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The effect of short-term intensive insulin therapy on circulating T cell subpopulations in patients with newly diagnosed type 2 diabetes mellitus

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ABSTRACT

Aims: To evaluate the effect of short-term intensive insulin therapy on circulating T cell subpopulations in patients with newly diagnosed type 2 diabetes mellitus (T2DM).

Methods: A total of 113 patients with T2DM and 28 normal subjects were enrolled. Demographic parameters and biochemical markers were collected at baseline, and flow cytometry was applied to determine the proportion of T cell subpopulations in participants. Then the patients underwent continuous subcutaneous insulin injection (CSII) treatment with euglycemia for 2 weeks, and the T cell subpopulations were measured again after CSII treatment.

Results: Compared with normal subjects, the proportion of Th1 cells and the ratio of Th1/Th2 increased, the proportion of Treg cells decreased in patients with T2DM ($p < 0.05$ for all). The ratio of Th1/Th2 was positively correlated with glycosylated hemoglobin A1c (HbA1c) and negatively correlated with high density lipoprotein cholesterol (HDL-C). Furthermore, there were negative associations between the proportion of Treg cells and fasting plasma glucose, HbA1c, triglyceride, low density lipoprotein cholesterol, and positive association between the proportion of Treg cells and HDL-C. After CSII treatment, the proportion of Th1 cells and the ratio of Th1/Th2 decreased ($p < 0.05$ for both), the proportion of Treg cells increased in patients with T2DM ($p < 0.05$).

Conclusions: Short-term intensive insulin therapy could modulate circulating T cell subpopulations in patients with T2DM, which might alleviate inflammatory responses caused by hyperglycemia.

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1. Introduction

Type 2 diabetes mellitus (T2DM) was described as a chronic low-grade inflammatory disease, characterized by increased circulating inflammatory cytokines and acute phase proteins that could contribute to systemic metabolic dysfunctions [1]. It was reported that pro-inflammatory cytokines, such as tumor necrosis factor-β (TNF-β), interleukin-2 (IL-2), and interferon-γ (IFN-γ), could activate intracellular transduction cascades that interfere with insulin signaling, and anti-inflammatory cytokines, such as interleukin-4 (IL-4) and interleukin-10 (IL-10), could increase insulin sensitivity [2–4]. Early studies on inflammatory regulation of diabetes focused on innate immunity, macrophages were the major inflammatory cell type in the glucose-utilizing tissues, such as liver and adipose tissue [5,6]. Recently, growing emphasis was placed on adaptive immune. It was believed that T cell subpopulations played a crucial role in the development of T2DM and its related complications [7].

Early short-term intensive insulin therapy was an effective strategy for patients with T2DM, which could improve glycemic control, preserve β cell function, and even prevent diabetic complications [8]. Despite these promising results, the data from published research was insufficient to reflect the changes of pathophysiology in patients with this therapy, particularly for the immune system. Our previous study found that insulin treatment had an anti-inflammatory effect in patients with newly diagnosed T2DM, which was independent of the reduction in blood glucose [9].

In order to elucidate the effects of insulin treatment on immune response, we evaluated the difference of circulating T cell subpopulations between patients with T2DM and normal subjects, and the effect of short-term intensive insulin therapy on circulating T cell subpopulations in patients with T2DM.

2. Patients and methods

2.1. Subjects

Patients with newly diagnosed T2DM were recruited in this study from the department of endocrinology and metabolism in Nanjing First Hospital between February 2016 and December 2017. T2DM was diagnosed in accordance with the WHO diagnostic criteria (1999) [10], and all patients never took anti-hyperglycemic therapy. In order to evaluate the effect of short-term intensive insulin therapy on circulating T cell subpopulations in patients with different blood glucose levels, we expanded the inclusion criteria of HbA1c. In the premise of ensuring patient safety, we included patients with hemoglobin A1c (HbA1c) > 7%. The key exclusion criteria were severe hepatic or renal dysfunction, acute diabetic complications, infections requiring antibiotic treatment, autoimmune disease, recent trauma or surgery, and taken systemic corticosteroids or any other drugs which might influence immune system in the last 3 months. Moreover, 28 age-matched subjects with normal blood glucose, lacked family history of T2DM as well as no clinical diagnosed diseases were recruited as control. The protocol and informed consent document were approved by the ethics committee of Nanjing First Hospital, and all participants gave written informed consent.

