

Elevated Interleukin-17 Levels in Patients with Newly Diagnosed Type 2 Diabetes Mellitus

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Abstract

Interleukin (IL)-17 is a newly described T cell-derived inflammatory cytokine. Recent studies have shown that inflammatory cytokines play important roles in the pathogenesis of type 2 diabetes mellitus (DM). However, whether IL-17 plays a prominent role in type 2 DM remains unclear. Here we measured serum concentrations of IL-17, and mRNA expression of IL-17 and its transcription factor ROR γ t in peripheral blood mononuclear cells (PBMC) from 30 patients with newly diagnosed type 2 DM and 20 healthy subjects. Results have shown that serum levels of IL-17 were significantly higher in patients than in healthy subjects (10.44 ± 6.47 vs. 2.99 ± 1.68 pg/mL, $P < 0.01$); and mRNA levels of IL-17 and its transcript factor ROR γ t were also upregulated in PBMC from patients ($P < 0.001$). Further, IL-17 mRNA expression was correlated with TNF- α mRNA expression in PBMC from patients ($r = 0.6988$, $P < 0.001$). These results indicated that IL-17 might contribute to the inflammation process in type 2 diabetes as an inflammation factor.

Keywords: Interleukin-17; Type 2 diabetes mellitus; Inflammation cytokines

Introduction

Inflammation is defined as the local physiological response to tissue injury and regulated by pro-inflammatory cytokines. Recently, chronic inflammation has received considerable attention as an important pathophysiological mechanism in type 2 diabetes mellitus (DM). Inflammatory cytokines have been postulated to be important pathogenic factors in the development of type 2 DM [1,2].

Interleukin (IL)-17 (IL-17A) is a newly identified inflammatory cytokine produced by activated and memory T lymphocytes [3]. It has pleiotropic activities including the induction of diverse inflammatory cytokines (e.g. IL-6 and TNF- α) and chemokines (e.g. CCL2/MCP-1, CXCL1/KC, and CXCL2/MIP-2) from a large variety of cells [4,5]. IL-17 acts as highly potent inflammatory cytokine that initiates tissue inflammation and induces the infiltration of other inflammatory cells into the target organs [6,7].

Increasing evidence suggests that IL-17 plays a crucial role in various inflammatory responses and autoimmune diseases [8-10]. Recent studies have shown that increased IL-17 levels were detected in STZ-induced diabetic animal models and non-obese diabetic (NOD) mice from insulinitis to diabetes [11,12]. However, to date, there are little published data evaluating the role of IL-17 in type 2 DM.

Based on these observations, in this study, we investigated mRNA levels of IL-17 and its transcription factor ROR γ t in peripheral blood mononuclear cells (PBMC) and serum concentrations of IL-17 from patients with newly diagnosed type 2 DM. Meanwhile, we analyzed the correlation between IL-17 mRNA expression and mRNA expression of inflammation cytokines involved in type 2 DM in PBMC from diabetic patients. Our results suggest that IL-17 might be involved in the pathogenesis of type 2 DM as an inflammation cytokine.

Materials and Methods

Subjects

Thirty patients with newly confirmed type 2 diabetes mellitus were

collected from out-patient department or epidemiology investigation. The control subjects, who were healthy examinees from the hospital, comprised 20 healthy non-diabetic subjects of similar age. This study conformed to the approved institutional guidelines. Informed consent was obtained from all participants. These patients have been not received the treatment of antidiabetic drugs before diagnosis with diabetes. Microvascular and macrovascular diseases were assessed by clinical history and examination including funduscopy through dilated pupils (retinopathy), proteinuria and creatinine (nephropathy), electrocardiogram and heart color ultrasound and peripheral vascular ultrasound (heart and peripheral vascular disease).

The diagnosis of type 2 diabetes was based on diagnostic criteria for diabetes of the World Health Organization (WHO) 1999. These are positive cases from any two of the following tests on different days: symptoms of diabetes mellitus plus casual plasma glucose concentration ≥ 11.1 mmol/L or fasting plasma glucose (FPG) ≥ 7.0 mmol/L or 2 h post-prandial plasma glucose (2h PPG) ≥ 11.1 mmol/L after a 75 g glucose load. All demographic and clinical data were extracted from patients' files. Body mass index (BMI) was calculated as the ratio of the weight in kilograms to the square of the height in meters (kg/m^2).

