The frequency of Tim-3 on circulating Tfh cells was increased in type 2 diabetes mellitus patients

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Abstract
Type 2 diabetes mellitus (T2DM) is a chronic, low-grade inflammation disease. T follicular helper (Tfh) cells and T cell immunoglobulin and mucin domain 3 (Tim-3) are implicated in many immune diseases. This study aims to explore whether Tim-3 expression on Tfh cells is associated with T2DM progression. White blood cells (WBCs) were harvested from 30 patients with T2DM and 20 healthy donors. The abundance of circulating Tfh cells (cTfh) and the frequency of Tim-3 were analyzed by flow cytometry. Levels of fasting plasma glucose (FPG), insulin, hemoglobin A1C (HbA1C), and fasting plasma C-peptide were measured. Body mass index (BMI) and diabetes duration were also recorded. Patients with T2DM had higher numbers of cTfh cells. In addition, cTfh cells showed a negative correlation with HbA1C and diabetes duration, a positive correlation with fasting plasma C-peptide. The frequency of Tim-3 on cTfh cells was higher among T2DM patients compared with healthy donors. The in vitro experiment showed that high glucose levels increased the abundance cTfh cells but had no effect on Tim-3 expression. Our results suggest that cTfh cells and associated Tim-3 frequency may contribute to the progression of T2DM, and high glucose levels may influence cTfh cells directly.

Keywords
T2DM, Tfh, Tim-3

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Introduction

There are around 425 million adults with diabetes worldwide, accounting for about 5.6% of the global population.¹ Of those, 90% is have type 2 diabetes mellitus (T2DM).² T2DM is characterized by “relative insulin deficiency,” with two key factors, insulin resistance and β cell dysfunction.³ Recent research has focused on the immunological mechanisms underlying T2DM. It is widely believed that chronic low-grade inflammation is related to insulin resistance in T2DM.⁴ However, most previous research has focused on the role of adipose cells,⁵,⁶ macrophages,⁷ regulatory T cells (Treg),⁸ natural killer cells (NK),⁹ Th cells (Th1, Th2, Th17),¹⁰ and corresponding pro-inflammatory factors such as TNF-α,¹¹ IL-1β,¹² IL-6,¹³ and IFN-γ.¹⁴ Importantly, the function of T follicular helper cells (Tfh) and their potential role in T2DM remains unexplored.

Tfh cells are a subset of T cells that promote B cell maturity and are identified by surface expression of CD4⁺ and CXCR5high.¹⁵ High expression of transcription factor B cell lymphoma 6 (BCL-6), interleukin-21 (IL-21) and the IL-6 receptor (IL-6R) are also observed on Tfh cells.¹⁶ Due to the relationship between Tfh cells and humoral immunity, there is a known link between increased abundance of Tfh cells and many autoimmune diseases, including type 1 diabetes mellitus (T1DM),¹⁷ myasthenia gravis (MG),¹⁸ systemic lupus erythematosus (SLE),¹⁹ and Graves’ disease.²⁰ However, the study of Tfh cells in T2DM is rare, and few studies have reported high numbers of Tfh cells patients with T2DM²¹ and diabetic nephropathy (DN),²² which is a major complication of diabetes. Accordingly, more in-depth and detailed research is needed to explore the correlation between Tfh cells and T2DM.

Tim-3 (T cell immunoglobulin and mucin domain 3) was initially identified as an immunosuppressive molecule,²³ and later found to be expressed in dendritic cells,²⁴ NK cells,²⁵ macrophages/monocytes,²⁶ activated T helper 17 cells²⁷ and cytotoxic T cells (CTL).²⁸ It has been reported that Tim-3 mediates immune tolerance in anti-tumor immunity,²⁹ and the anti-tumor efficacy of Tim-3 inhibition has been demonstrated in mice.³⁰ Recent research showed a potential link between Tim-3 and T2DM characterized by elevation of peripheral blood galectin-9 (the ligand of Tim-3) in patients with T2DM.³¹ Tim-3 upregulation has also been observed in renal tissue in DN.³² However, further study of the correlation between Tim-3 expression on Tfh cells and T2DM is required.

We analyzed the abundance of cTfh cells and the frequency of Tim-3 on cTfh cells in patients with T2DM and healthy donors, and further explored the potential relationship with T2DM pathology. To further validate the authenticity of our results, we designed an in vitro experiment with high and low glucose stimulation of lymphocytes from healthy donors.

