), between patients with T2D and healthy subjects.

Serum biochemical analysis revealed that the serum levels of FBS, insulin, triglycerides (TG), and total cholesterol (TC) were higher in patients with T2D than in healthy subjects ($P<0.001$). Furthermore, HbA1c and homeostatic model assessment of IR (HOMA-IR) were higher in patients with T2D than in healthy subjects ($P<0.001$). Nevertheless, no significant difference was observed between the two groups regarding high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), urea, and creatinine (Table 3).

Increased Serum Levels of Interleukin-29 in Patients With Type 2 Diabetes

The mean concentration of IL-29 was 190 ± 53.42 pg/mL in the T2D group and 69 ± 35.15 pg/mL in the control group (Figure 1). Moreover, the difference in serum IL-29 levels was considered statistically significant between the two groups ($P<0.001$).

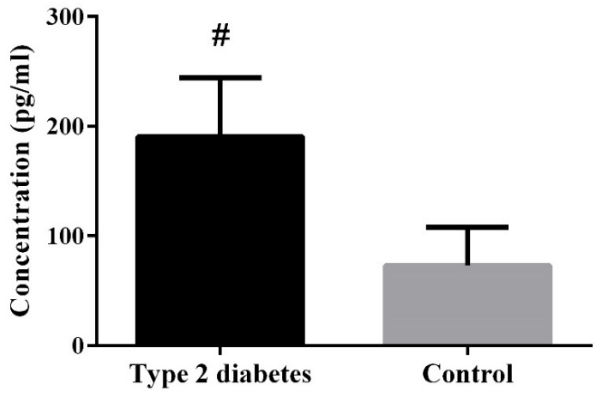

Figure 1. Concentration of Serum IL-29 in Patients With T2D and Healthy Subjects
Note. T2D: Type 2 diabetes; IL: Interleukin. Data are expressed as means ± standard deviations. # $P<0.001$

Table 2. Anthropometric Indices of the Study Population

Variable	Healthy Subjects (n = 30)	Patients With T2D (n = 30)	*P*-Value
Female/male	14/16	15/15	0.341
Age (year)	48.27 ± 6.33	50.41 ± 5.77	0.062
Weight (kg)	75.43 ± 7.44	72.71 ± 6.91	0.105
Height (cm)	171.25 ± 5.91	170.9 ± 8.41	0.192
BMI (kg/m^2)	25.57 ± 2.21	24.05 ± 3.58	0.026
SBP (mmHg)	121.1 ± 4.43	118.62 ± 4.51	0.088
DBP (mmHg)	81.8 ± 2.98	79.56 ± 4.35	0.089

Note. Data are expressed as means ± standard deviations. BMI: Body mass index; DBP: Diastolic blood pressure; SBP: Systolic blood pressure; T2D: Type 2 diabetes.

Overexpression of the Toll-Like Receptor-4, Toll-Interleukin-1 Receptor-Domain-Containing Adapter-Inducing Interferon-β, Interferon Regulatory Factor 3, and Interleukin 29 in Patients With Type 2 Diabetes

Based on the results (Figure 2 a-d), the expression of the *IL-29* gene in the PBMCs of patients with T2D was significantly higher than in healthy subjects ($P<0.001$). In addition, the expression of TLR-4 and its downstream elements (i.e., TRIF and IRF3) was upregulated in the PBMCs of patients with T2D as compared with healthy subjects ($P<0.001$).

Correlation of Overexpression of Interleukin 29 With Induced Toll-Like Receptor 4 Signaling Pathway

Pearson's correlation analysis (Table 4) demonstrated that serum IL-29 protein levels were positively correlated with the expression of *IL-29* and *TLR-4* genes ($P<0.001$). In addition, there was a positive correlation between downstream elements of the TLR-4 pathway, including *TRIF* gene expression with IL-29 protein levels ($P=0.008$) and *IRF3* gene expression with IL-29 protein levels ($P=0.007$).