Clinical and biochemical measurements

Venous blood samples were collected from each subject in the morning after a 12 h fasting. The collection tubes were promptly centrifuged, and serum was separated and stored at -80°C for the measurement of cytokines. Fasting plasma glucose (FPG), total cholesterol (TC), triglycerides (TG), high density lipoprotein (HDL)

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cholesterol, and glycosylated haemoglobin (HbA1c) were measured by using routine clinical laboratory procedures. Insulin was determined by radioimmunoassay Kit (DPC, Los Angeles, CA). The insulin sensitivity was determined by Homeostasis Model Assessment Model (HOMA) index with formula: $HOMA-IR = \text{fasting insulin } (\mu\text{U/ml}) \times \text{fasting glucose (mmol/l)} / 22.5$ [13]. Low HOMA-IR values indicate high insulin sensitivity, whereas high HOMA values indicate low insulin sensitivity (insulin resistance).

PBMC Isolation from blood samples

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized peripheral blood obtained from normal subjects and patients by density gradient centrifugation using Ficoll-Hypaque (density: 1.077; Pharmacia, Dübendorf, Switzerland). Mononuclear cells at the interface were carefully transferred into a Pasteur Pipette, and washed twice in PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4 , 2 mmol/L KH_2PO_4). Cells were suspended at a density of 2×10^6 cells/ml and used for RNA isolation.

ELISA for serum IL-17

Serum IL-17 levels were assayed by ELISA Kit (Human IL-17 immunoassay, eBioscience, San Diego, USA) according to the manufacturer's instructions. The optical density was measured at 450 nm with an automatic ELISA reader. The minimum detection limit was 4 pg/ml for IL-17. To minimize the effect of inter-assay variation, samples from diabetes patients and controls were equally represented on each ELISA plate. All samples were analyzed in duplicates, and the mean of the duplicates was used for the statistical analysis. The intra-assay and inter-assay coefficients of variation for IL-17 were 6.4 and 14.3%, respectively.

Quantitative real time PCR

Total RNA was isolated from cell pellets using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genomic DNA was removed from total RNA using the RNase-free DNase set (Qiagen, Hilden, Germany). The first strand cDNA was synthesized by using the cDNA synthesis kit (Promega, Madison, WI). All reactions were performed on ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). The gene expression levels were analyzed by real time PCR using SYBR Green master mix (Applied Biosystems, Foster City, USA). The PCR conditions comprised an initial holding at 50°C for 2 min, and 95°C for 10 min followed by a two-step PCR program consisting of 95°C for 15 s, and 60°C for 60 s for 40 cycles. For each sample, mRNA expression level was normalized to the level of GAPDH gene. The sequences of primers were showed in the Table 1.

Statistical analysis

Data were expressed as mean \pm standard deviation (SD) or median (interquintile range). Differences between groups were tested with the Student's *t*-test or the Mann-Whitney *U* test for skewed

data. Spearman's correlation coefficient by rank test was calculated to assess the correlations between variables. The values of $P < 0.05$ were considered significant.

Results

Basic clinical characteristics of patients

The clinical and demographic data of patients with type 2 DM in this study were shown in Table 2. Age and sex distributions were similar between patients and control subjects. Among the 30 recruited patients, 16 were men and 14 were women. The average age of the patients was 53.24 ± 7.54 years. Healthy controls were 20 subjects with mean age 49.48 ± 6.54 years, including male 12 and female 8 subjects. The diabetic patients were characterized by markedly higher levels of fasting blood glucose (FBG), glycosylated haemoglobin (HbA1c), C-peptide and C-reactive protein (CRP) compared to control subjects.