Methods

Blood samples data

30 T2DM patient blood samples from the Endocrinology Department of Qilu Hospital and 20 healthy donor blood samples from the clinical lab of Qilu Hospital were obtained (the sample size was not selected by any power calculation which is the limitation of our study). White blood cells (WBCs) were collected using red blood cell lysis buffer (Solarbio, USA). All samples were negative for specific virus infection or other diseases which could affect the immune system. Our study was approved by the medical ethics committee of Shandong University. Informed consent was obtained from each patient. Baseline characteristics are shown in Table 1.

| Characteristics                  | T2DM patients (n = 30) | Healthy donors (n = 20) |
|---------------------------------|------------------------|-------------------------|
| Male, Female                    | 18, 12                 | 11, 9                   |
| Age, years (range)              | 60 (42–76)             | 56 (39–67)              |
| Fasting plasma glucose (mmol/L) | 10.27 ± 0.5750         | 4.21 ± 0.4023           |
| Fasting plasma C-peptide (ng/ml)| 1.10 ± 0.1096          | 3.14 ± 0.3081           |
| BMI (kg/m²)                     | 25.90 ± 0.9927         | 23.02 ± 0.3946          |
| HbA1C (%)                       | 7.82 ± 0.4091          | 5.254 ± 0.982           |
| Insulin (uIU/mL)                | 12.16 ± 1.6490         | 10.82 ± 5.09            |
| Diabetes duration (years)       | 10.80 ± 1.2300         | –                       |

Table 1. Clinical characteristics of blood samples.
Flow cytometry (FCM) detection

Isolated WBCs were stained with corresponding antibodies (anti-CD4 (ebioscience, USA), anti-Tim-3 (ebioscience, USA), anti-CXCR5 (ebioscience, USA). Cells were incubated with antibodies for 30 min at room temperature and analyzed by FACS Aria II (Figures 1–3).

Statistical analysis

Statistical analysis was performed using Prism GraphPad Software (version 6.0). Comparison between two groups was calculated by unpaired t-test. In the correlation section, the Pearson correlation analysis was used. \( p < 0.05 \) was considered statistically significant.

Results

cTfh cells are more abundant in patients with T2DM than healthy donors

We first analyzed the abundance of cTfh cells from patients with T2DM and healthy donors. The cTfh cells were marked with CD4 and CXCR5. As shown in Figure 1(a), T2DM patients (33.88 ± 1.042%) displayed more cTfh cells than healthy donors (27.72 ± 1.356%; \( p < 0.001 \)).

**Figure 1.** The circulating Tfh cells in T2DM patients and controls. WBCs were isolated from patients \((n = 30)\) and controls \((n = 20)\): (a) the scatter diagram for Tfh cells marked with CD4\(^+\)CXCR5\(^+\) is shown on the left and the statistical chart of healthy donors \((27.72 ± 1.356\%)\) and T2DM patients \((33.88 ± 1.042\%)\) is shown on the right and (b) correlation analysis for Tfh cells and T2DM characters including FPG, BMI, insulin, HbA1C, diabetes duration, and fasting plasma C-peptide.

Source. The correlation r-value and corresponding p-value is shown at the top left corner of each statistical picture. ***p-value < 0.001.

**Flow cytometry (FCM) detection**

The lymphocytes from 6 healthy donors were isolated by EZ-Sep™ lymphocyte separation (Dakewe, China). The cells were cultured in DMEM sugar-free medium and stimulated by high concentration \((25 \text{ mmol/L})\) and low concentration \((5 \text{ mmol/L})\) glucose in cell culture medium for 24 h. The abundance of Tfh \((\text{CD4}^+\text{CXCR5}^+)\) and the frequency of Tim3 on cTfh cells were analyzed by flow cytometry.

**In vitro experiment**

The lymphocytes from 6 healthy donors were isolated by EZ-Sep™ lymphocyte separation (Dakewe, China). The cells were cultured in DMEM sugar-free medium and stimulated by high concentration \((25 \text{ mmol/L})\) and low concentration \((5 \text{ mmol/L})\) glucose in cell culture medium for 24 h. The abundance of Tfh \((\text{CD4}^+\text{CXCR5}^+)\) and the frequency of Tim3 on cTfh cells were analyzed by flow cytometry.
The relationship between cTfh and characteristics of T2DM

We next evaluated the correlation between Tfh cells and T2DM characteristics as shown in Table 1 (fasting plasma glucose (FPG); body mass index (BMI); insulin; HbA1C; diabetes duration; fasting plasma C-peptide). No significant correlation was found between cTfh cells and FPG, BMI, or insulin. A negative correlation was found between cTfh cells and HbA1C (Figure 1(b), $r = -0.4424$, $p$-value $=0.0392$) and diabetes duration (Figure 1(b), $r = -0.4020$, $p$-value $=0.0339$). HbA1C can provide insight into blood glucose control over recent months in patients with T2DM. A strong positive correlation was also found between cTfh cells and fasting plasma C-peptide (Figure 1(b), $r = 0.6223$, $p$-value $=0.0044$).

The frequency of Tim-3 on cTfh cells and the relationship with T2DM

We analyzed the frequency Tim-3 on cTfh cells (CD4$^+$CXCR5$^+$) by flow cytometry. As shown in Figure 2(a), the cTfh cells from T2DM patients (11.94 ± 1.270%) expressed much more Tim-3 than those from healthy donors (7.02 ± 0.773%) ($p$-value $<0.01$). However, no significant correlations between the frequency of Tim-3 on cTfh cells and T2DM characters in the correlation analysis (Figure 2(b)).