Association Between Interleukin-29 and Toll-Like Receptor-4 Signaling Pathway With Anthropometric and Biochemical Parameters

Table 5 presents the correlation between IL-29 and the TLR-4 signaling pathway with anthropometric data and biochemical parameters. A positive correlation was observed between TG and TC with inflammatory markers,

Table 3. Biochemical Parameters of the Study Population

Variable	Healthy Subjects (n = 30)	Patients With T2D (n = 30)	*P*-Value
FBS (mg/dL)	91.62 ± 5.35	166.45 ± 55.30	<0.001
Insulin (μIU/mL)	4.01 ± 1.49	12.77 ± 6.03	<0.001
HOMA-IR	0.91 ± 0.43	5.25 ± 4.22	<0.001
HbA1c (%)	5.80 ± 0.62	8.41 ± 2.21	<0.001
TG (mg/dL)	135.31 ± 49.10	267.80 ± 75.12	<0.001
TC (mg/dL)	155.50 ± 24.02	212.96 ± 40.71	<0.001
LDL-C (mg/dL)	93.60 ± 19.50	107.11 ± 27.43	0.066
HDL-C (mg/dL)	47.0 ± 10.62	43.5 ± 8.91	0.081
Urea (mg/dL)	22.79 ± 5.63	26.8 ± 6.35	0.079
Creatinine (mg/dL)	1.04 ± 0.14	1.03 ± 0.22	0.095

Note. Data are expressed as means ± standard deviations. FBS: Fasting blood sugar; HOMA-IR: Homeostatic model assessment of insulin resistance; HbA1c: Hemoglobin A1c; TG: Triglyceride; TC: Total cholesterol; LDL-C: Low-density lipoprotein cholesterol; HDL-C: High-density lipoprotein cholesterol.

Table 4. Correlation Coefficients of Serum IL-29 Protein Levels With the Expression of *IL-29*, *TLR-4*, *TRIF*, and *IRF3* Genes

Variable	*IL-29* Gene Expression	*TLR-4* Gene Expression	*TRIF* Gene Expression	*IRF3* Gene Expression
IL-29 protein levels	$P<0.001$ r = 0.245	$P<0.001$ r = 0.187	$P=0.008$ r = 0.286	$P=0.007$ r = 0.335

Note. IL-29: Interleukin-29; IRF3: Interferon regulatory factor 3; TLR-4: Toll-like receptor-4; TRIF: TIR-domain-containing adapter molecule 1; IRF3: Interferon Regulatory Factor 3.

Table 5. Correlation Coefficients of IL-29 Protein Levels and Gene Expression of *IL-29*, *TLR-4*, *TRIF*, and *IRF3* With Anthropometric and Biochemical Parameters

Variable	*IL-29* Protein Levels	*IL-29* gene Expression	*TLR-4* Gene Expression	*TRIF* Gene Expression	*IRF3* Gene Expression
Age	0.13	0.105	0.043	0.110	0.29
Weight	0.22	0.35	0.04	0.15	0.05
Height	0.27	0.14	0.20	0.27	0.02
BMI	**0.45**$^{+}$	**0.37**$^{+}$	**0.24***	0.11	0.18
SBP	0.17	0.03	0.23	0.37	0.22
DBP	0.02	0.01	0.21	0.23	0.27
FBS	**0.23**$^{\#}$	**0.37**$^{\#}$	**0.28***	0.28	0.21
Insulin	**0.32**$^{+}$	**0.29**$^{\#}$	**0.31**$^{+}$	0.22	0.29
HOMA-IR	**0.40**$^{\#}$	**0.45**$^{\#}$	**0.29**$^{\#}$	**0.34***	0.21
HbA1c	**0.39**$^{+}$	**0.30***	**0.20***	0.20	0.30
TG	**0.20**$^{+}$	**0.23**$^{\#}$	**0.38**$^{+}$	**0.21**$^{+}$	**0.18***
TC	**0.19**$^{\#}$	**0.27**$^{\#}$	**0.48***	**0.33***	**0.19**$^{+}$
LDL-C	0.11	0.29	0.20	0.25	0.29
HDL-C	0.37	0.37	0.21	0.21	0.02
Urea	0.18	0.23	0.29	0.03	0.11
Creatinine	0.14	0.29	0.04	0.04	0.29

Note. Data are expressed as means ± SD. BMI: Body mass index; DBP: Diastolic blood pressure; FBS: Fasting blood glucose; HDL-C: High-density lipoprotein cholesterol; HOMA-IR: Homeostasis model assessment of insulin resistance; IL-29: Interleukin-29; IRF3: Interferon regulatory factor 3; LDL-C: Low-density lipoprotein cholesterol; SBP: Systolic blood pressure; TC: Total cholesterol; TG: Triglycerides; TLR-4: Toll-like receptor-4; TRIF: TIR-domain-containing adapter-inducing interferon-β. * $P<0.05$, $^{+}$ $P<0.01$, $^{\#}$ $P<0.001$. The bold numbers indicate that this correlation is statistically significant.