To access liver and renal tubular function, we examined some clinical parameters including alanine aminotransferase (ALT), blood

Clinical features	DM	CON
N (M/F)	30 (16/14)	20 (12/8)
Age (years)	53.24 ± 7.54	49.48 ± 6.54
BMI (kg/m ²)	25.36 ± 4.23	24.17 ± 2.9
SBP (mm Hg)	136.57 ± 17.11	128.73 ± 9.2
DBP (mm Hg)	80.29 ± 10.28	78.23 ± 5.84
FBG (mmol/l)	$7.10 \pm 1.23^*$	4.83 ± 0.86
2h FBG (mmol/l)	11.53 ± 1.77	-
HbA1c (%)	$8.91 \pm 1.93^*$	4.92 ± 1.21
TC (mmol/l)	4.49 ± 0.73	4.27 ± 1.26
Triglyceride (mmol/l)	2.12 ± 1.48	1.63 ± 0.78
HDL (mmol/l)	1.22 ± 0.22	1.24 ± 0.61
LDL (mmol/l)	2.63 ± 0.66	2.18 ± 0.94
CRP (mg/l)	$2.92 \pm 0.46^*$	1.26 ± 0.38
C-peptide (ng/ml)	$2.81 \pm 0.97^*$	1.21 ± 0.32
2h C-peptide (ng/ml)	4.80 ± 1.26	-
ALT (IU/l)	24 ± 9.71	21.82 ± 7.36
Cr ($\mu\text{mol/l}$)	62.05 ± 18.54	64.32 ± 12.61
BUN (mmol/l)	5.59 ± 1.45	4.89 ± 1.05
UA ($\mu\text{mol/l}$)	261.33 ± 80.62	248.28 ± 74.23
Ualb (mg/l)	$23.7 (16.85-28.7)$	$14.85 (8.24-21.81)$
Ualb/Ucr (g/mol)	$1.88 (1.26-2.16)$	$1.13 (0.62-1.57)$

DM: Type 2 Diabetic Patients; CON: Healthy Controls; F: Female Subjects; M: Male Subjects; BMI: Body Mass Index; SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure; FBG: Fasting Blood Glucose; 2h FBG: Blood Glucose After Meal Two Hours; HbA1c: Glycosylated Haemoglobin; TC: Total Cholesterol; HDL: High-Density Lipoproteins; LDL: Low-Density Lipoproteins; CRP: C-Reactive Protein; 2h C-peptide: 2h Postprandial C-Peptide; ALT: Alanine Aminotransferase; Cr: Blood Creatinine; BUN: Blood Urea Nitrogen; UA: Blood Uric Acid; Ualb: Urinary Microquantitative Albumin; Ualb/Ucr: Urinary Albumin/Creatinine Ratio. Data are expressed as mean \pm SD or median (interquintile range); Differences between CON and DM: * $P < 0.05$

Table 2: Clinical features of type 2 diabetic patients.

Gene	Acc Num	Forward Primer (5'-3')	Reverse Primer (5'-3')
GAPDH	NM_002046	GAAGGTGAAGGTCCGGAGTC	GAAGATGGTGATGGGATTTTC
IL-17	NM_002190	AATCTCCACCGCAATGAGGA	ACGTTCCCATCAGCGTTGA
RORyt	NM_001001523	ACCGATGTGGACTTCGTTTTG	CGGTGTGCTGCGGAAACT
IL-6	NM_000600	GTGGCTGCAGGACATGACAA	TGAGGTCGCCATCTACATTT
TNF- α	NM_000594	GAGATCAATCGGCCGACTA	CGTTTGGGAAGTTGGATGT
IL-1 β	NM_000576	CTTTGAAGCTGATGGCCCTAAA	AGTGGTGGTCGGAGATTCGT

Table 1: The sequence of primers for real time PCR.

creatinine (Cr), blood urea nitrogen (BUN), blood uric acid (UA), urinary microquantitative albumin (Ualb) and urinary albumin/creatinine ratio (Ualb/Ucr). These clinical parameters reflecting liver and renal function did not exceed the upper limits of normal range in patients with type 2 diabetes (data not shown).

In all of patients, glutamic acid decarboxylase antibody (GAD) and islet cell antibody (ICA) and insulin antibody (IAA) were negative. No patients have apparent diabetic complications including microvascular and macrovascular complications.