2.2. Study design

All the enrolled patients were admitted to hospital. During the admission assessment of patients and the first visit of control participants, height, weight, and blood pressure were measured by trained and certified nurse using standard protocols and techniques. Body mass index (BMI) was calculated as weight divided by the square of height (kg/m^2).

During the study period, all patients were given diet and exercise instructions, and received continuous subcutaneous insulin injection (CSII) of insulin aspart (Novo Nordisk, Bagsvaerd, Denmark) via Medtronic insulin pumps (Northridge, CA). Initial insulin doses were calculated as 0.4–0.5 IU/kg and were equally administered as basal and bolus injection. Insulin doses were subsequently titrated by the treating physician according to blood glucose values obtained by self-monitoring. The capillary blood glucose values were monitored at 7 time-points: 07:00, 09:00, 11:00, 13:00, 17:00, 19:00, and 22:00, respectively. The glycemic target were defined as fasting blood glucose levels of less than 6.1 mmol/L and blood glucose at 2 h after each of three meals of less than 8.0 mmol/L [8], and the rate of glycemic target achievement should ≥75%. After the glycemic target was achieved, CSII treatment was maintained for 2 weeks. In addition, patients with a history of hypertension or dyslipidemia were told to continue taking their drugs.

2.3. Laboratory analyses

After an overnight fast, fasting blood samples were collected for the measurement of fasting plasma glucose (FPG), glycosylated serum protein (GSP), HbA1c and lipid profiles in all participants before treatment. Fasting blood samples were also collected for the measurement of these biochemical markers in all T2DM patients at the end of treatment (24-hour after insulin cessation). The blood glucose level was assessed by glucose oxidase method using an automatic biochemistry analyzer (HITACHI-7180, Tokyo, Japan), fasting and postprandial capillary blood glucose was monitored by blood glucose meter (OneTouch® UltraVue™, Johnson, USA), HbA1c was measured by high-pressure liquid chromatography (BIO-RAD D-10™, California, USA), and lipid profiles were assessed by enzymatic colorimetric assay using an automatic biochemistry analyzer (HITACHI-7180, Tokyo, Japan). All these tests were done in the clinical laboratory of Nanjing First Hospital.

2.4. Flow cytometry analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by Ficoll-Hypaque density gradient separation. Immuno-phenotype of the PBMCs was performed by a BD FACSCcanto™ II flow cytometer (BD Biosciences, USA)

with BD FACSDiva™ software v6.1.2 (BD Biosciences, USA) for data evaluation. PBMCs were washed and stained, then four color flow cytometry analysis was performed. $CD8^+$ T cells were stained with anti-CD3-APC and anti-CD8-PE-Cy7 antibodies, Th1 cells were stained with anti-CD4-PerCP-Cy5-5 and anti-IFN-γ-FITC antibodies, Th2 cells were stained with anti-CD4-PerCP-Cy5-5 and anti-IL-4-PE antibodies, and Treg cells were stained with anti-CD4-FITC, anti-CD25-PE and anti-FOXP3-APC antibodies. All the antibodies used in Flow cytometry analysis were purchased from BD Biosciences (San Diego, CA, USA).

2.5. Statistical analyses

All analyses were conducted using SPSS 22.0. All variables were tested for normal distribution of the data. Data are presented as means ± standard error (SE), or as median and interquartile range (IQR). Differences between the groups examined using the student's unpaired *t*-test or Mann-Whitney *U* test. Spearman analysis in non-parametric variables were performed to identify the clinical and metabolic parameter that correlate with the T cell subpopulations. All comparisons were 2-sided at the 5% significance level. P value < 0.05 was considered to be statistically significant.

