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Original Article

Detection Th1/Treg cells and serum cytokines in fulminant type 1 diabetes

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Abstract: Objective: To investigate the role of Th1/Treg cells and various cytokines in fulminant type 1 diabetes mellitus (FT1DM). Methods: 20 cases of type 2 diabetes mellitus (DM), 18 classic type 1 DM, 20 FT1DM and 20 normal controls, in our hospital were enrolled in the study group. We determined the imbalance of Th1/Treg cells and compared the changes within different cytokines, such as TNF-α, IL-6, IL-1β, IL-8, MIP-1β, IL-12p70, IP-10, IFN-γ, IL-9, IL-15, IL-17A, IL-10, MCP-1, MIP-1a, fractalkine, and MCP-3. Results: We found that $CD4^+CD25^+Foxp3^+$ cells strikingly decreased in type 1 DM and type 2 DM. We also observed the cytokines associated with inflammation (TNF-α, IL-6, IL-1β, IL-8, MIP-1β) were significantly higher in FT1DM cases compared with those in classic type 1 DM cases ($P<0.05$). Th1 associated cytokines (IL-12p70, IP-10, IFN-γ, and IL-9) were also significantly increased in FT1DM cases compared with those in classic type 1 DM cases ($P<0.05$). There were no obvious differences in cytokines status (IL-15, IL-17A, IL-10, MCP-1, MIP-1a, fractalkine, and MCP-3) among controls, type 2 DM, classic type 1 DM and FT1DM cases ($P>0.05$). Conclusions: Our data offer evidence for the imbalance of Th1/Treg cells and changed cytokines in FT1DM patients, which may have significant impacts on the prevention of the disease and treatment of the patients.

Keywords: Cytokine status, fulminant type 1 diabetes mellitus, Th1/Treg

Introduction

Diabetes mellitus (DM) is common metabolic disease across the world. Diabetic complications include microvascular complications, such as retinopathy, neuropathy and nephropathy, and macrovascular complications [1]. The incidence of type 1 diabetes (T1DM) has risen considerably in the past 20 years, but the exact etiology was not clear. Genetic, immunologic, and environmental factors may contribute to the pathogenesis of disease [2-4]. T1DM is almost due to T-cell mediated pancreatic islet β-cell destruction [5]. Fulminant type 1 diabetes mellitus (FT1DM) is a subtype of T1DM first reported in 2000; a new clinical entity featured by acute destruction of pancreatic beta cells [6]. This disease is characterized by abrupt onset of diabetic symptoms, rapid progression to ketosis or ketoacidosis (usually less than 1 week), remarkably reduced pancreatic enzyme levels [7]. The prevalence was estimated to be 8.9% in all T1DM patients and 0.2% in newly diagnosed all diabetic patients [8]. Clinical consequences of FT1DM could be fatal when timely medications are not provided, suggesting the particular importance of early diagnosis of the disease. On the other hand, flu-like symptoms (such as fever, headache and cough) and abdominal symptoms (such as vomiting and diarrhea) were observed in most FT1DM patients. In addition, common forms of diabetes such as classic type 1 diabetes (T1DM) share many clinical manifestations without F1TDM; the death of the patients is almost inevitable, highlighting the critical importance of early diagnosis of the disease.

In F1TDM, insulin deficiency is involving in the destruction of the β-cells of the pancreas, which may be induced by the activation of autoaggressive T helper (Th) lymphocytes and macrophages. Th1 cells are an important source of interferon-gamma (IFN-γ) and also form a major component of cellular immune response. Th1 cells also secret cytokines such as IL-12p70, IP-10, IFN-γ, and IL-9. $CD4^+CD25^+$Tregs represent one of the important subsets of Tregs,

Table 1. Patient characteristics of controls and DM cases

Parameters	Control	T2DM	CT1DM	FT1DM
N (male/female)	13/7	10/10	6/12	7/13
Age (year)	44.6±2.7	46.2±3.1	40.7±2.8	42.5±1.5
BMI (kg/m^2)	22.41±1.7	26.2±2.91	21.5±1.84	21.7±2.43
HDL (mmol/L)	1.45±0.31	1.378±0.24	1.759±0.31	1.598±0.27
LDL (mmol/L)	1.95±0.39	2.24±0.36	2.31±0.801	2.09±0.93
HbA1C (%)	5.64±0.35	8.57±0.89	8.606±0.40	6.48±0.36
CP (0) (ng/ml)	1.36±0.55	1.95±0.72	0.78±0.56	0.03±0.02
CP (30) (ng/ml)	3.92±1.57	3.10±1.34	1.12±0.82	0.03±0.01
CP (120) (ng/ml)	3.78±1.28	4.88±1.55	1.32±0.42	0.06±0.04

BMI: Body Mass Index; HDL: High density lipoprotein; LDL: Low density lipoprotein ; HbA1C: Glycosylated hemoglobin A1C; CP: C peptide.

which play a key role in preventing autoimmunity and immune diseases [9].