Serum levels of IL-17 in patients with type 2 diabetes

To investigate the role of IL-17 in type 2 diabetes, we examined serum concentrations of IL-17 in patients with newly diagnosed type 2 diabetes. As shown in Figure 1, serum IL-17 levels in patients with type 2 diabetes were significantly elevated compared to healthy subjects (10.44 ± 6.47 vs 2.99 ± 1.68 pg/mL, $P < 0.01$).

mRNA expression of IL-17 and ROR γ t in PBMC from diabetic patients

PBMC is a major source of IL-17. To test whether serum IL-17 is secreted by PBMC, we further investigated mRNA levels of IL-17 and its upstream regulator ROR γ t in PBMC from patients with type 2 diabetes. As shown in Figure 2A, mRNA levels of IL-17 were dramatically higher in diabetic patients than in control subjects ($P < 0.001$); and the expression of ROR γ t gene in diabetic patients were also markedly increased compared with control subjects ($P < 0.001$).

Relationship between IL-17 and IL-6 or TNF- α or IL-1 β mRNA expression levels in PBMC from diabetic patients

To clarify the relationship between IL-17 and other inflammation cytokines on mRNA levels, we first examined mRNA expression of inflammation cytokines such as IL-6, TNF- α and IL-1 β in PBMC from diabetic patients using real time PCR. Our results showed mRNA levels of TNF- α was significantly upregulated in patients with type 2 DM (Figure 2B). Although mRNA levels of IL-6 and IL-1 β were increased, but no statistical difference was observed between control and type 2 DM group. We further analyzed the correlation between mRNA expression of IL-17 and inflammation cytokines including IL-6, TNF- α and IL-1 β . No significant relationship between the expression of IL-17 gene and that of IL-6 or IL-1 β gene was observed in PBMC from diabetic patients (Figure 3A and Figure 3C). However, there was significant correlation between IL-17 gene expression and TNF- α gene expression in PBMC

from diabetic patients ($r = 0.6988$, $P < 0.001$) (Figure 3B).

Discussion

This study was designed to investigate IL-17 levels in patients with newly diagnosed type 2 diabetes. There are several notable findings in the present study. First, we found that serum levels of IL-17 in newly diagnosed patients with type 2 DM were significantly increased compared to healthy subjects. Second, we demonstrated that mRNA expression of IL-17 and its transcription regulator ROR γ t were markedly upregulated in PBMC from diabetic patients. Final, our data showed that IL-17 gene expression correlated with TNF- α gene expression in PBMC from diabetic patients. These results suggested that IL-17 might participate in the inflammatory process of type 2 diabetes and have a crucial role in the pathogenesis of type 2 DM.

Chronic low-grade inflammation is closely involved in the pathogenesis of type 2 diabetes. Elevated concentrations of various inflammatory markers such as tumor necrosis factor- α (TNF- α) and interleukin (IL)-6 and IL-1 β have been reported in patients with type 2 diabetes [14-16]. There is evidence that inflammatory mediators not only represent markers of metabolic aberrations [17,18] but may also contribute to insulin resistance and β -cell dysfunction [19]. TNF- α as well as IL-6 are considered the major adipocyte cytokines [20]. Both can alter insulin sensitivity by triggering different key steps in the insulin signaling pathway [21-23]. IL-1 β negatively affects β -cell function and plays a role in the pathogenesis of progressive β -cell failure in type 2 diabetes [24]. Our results demonstrated that mRNA expression of TNF- α , IL-1 β and IL-6 were increased at different levels in PBMC from patients with newly diagnosed type 2 diabetes. Moreover, we found that IL-17 gene expression correlated with TNF- α gene expression in PBMC from diabetic patients. These results suggested IL-17 might participate in the pathogenesis of type 2 DM together with other inflammation cytokines.