3. Results

3.1. Baseline characteristics

The study population consisted of 113 patients with T2DM and 28 normal subjects (see Fig. 1). There were no significant differences in age, gender, diastolic blood pressure (DBP), total cholesterol (TC) between the T2DM and control groups ($p > 0.05$ for all). However, compared with control group, the levels of BMI, systolic blood pressure (SBP), FPG, HbA1c, triglyceride (TG), and low density lipoprotein cholesterol (LDL-C) increased, and the level of HDL-C decreased in patients with T2DM (all $p < 0.05$) (Table 1).

3.2. The distribution of T cell subpopulations in patients with T2DM

The proportions of T cell subpopulations in the peripheral blood were evaluated by flow cytometry. And compared with control group, the proportion of Th1 cells and the ratio of Th1/Th2 increased, the proportion of Treg cells decreased in patients with T2DM ($p < 0.05$ for all) (Fig. 2).

3.3. The relationship between T cell subpopulations and clinical parameter

Spearman's rank correlation analysis revealed a positive correlation between the ratio of Th1/Th2 and HbA1c ($r = 0.221$, $p = 0.008$), and a negative correlation between the ratio of Th1/Th2 and HDL-C ($r = -0.202$, $p = 0.016$). Furthermore, negative associations between the proportion of Treg cells and FPG ($r = -0.370$, $p < 0.001$), HbA1c ($r = -0.395$, $p < 0.001$), TG ($r = -0.452$, $p < 0.001$), LDL-C ($r = -0.381$, $p < 0.001$), and positive association between the proportion of Treg cells and HDL-C ($r = 0.189$, $p = 0.023$) were observed (Fig. 3).

3.4. Regression analysis of associations between T cell subpopulations and clinical parameter

To confirm the factors which influenced T cell subpopulations, multiple stepwise regression analysis was applied to the data from all subjects. With the ratio of Th1/Th2 and the proportion of Treg cells as dependent variable, age,