Inflammation is closely associated with the metabolic diseases, including T1DM. Increased inflammatory reaction may exacerbate the blood glucose control. Previous studies also showed that certain inflammatory markers changed a lot in DM cases compared with those in normal controls. Studies also showed that inflammation has a link between microvascular complications and subclinical atherosclerosis in T1DM [10]. The alteration of inflammatory markers may be an indicator of T1DM severity/risk.

In this study, the aim of this research was to evaluate the balance between T helper 1 and regulatory T cells which may facilitate the sighting of new therapeutic strategies in the treatment of type 1 DM. We also compared the differences of various cytokines status in FT1DM cases with normal controls, type 2 DM (T2DM), classic type 1 DM (CT1DM).

Materials and methods

Monoclonal antibodies and reagents

PE-anti-hFoxp3 (PCH101) mAb, FITC-anti-hCD-25 mAb (M-A251), and PE-anti-hIFN-γ mAb were purchased from eBioscience (San Diego, CA), PE-anti-CD25 mAb (BC96), and isotype control monoclonal antibodies (mAbs) were purchased from Biolegend.

Patient population

Fresh peripheral blood samples from patients with T2D, CT1DM, FT1DM and control subjects were collected at Traditional Chinese Medicine Hospital of Jiangsu Province. The cases were enrolled after diagnosed as DM cases the first time. The characteristics of study cohort are provided in **Table 1**.

For normal controls, CT1DM, FT1DM, T2DM patients, venous blood samples were obtained after overnight fasting for at least 8 h and subjected to routine cytokines status measurements. The study protocol was approved by the institutional review board. All participants gave informed consent prior to the study.

Flow cytometry

Peripheral blood mononuclear cells (PBMCs) from controls and patients were isolated by Ficoll-hypaque gradient centrifugation. Evaluation of Tregs and Th1 cells was done by standard 2-color flow cytometry as previously described. Phycoerythrin-Cyanine 5 (PC5)-labeled anti-CD4, fluorescein isothiocyanate (FITC)-labeled anti-CD25, and For Foxp3 staining, cells were fixed and permeabilized with fix/Perm solution (eBioscience) and stained with Foxp3 mAb, according to the instruction provided by the manufacturer (eBioscience, San Diego, CA). Negative control fluorescence was assessed using the isotype-matched control mAb.

Cytokines measurements

Three milliliters of cubical vein blood was collected after fasting for at least 12 h, left standing for 1 hour at 4°C, and then centrifuged 2000 rpm for 20 minutes; 0.5 mL of the upper serum was collected and stored at -80°C for later testing. Serum cytokines profiles (TNF-α, IL-6, IL-1β, IL-8, MIP-1β, IL-12p70, IP-10, IFN-γ, IL-9, L-15, IL-17A, IL-10, MCP-1, MIP-1a, fractalkine, and MCP-3) were determined using the Luminex detection method, which is based on LiquiChip technology according to the instruction provided by the manufacturer (Milliplex). Detection equipment for the Luminex200 (Luminex Co. Ltd., USA), according to the kit instructions.

Data collection

We earnestly extracted the clinical findings of the patients from normal controls, T2DM, C1-T2DM and FT1DM cases. Demographic and