Here we demonstrated that the expression of IL-17 is increased in patients with newly diagnosed type 2 DM. The exact role of IL-17 in the pathogenesis type 2 DM has not been explored. IL-17 is capable of inducing the expression of pro-inflammatory cytokines such as TNF- α , IL-1 β , IL-6, chemokines and adhesion molecules, which mediate tissue infiltration and tissue destruction. In addition to stimulating the secretion of other pro-inflammatory cytokines, IL-17 is involved in the induction of potentially harmful mediators of inflammation, such as free radical nitric oxide [25]. Recent studies have described an ongoing process of β cell destruction by apoptosis in animal models of type 2 diabetes and in human type 2 DM [26,27]. In human islets, IL-1 β has been shown to impair insulin release and to promote the apoptosis of β cell [28]. Several studies have pointed to the evidence that some cytokines such as TNF- α are involved in the apoptosis of β cell in synergy with IL-1 β [29]. Further, Miljkovic D reported IL-17 could augment the expression of inducible nitric oxide synthase (iNOS) and cause NO-dependent toxicity in MIN6 cells and mouse pancreatic islets [11]. Therefore, it is possible that IL-17 might participate in the local inflammation and lead to the destruction of β cell in the pancreas in synergy with these inflammation cytokines.

In the study, our data indicated that IL-17 may be involved in the inflammation process of type 2 diabetes. But whether IL-17 is involved in the progression of type 2 diabetes is not conclusively clarified. Several issues are still unsolved. Recent study reported that the plasma level of IL-17 is increased in obese women [30]. Obesity is considered the main environmental cause for the development of insulin resistance and type 2 DM. Obesity is defined as BMI > 25 kg/m 2 in Asian. In our

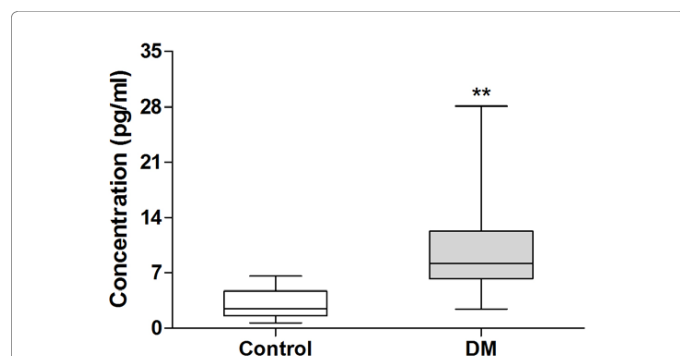


Figure 1: Serum IL-17 levels in patients with type 2 diabetes. Serum were collected and measured for the concentration of the indicated cytokines using ELISA kits. DM: type 2 diabetic patients; Control: Healthy controls. Asterisks represent statistical differences between groups; $**P < 0.01$.