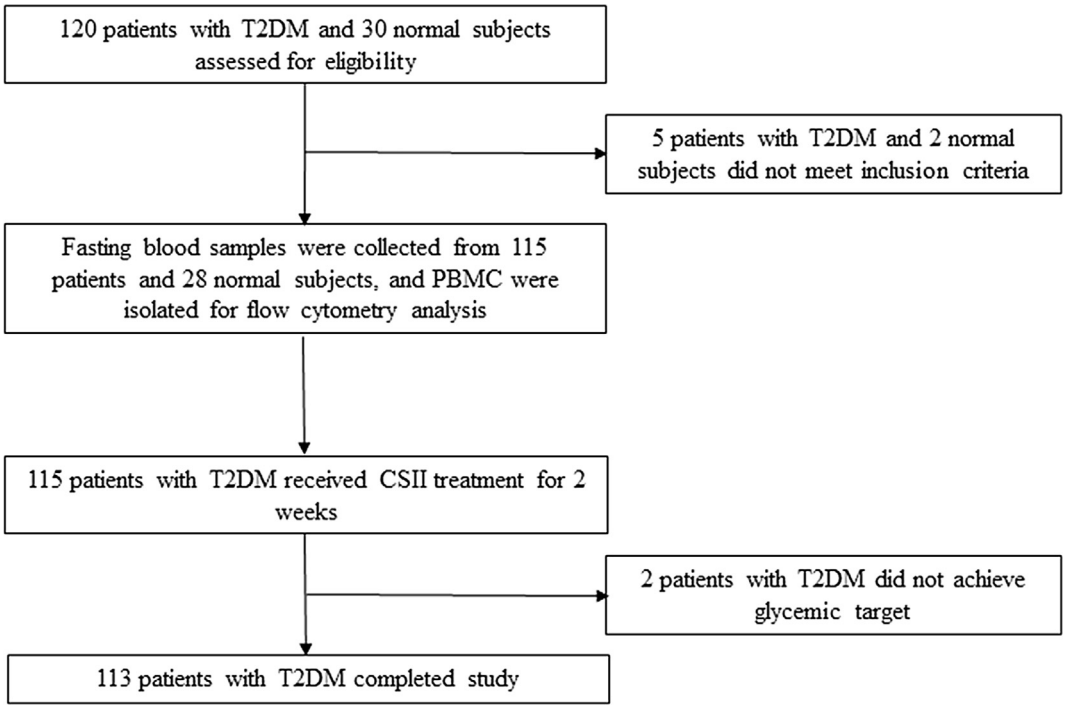

Fig. 1 – Trial profile.

Table 1 – Clinical characteristics of patients with T2DM and normal subjects.

	T2DM (n = 113)	Normal (n = 28)
Age, years	60.2 ± 12.4	56.2 ± 10.0
Gender, male/female	68/45	15/13
BMI, kg/m^2	24.71 ± 4.25**	22.93 ± 2.18
SBP, mmHg	130.00 (120.00, 140.00)**	120.00 (110.00, 125.75)
DBP, mmHg	80.00 (72.00, 80.00)	80.00 (70.00, 88.75)
FPG, mmol/L	7.70 (6.20, 9.90)**	5.09 (4.69, 5.35)
HbA1c, %	9.30 (8.30, 10.0)**	5.00 (4.40, 5.40)
TC, mmol/L	4.15 (2.88, 5.01)	4.47 (3.87, 4.96)
TG, mmol/L	2.38 (1.47, 3.60)**	1.19 (0.99, 1.28)
LDL-C, mmol/L	2.54 (1.93, 2.99)	2.22 (1.75, 2.60)
HDL-C, mmol/L	1.32 (1.11, 1.70)**	1.36 (1.21, 1.52)
CD3$^+$CD8$^+$ T cells, %	32.96 (25.00, 40.65)	34.50 (27.47, 40.25)
Th1 cells, %	20.70 (16.20, 28.15)**	16.30 (14.22, 21.55)
Th2 cells, %	1.20 (0.90, 1.60)	1.40 (0.92, 1.70)
Th1/Th2 ratio	17.81 (13.30, 23.02)**	13.47 (9.97, 16.26)
Treg cells, %	0.80 (0.50, 1.10)**	2.25 (1.52, 3.00)
Medication for dyslipidemia, %	20.35 (23/90)	–
Medication for hypertension, %	28.31 (32/81)	–

Abbreviation: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting plasma glucose; HbA1c, hemoglobin A1c; TC, total cholesterol; TG, triglyceride; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; Th1 cells, type 1 T help cells; Th2, type 2 T help cells. *, $p < 0.05$ vs. control participants; **$p < 0.01$ vs. control participants.

gender, BMI, SBP, DBP, FPG, HbA1c, TC, TG, LDL-C and HDL-C as independent variable, multiple stepwise regression analysis revealed a significant positive association between the ratio of Th1/Th2 and HbA1c ($p = 0.018$), and a negative association between the proportion of Treg cells and TG ($p = 0.043$) (Table 2).

3.5. *The changes of T cells subpopulations in T2DM after CSII treatment*

After 2 weeks of CSII treatment, a significant reduction of FPG and GSP were observed in patients with T2DM ($p < 0.05$ for all). Moreover, CSII treatment could decrease the proportion of Th1 cells and the ratio of Th1/Th2 ($p < 0.05$ for all), increase the proportion of Treg cells in patients with T2DM ($p < 0.05$) (Table 3).

3.6. *Comparison of T cells subpopulations between groups with different HbA1c levels*

According to HbA1c levels, all diabetic patients were divided into two groups: HbA1c < 9% and HbA1c ≥ 9%. An increased trend in Th1 cells were found in patients with HbA1c ≥ 9% compared with HbA1c < 9% ($p < 0.05$). After CSII treatment, the proportion of Treg cells increased in patients with HbA1c < 9% ($p < 0.05$). While in patients with HbA1c ≥ 9%, aside from the proportion of Treg cells increased, the proportion of Th1 cells significantly decreased ($p < 0.05$ for all) (Table 4).