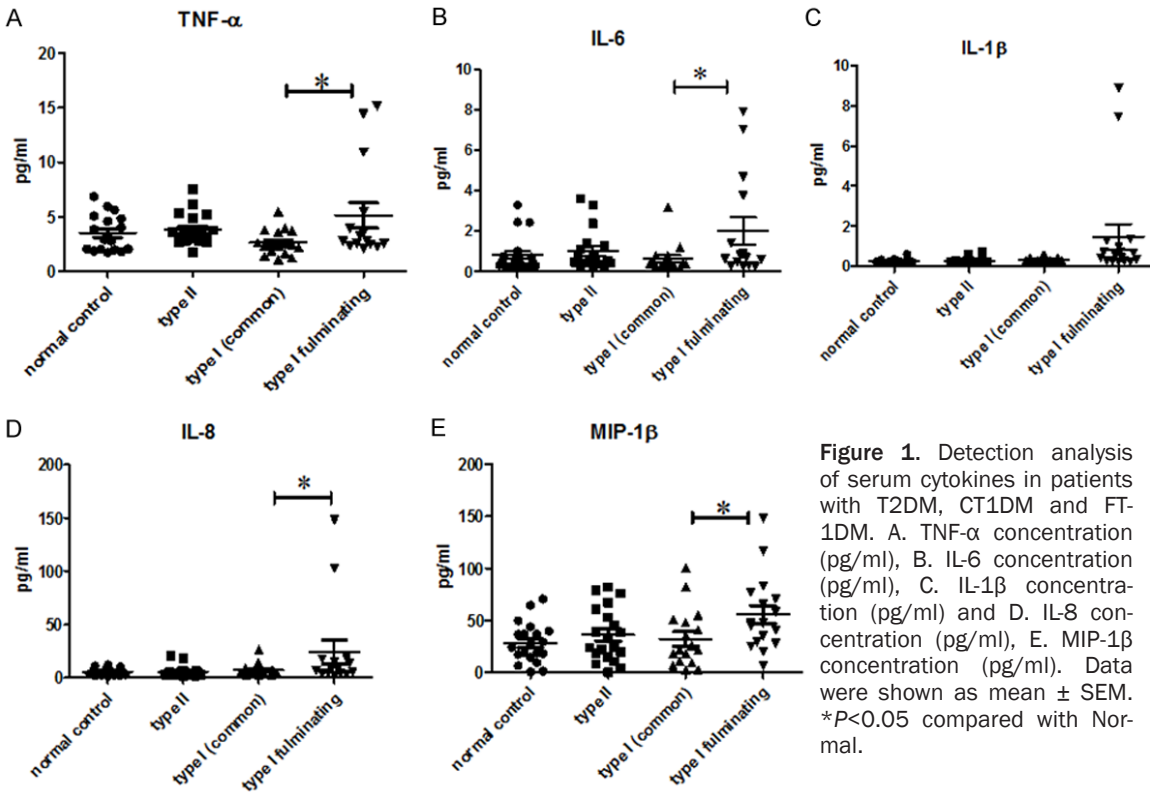

Figure 1. Detection analysis of serum cytokines in patients with T2DM, CT1DM and FT-1DM. A. TNF-α concentration (pg/ml), B. IL-6 concentration (pg/ml), C. IL-1β concentration (pg/ml) and D. IL-8 concentration (pg/ml), E. MIP-1β concentration (pg/ml). Data were shown as mean ± SEM. **P*<0.05 compared with Normal.

clinical data were reviewed respectively for age, gender, disease course, blood glucose level and complications.

Statistical analysis

Continuous variables were expressed as means ± standard deviation (SD), Significant differences between two groups or multiple groups were analyzed by using the Student t test or one-way ANOWAY test. P<0.05 was considered statistically significant, except where otherwise specified.

Results

Patient's characteristics

A total of normal controls (n=20) and DM cases (Type 2 DM, n=20, Classic type 1 DM, n=18, fulminant type 1 DM (FT1DM), n=12) were enrolled in our study. The mean age was 44.5.6 ± 2.7 and 42.5 ± 2.5 years for normal controls and DM cases, respectively. No marked differences of age, male/female ratio, disease course and ratio of complications were observed amomg T2DM, CT1DM and FT1DM cases (**Table 1**).

Imbalance of Th1/Tregs in patients with T1D patients

$CD4^+CD25^+$Tregs represent one of the important subsets of Tregs, which play a key role in preventing autoimmunity and immune diseases. Flow cytometry was used to detect Th1/Treg cells in peripheral blood of patients. We found that $CD4^+CD25^+$Tregs significantly decreased in CT1DM, FT1DM and T2DM patients, and different types had no significant difference (**Figure 5**). As shown in **Figure 6**, Th1 cells increased in T1DM (CT1DM, FT1DM) evidenced by promotion of $CD8^+IFN\text{-}\gamma^+$ and $CD4^+IFN\text{-}\gamma^+$. Cells with chemotaxis function such as $CXCR3CD4^+$ and $CXCR3CD8^+$ increased obviously in CT1DM, FT1DM shown in **Figure 7**. These data strongly indicate that the remarkable imbalance of Th1/Tregs subsets presents in T1D patients.