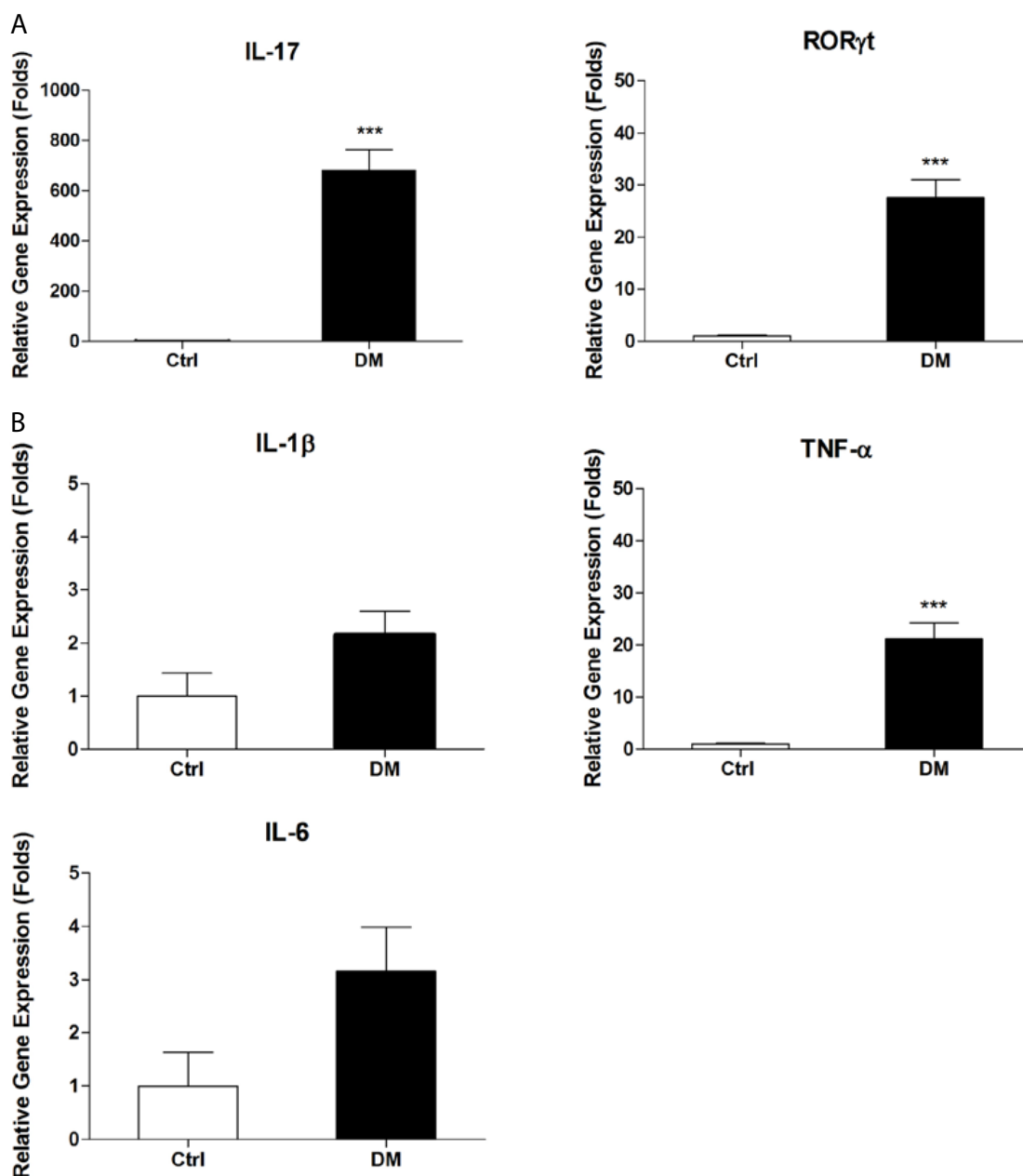


Figure 2: Gene expression in peripheral blood mononuclear cells from patients with type 2 DM. PBMC were separated from patients with type 2 diabetes and healthy controls and subjected to real time PCR analysis for mRNA expression of IL-17 and its transcript factor ROR γ t, IL-6, TNF- α and IL-1 β . Data are presented as relative expression of genes in reference to GAPDH. DM: type 2 diabetic patients; Control: Healthy controls. Data are representative of three experiments. Asterisks represent statistical differences between groups; *** P <0.001.

experiment, the average level of BMI in all patients is about 25.42 kg/m²; and BMI levels were similar between diabetic patients and health subjects. As a consequence of narrow selection criteria, the subdivision of the patients' groups was hardly enough to make clear estimations. Further study should be performed in a large number of diabetic patients including diabetic patients with low-level BMI (BMI<25) and diabetic patients with high-level BMI (BMI \geq 25) in order to investigate the relationship between IL-17 and clinical parameters. Moreover, further studies is required to investigate the circulating levels of IL-17 in groups with impaired glucose tolerance (IGT) and diabetic patients with complications. Serum IL-17 level might be useful to predict an

early detection of risk for type 2 diabetes, as well as being potential marker of established diabetic complications. On the other hand, we need to further explore the detailed mechanism of IL-17 involved in the pathogenesis of type 2 diabetes.

In conclusion, we demonstrated that serum levels and mRNA levels of IL-17 are increased in patients with newly diagnosed type 2 DM. Our results suggested IL-17 might promote the inflammatory state of patients, and participate in the pathogenesis of type 2 DM. Further studies are necessary to clarify the crucial role of IL-17 in the pathogenesis of type 2 DM and whether IL-17 is a prognostic factor for the development of type 2 DM.