4. Discussion

In the present study, we found an imbalance of Th1, Th2 and Treg cells in patients with T2DM compared with health subjects, which were associated with glucose-lipid metabolism disorder. For the first time we found that after 2 weeks of CSII treatment, the imbalance of Th1, Th2 and Treg cells could be rebuilt with the improvement of glycemic control in patients with newly diagnosed T2DM.

CD4$^+$ T cells could be divided into pro-inflammatory Th1, Th17, and anti-inflammatory Th2 and Treg subtypes based on their functionality and cytokine production [11]. Once activated, Th1 and Th2 cells showed many inflammatory features, such as releasing large amount of cytokines. Specifically, Th1 cells produce IFN-γ and TNF-β, trigger cell mediated immunity, which promote inflammatory process and insulin resistance in metabolic organs, such as adipose tissue, liver, and muscle [12–14]. In contrast, Th2 cells produce IL-4 and IL-13, which could attenuate adipose tissue inflammation, maintain adipose tissue metabolic functions [15]. Treg cells are involved in preventing excessive inflammatory responses and limiting tissue impairment by controlling immune cell activation and producing anti-inflammatory cytokines, such as IL-10 and transforming growth factor-β (TGF-β) [16]. It was reported that Th1 cells increased in adipose tissue and peripheral blood of patients with T2DM, and were correlated with insulin resistance [17]. While the amount of Treg cells decreased in patients with T2DM [18].

After 2 weeks of CSII treatment, we found that a significant improvement of glycemic control could be achieved in patients with newly diagnosed T2DM. Following with the improvement of glycemic control, the proportion of Th1 cells and the ratio of Th1/Th2 decreased, the proportion of Treg cells increased in patients with T2DM. These results showed that short-term intensive insulin therapy could improve glycemic control and modulate immune system imbalance. After stratified by HbA1c, only the proportion of Treg cells increased with statistical significance in patients with low HbA1c; while in patients with high HbA1c, the proportion of Th1 cells decreased, and the proportion of Treg cells increased