High glucose increases the abundance of Tfh cells in vitro

Since the fundamental characteristic of T2DM is hyperglycemia, we stimulated lymphocytes with low- and high-concentration glucose for 24h in
vitro and performed flow cytometry to detect the amount of cTfh cells and Tim-3 frequency. High-concentration glucose increased the amount of cTfh cells (Figure 3(a)); however, the frequency of Tim-3 on cTfh cells was not influenced by the concentration of glucose (Figure 3(b)).

**Discussion**

We examined the number and role of cTfh cells and associated Tim-3 frequency in patients with T2DM. Our results demonstrate an increase in the amount of cTfh cells in T2DM patients compared with healthy donors (Figure 1(a)). Tfh cells are a subset of CD4+ T cells that promote the B cell maturity. Recent studies have identified the role of chronic inflammation in the progression of T2DM. B cells are responsible for the release of cytokines such as IL-6 and TNF-α in T2DM, which is closely related to chronic inflammation and insulin resistance. Therefore, it appears that elevation of Tfh cells in T2DM may promote B cells to release pro-inflammatory cytokines and aggravate the progression of T2DM.

The correlation between T2DM characteristics and cTfh cells (Figure 1(b)) has been identified. The negative correlation between HbA1c and cTfh cells indicates that poor control of blood glucose levels decreases the amount of cTfh cells. However, the reason why the abundance of cTfh cells correlates with HbA1c but not FBG remains unclear. cTfh cell number is related to chronic inflammation, and we illustrated a clear relationship with T2DM. However, the negative correlation between diabetes duration and cTfh cells is associated with the body’s adaption to the long-term diabetes duration that reduced the amount of cTfh cells, although the detailed mechanisms need to be further explored. Fasting plasma C-peptide testing is more accurate to reflect the insulin released level by islet B cells and the body’s insulin resistance level than plasma insulin testing in T2DM patients, especially those who rely on insulin injections. Therefore, the positive correlation between plasma C-peptide level rather than insulin level and cTfh cells indicates that more cTfh cells leads to higher insulin resistance. In contrast.
to Wang et al.’s study, which showed a correlation between BMI of T2DM patients and the amount of cTfh cells,21 our results showed no significant correlation between the two parameters. This difference might be due to sample variation.

Our findings also indicate that the frequency of Tim-3 on cTfh cells is higher in patients with T2DM compared with healthy controls. Tim-3 is closely related to the immune tolerance.23,38 Our previous studies indicated that the expression of Tim-3 on CD14+ cells is down-regulated in T2DM patients39 due to high Tim-3 expression that promotes the transformation of macrophages to M2 with mainly anti-inflammatory effects.40 In contrast with macrophages, we identified elevated frequency of Tim-3 on cTfh cells (Figure 2(a)). The body’s adaption to chronic inflammation and negative feedback regulation for the decline of Tim-3 expression on macrophages may illustrate this phenomenon. Interestingly, Kurose et al. found that expression of Gal-9 (galectin-9), the ligand of Tim-3, increased in the blood of patients with T2DM due to renal lesions.31 This is a novel research pathway especially in patients with diabetic nephropathy. We found no significant correlation between T2DM characteristics and Tim-3 frequency on cTfh cells (Figure 2(b)); therefore, the factors that are directly correlated with Tim-3 expression on cTfh cells in T2DM remain unclear.

We also showed that high glucose concentration could directly stimulate cTfh cell activation and increase the differentiation of CD4+ cells into Tfh cells (Figure 3(a)). Conversely, there is no direct relationship between the concentration of glucose and the frequency of Tim-3 on cTfh cells (Figure 3(b)). This indicates that it is the chronic inflammation and aberrant immune environment in T2DM that cause the change of Tim-3 expression rather than high glucose concentration. Importantly, further research is needed to explore the detailed mechanisms relating to what factors can directly impact Tim-3 expression in T2DM.

However, there are also some limitations of this study. Firstly, the sample size wasn’t calculated and justified by professional statistical software in this study; secondly, we just detected the frequency of Tim-3 on cTfh cells, the ligand of Tim-3 and the Tim-3 function was not identified; finally, limited by the volume of peripheral blood collection, we were unable to detect the downstream signaling pathways of Tim-3.

Conclusion
Our research provides a new perspective for the understanding of T2DM. Elevated cTfh cells and Tim-3 frequency may be a valuable avenue in order to explore the complex pathophysiological changes underlying T2DM.

Declaration of conflicting interests
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Ethical approval
Ethical approval for this study was obtained from The Ethics Committee of Qilu Hospital of Shandong University (2019027).

Informed consent
Written informed consent was obtained from all subjects before the study.

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