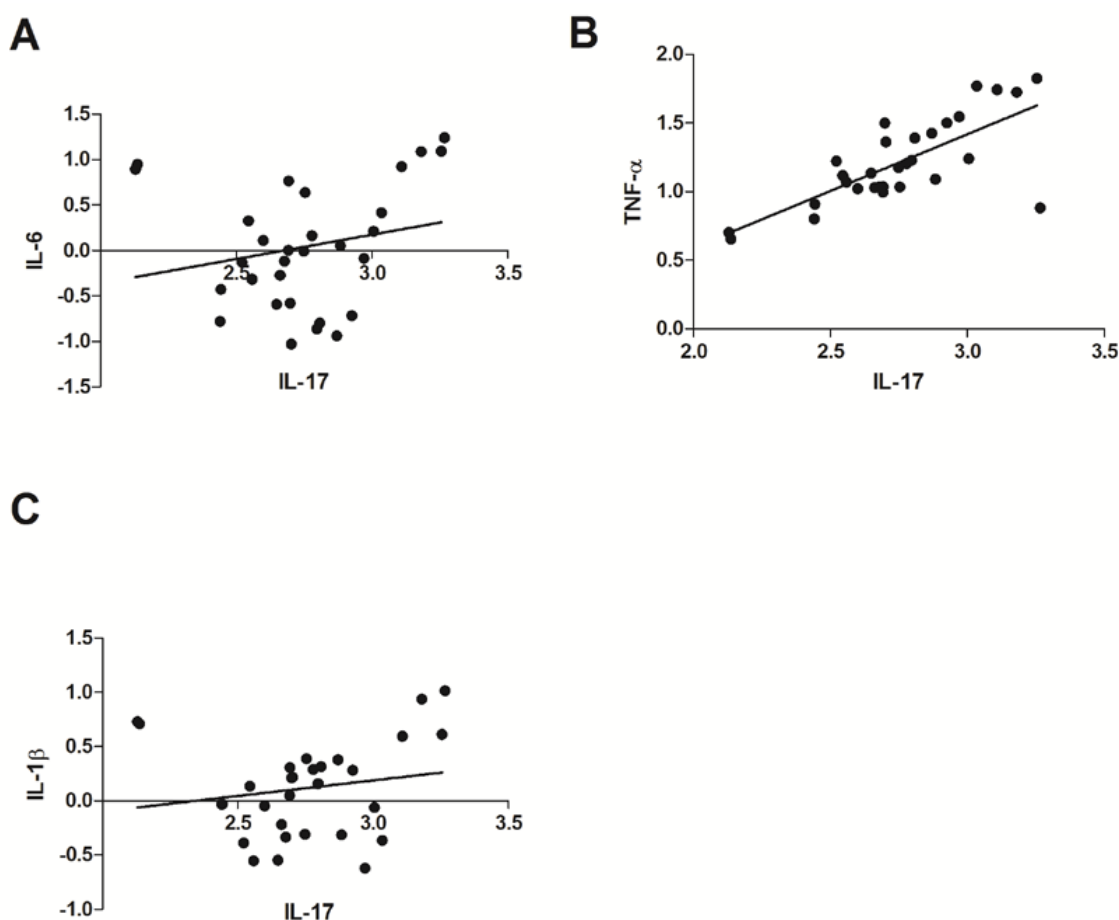


Figure 3: Relationships between mRNA expression of IL-17 and inflammation cytokines in peripheral blood mononuclear cells from patients with type 2 DM. A: Relationship between expression levels of IL-17 and IL-6 in PBMC from diabetic patients ($r=0.2529$, $P=0.1775$); B: Relationship between expression levels of IL-17 and TNF- α in PBMC from diabetic patients ($r=0.6988$, $P<0.001$); C: Relationship between expression levels of IL-17 and IL-1 β in PBMC from diabetic patients ($r=0.2516$, $P=0.1798$). r =correlation coefficient; The P values were calculated by Spearman's correlation coefficient by rank test.

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