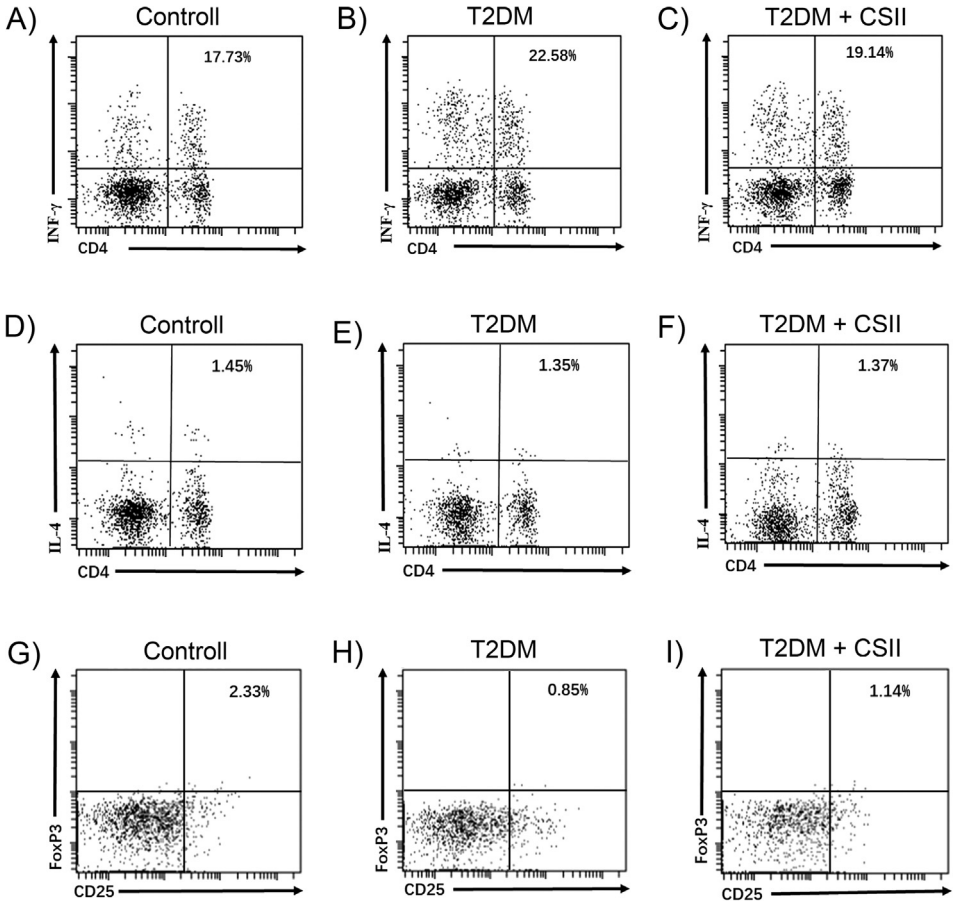

Fig. 2 – Representative FACS dot-plots used for the quantification of T cell subpopulations. PBMCs were stained with anti - CD4 – PerCP - Cy5 - 5, anti - CD25 - PE, anti - FOXP3 - APC, anti - IL - 4 - PE and anti - INF - γ - FITC. Th1 cells were defined as $CD4^+$ IFN - γ^+ cells in control subjects (A), patients with T2DM (B), and patients with T2DM after CSII treatment (C), Th2 cells were defined as $CD4^+$ IL - 4^+ cells in control subjects (D), patients with T2DM (E), and patients with T2DM after CSII treatment (F), and Tregs cells were defined as $CD4^+$ $CD25^+$ $FOXP3^+$ cells in control subjects (G), patients with T2DM (H), and patients with T2DM after CSII treatment (I).

after CSII treatment. Treg cells could inhibit the inflammatory response by various pathways, such as surpassing cytokine secretion, modulating the microenvironment and changing the expression of surface receptors [19]. In diet-induced obesity and T2DM rodent models, induction of Treg cells could significantly mitigated inflammation and autoimmune reactions, improve insulin sensitivity, lower blood glucose, ameliorate metabolic and cytokine abnormalities, and reduce end-organ complications [20,21]. Our research indicated that there were immunomodulatory effects with short-term intensive insulin therapy, which might bring more benefits, such as attenuated tissue impairment.

The mechanisms underlying the immunomodulatory effects of short-term intensive insulin therapy remained unclear. It was reported that hyperglycemia activate inflammatory pathways and induce intracellular oxidative stress, intensive insulin therapy could reverse hyperglycemia more quickly, suppress the production of reactive oxygen species (ROS) [22,23]. Intensive insulin therapy also increase the release of anti-inflammatory cytokines and upregulate mRNA expression of anti-inflammatory signal transcription factors [24,25]. Moreover, insulin resistance and dyslipidemia promote the release of inflammatory cytokines, which was related to the inflammatory responses in patients with diabetes, and intensive insulin therapy was associated with the improvement of dyslipidemia and insulin resistance [26–28]. Additionally, in our previous study, insulin treatment in newly diagnosed type 2 diabetes was associated with a significantly decreased of inflammatory markers, such as high-sensitivity C-reactive protein (hs-CRP) and interleukin-6 (IL-6), indicated that insulin treatment also had anti-inflammatory effects [9].

In conclusion, immune system imbalance were related to glucose-lipid metabolism disorder in patients with T2DM. Short-term intensive insulin therapy rebuilt the balance of immune cells in patients with T2DM. However, the mechanisms of short-term intensive insulin therapy in immunomodulatory should be further evaluated, and large scale, randomized prospective studies will be warrant to confirm the influence of short-term intensive insulin therapy on T cells in patients with T2DM.