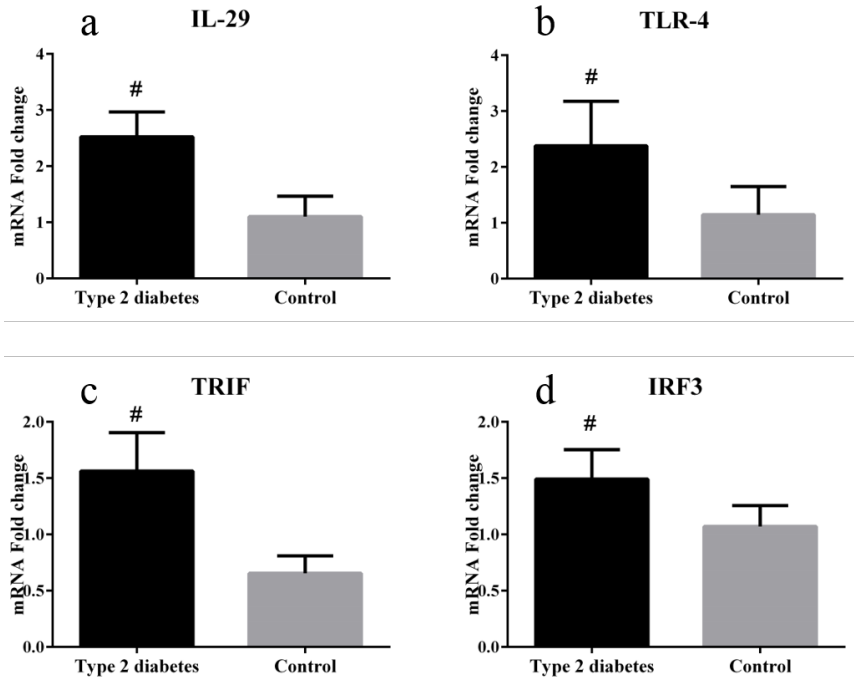

Figure 2. The mRNA Fold Change ($2^{-\Delta\Delta Ct}$) of IL-29 **(a)**, TLR-4 **(b)**, TRIF **(c)**, and IRF3 **(d)** in the PBMCs of Patients With T2D and Healthy Subjects
Note. mRNA: Messenger ribonucleic acid; IL: Interleukin; TLR-4: Toll-like receptor-4; TRIF: TIR-domain-containing adapter-inducing interferon-β; IRF3: Interferon Regulatory Factor 3; PBMC: Peripheral blood mononuclear cell; T2D: Type 2 Diabetes. Data are expressed as means ± standard deviations. $^{\#}$ $P<0.001$

including IL-29, TLR-4, TRIF, and IRF3. Additionally, the expression of IL-29 and TLR-4 was positively associated with HOMA-IR, FBS, HbA1c, and insulin levels.

Moreover, BMI was positively correlated with IL-29 and TLR-4 among study participants. However, no significant correlation was found between inflammatory markers and other anthropometric and biochemical parameters, such as body weight, height, SBP, DBP, HDL-C, LDL-C, urea, and creatinine.

Discussion

IR is recognized as a primary contributor to the development and progression of T2D. Adipose tissue macrophages secrete adipokines, which promote IR by inducing inflammation and disrupting the insulin signaling pathway (22, 23). Within adipocytes, TLR-4 activation enhances the expression of multiple pro-inflammatory mediators, including interferons, by modulating intracellular signaling cascades (24). According to recent

research, IL-29 overexpression plays a crucial role in the development of IR in obesity (8). However, its precise involvement in the pathogenesis of T2D remains unclear. Therefore, this study evaluated the relationship between IL-29 expression and the TLR-4 signaling pathway, which are closely associated with T2D and IR. Accordingly, the gene expression and serum protein levels of IL-29 in T2D patients and healthy individuals were examined, alongside the gene expression of key components of the TLR-4 signaling pathway.