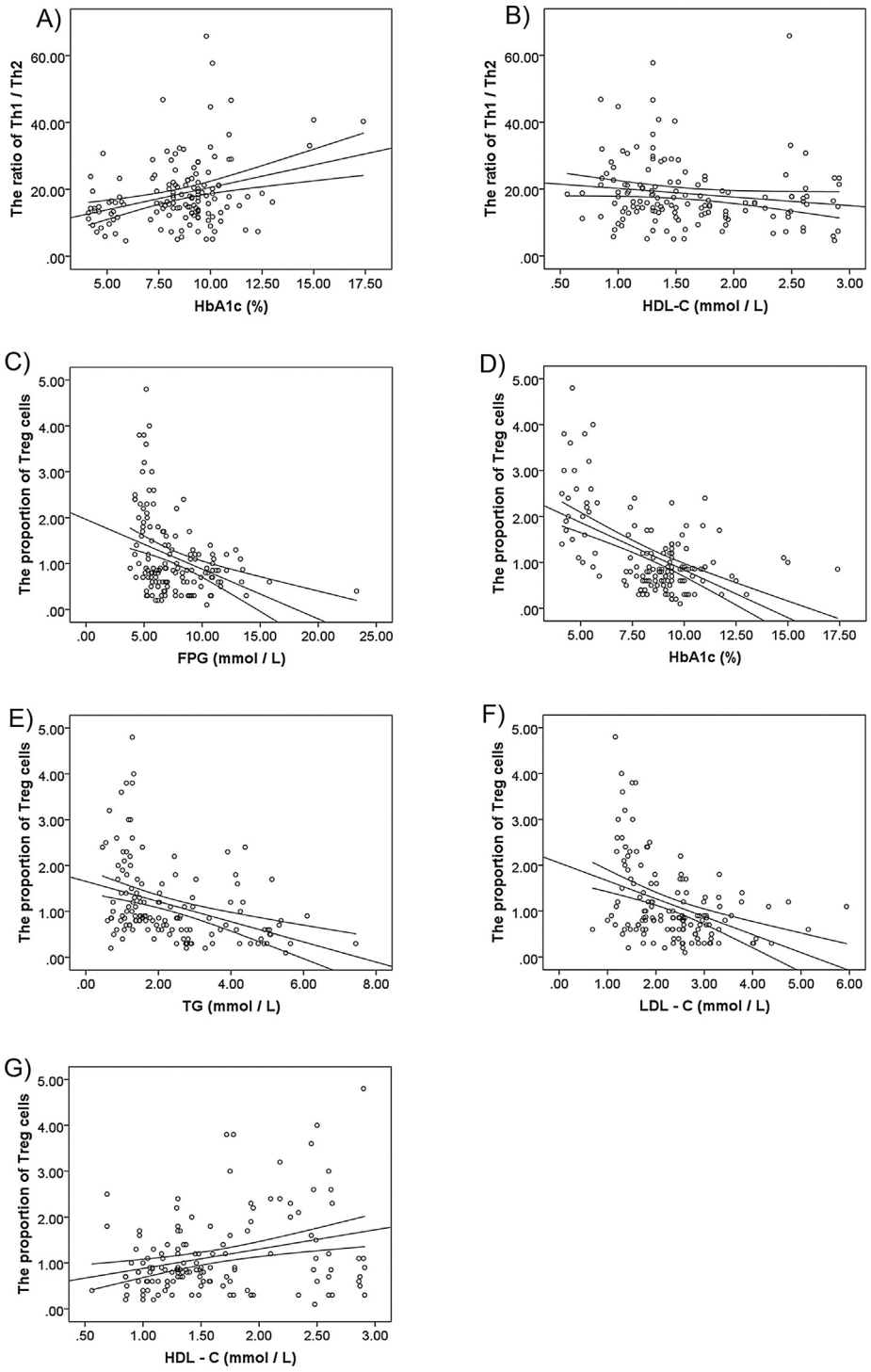

Fig. 3 – Linear correlation between different T cell subpopulations and clinical parameter. The relationship between the ratio of Th1 / Th2 and HbA1c (A), HDL - C (B). The relationship between the proportion of Treg cells and FPG (C), HbA1c (D), TG (E), LDL - C (G), HDL - C (H).

Table 2 – Multiple regression analysis between T cell subtypes and correlative variables.

Dependent variable	Independent variable	B	SE	β	t	p	95%CI for B
Th1/Th2 ratio	HbA1c	1.459	0.608	0.222	2.401	0.018	0.255–2.663
Treg cells	TG	−0.063	0.031	−0.191	−2.045	0.043	−0.123 to −0.002

Abbreviation: △, the difference between before and after CSII treatment; GSP, glycosylated serum protein.

Table 3 – Clinical characteristics of patients with T2DM before and after CSII treatment.

	Baseline	After CSII treatment
FPG, mmol/L	7.70 (6.20, 9.90)	6.17 ± 1.00**
GSP, μmol/L	353.04 (295.38, 400.31)	338.04 (300.00, 372.17)**
CD3 + CD8 + T cells	32.96 (25.00, 40.65)	31.20 (30.74, 32.90)**
Th1 cells, %	20.70 (16.20, 28.15)	18.10 (16.80, 21.50)**
Th2 cells, %	1.20 (0.90, 1.60)	1.39 (1.00, 1.50)
Th1/Th2 ratio	17.81 (13.30, 23.02)	14.75 (11.93, 20.66)*
Treg cells, %	0.80 (0.50, 1.10)	1.10 (1.00, 1.20)**

Abbreviation: FPG, fasting plasma glucose; GSP, glycosylated serum protein; Th1 cells, type 1 T help cells; Th2, type 2 T help cells. *$p < 0.05$ vs. baseline; **$p < 0.01$ vs. baseline.

Table 4 – The T cell subpopulations between groups with different HbA1c levels.

	HbA1c < 9%		HbA1c ≥ 9%	
	Baseline	After CSII treatment	Baseline	After CSII treatment
FPG, mmol/L	6.95 (6.20, 8.95)	6.00 (5.40, 6.20)**	7.90 (5.90, 10.75)	6.20 (5.45, 7.15)**
GSP, μmol/L	316.96 (289.92, 356.72)	324.87 (387.33, 353.62)	366.46 (303.57, 427.38)#	345.95 (317.89, 376.13)**
$CD3^+$ $CD8^+$ T cells, %	34.65 (26.55, 44.60)	31.41 (30.82, 35.98)	30.70 (24.70, 39.65)	31.08 (30.40, 31.67)
Th1 cells, %	19.25 (14.55, 25.67)	18.57 (16.82, 22.00)	22.50 (18.50, 22.95)#	17.88 (16.77, 21.50)**
Th2 cells, %	1.00 (0.80, 1.35)	1.38 (1.00, 1.50)	1.35 (1.00, 1.75)	1.40 (1.05, 1.50)
Th1/Th2 ratio	17.84 (14.40, 22.77)	15.90 (12.20, 20.44)	17.81 (13.03, 23.96)	14.17 (11.66, 23.12)
Treg cells, %	0.80 (0.52, 1.05)	1.06 (0.99, 1.17)**	0.85 (0.50, 1.10)	1.10 (1.00, 1.25)**

Abbreviation: FPG, fasting plasma glucose; GSP, glycosylated serum protein; Th1 cells, type 1 T help cells; Th2, type 2 T help cells. *, $p < 0.05$ vs. baseline; **$p < 0.01$ vs. baseline; #$p < 0.05$ vs. HbA1c < 9% group.

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Disclosures: The authors declare that they have no conflict of interest.

Compliance with ethics guidelines: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Data availability: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Thanking participants: We thank the participants for their involvement in the study.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diabres.2019.02.007.

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