Our findings demonstrated a notable increase in IL-29 expression at both transcriptional and translational levels in PBMCs and the serum of diabetic individuals compared to healthy controls, suggesting that IL-29 may play a pro-inflammatory role in the progression of T2D. Similar results were reported in a recent study, indicating that IL-29 contributes to IR by mediating interactions between macrophages and adipocytes. Additionally, IL-29 is believed to enhance inflammation in adipocytes and synovial fibroblasts by regulating IL-1β, MCP-1, and IL-8 levels (8, 25, 26).

Our results also revealed increased TLR-4 expression during diabetes progression, which is consistent with those of prior research showing elevated TLR-4 levels in the PBMCs of diabetic neuropathy patients (27). Furthermore, the expression of TRIF and IRF3 (key downstream components of the TLR-4 signaling pathway) was significantly upregulated in the diabetic group compared to healthy subjects.

Considering the role of the TLR signaling pathway in glucose homeostasis, it has been hypothesized that TRIF signaling is essential for normal β-cell function. Previous in vitro and in vivo studies suggested that TRIF knockdown leads to hyperglycemia and β-cell dysfunction (28). However, our findings contradict this notion, as higher TRIF expression was observed in diabetic patients. Additionally, our results regarding IRF3 expression align with previous findings by Wang et al, demonstrating that IRF3 promotes IR by inhibiting the inhibitory kappa B kinase beta/nuclear factor kappa B axis (29).

Our findings further confirmed the upregulation of IL-29 expression in the serum and PBMCs of diabetic individuals, which conforms to the results of an in vitro study by Xu et al, indicating a positive association between IL-29 and TLR-4 in RAW264.7 cells during rheumatoid arthritis pathogenesis (30). Likewise, TLR-4 overexpression was linked to inflammatory markers, such as IL-29 and tumor necrosis factor-alpha, reinforcing the role of IL-29 in driving inflammation. These findings suggest that IL-29 plays a crucial role in the development of IR in T2D patients.

The correlation between IL-29 serum levels and significant components of the TLR-4 pathway, including TLR-4, TRIF, and IRF3 was analyzed to investigate the involvement of inflammation in TLR-4 pathway activation. Our results showed a positive association between IL-29 and elements of the TLR-4 signaling pathway, further supporting the hypothesis that the IL-29–TLR-4 axis in PBMCs contributes to IR by exacerbating inflammation in adipose tissue (26, 30).

Based on our findings, a positive association was observed between TG and TC with inflammatory parameters, including IL-29, TLR-4, TRIF, and IRF3. There was also a positive correlation between IL-29. Similarly, our findings revealed a positive correlation between TG and TC with inflammatory markers (IL-29, TLR-4, TRIF, and IRF3). In addition, IL-29 and TLR-4 were positively associated with HOMA-IR, FBS, HbA1c, and insulin levels. The observed correlation between BMI and IL-29/TLR-4 suggests that IL-29/TLR-4 pathway activation could contribute to IR in obese individuals. The correlation between HOMA-IR and IL-29 serum levels aligns with the findings of previous research, identifying IL-29 as a key player in inflammation-induced IR (8, 31, 32).

However, no significant correlations were found between inflammatory markers and HDL-C, LDL-C, urea, or creatinine. While inflammatory factors are known to be involved in dyslipidemia and adipose tissue inflammation, our findings indicate that IL-29 may not directly contribute to lipoprotein metabolism (19). Further research is needed to determine whether IL-29 overexpression influences lipid profile disturbances. It is noteworthy that expanding the sample size in future studies can strengthen the statistical power to confirm or refute these findings.

Conclusion

In general, the correlation between IL-29 and TLR-4 pathway components supports the idea that IL-29 enhances inflammation via TLR-4 activation in the PBMCs of T2D patients. Moreover, IL-29 and the TLR-4 pathway could serve as potential biomarkers for inflammation-induced IR, while targeting their expression may offer new therapeutic strategies for diabetes management. It I expected that further studies focusing on molecular mechanisms underlying these interactions will provide deeper insights into the role of IL-29 and the TLR-4 pathway in T2D pathogenesis.

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Nil.

Authors' Contribution

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Competing Interests
The authors have no conflict of interests to disclose.

Ethical Approval
This study was conducted according to the ethical principles outlined in the Declaration of Helsinki. In addition, ethical approval was obtained from Shahrekord University of Medical Sciences (No. IR SKUMS.REC.1399.007).

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