Cytokines status among DM cases

Cytokines (TNF-α, IL-6, IL-1β, IL-8, MIP-1β, IL-12p70, IP-10, IFN-γ, IL-9, L-15, IL-17A, IL-10, MCP-1, MIP-1a, Fractalkine, and MCP-3) were assessed in the peripheral serum from the different DM groups and normal controls. The

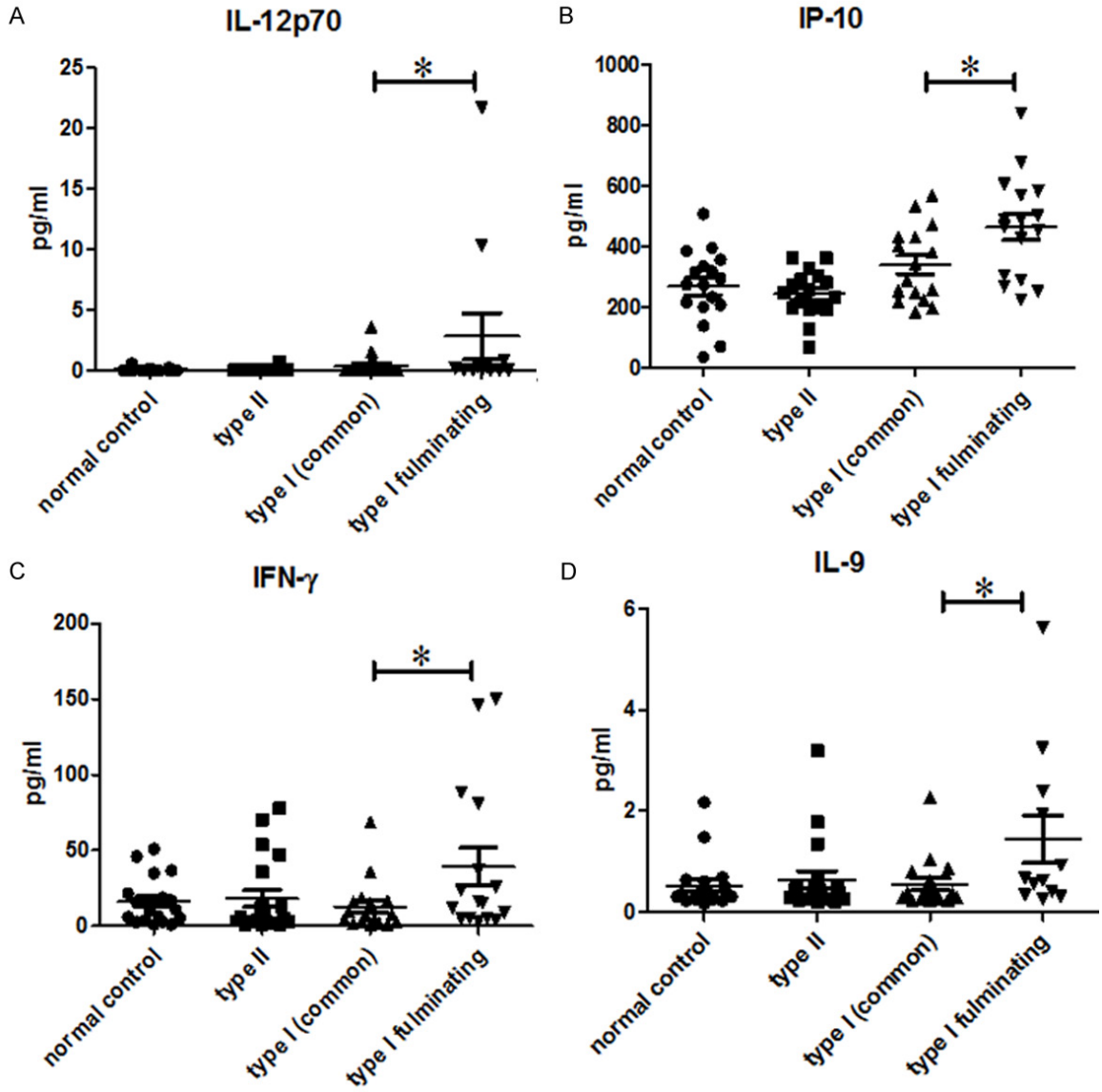

Figure 2. Detection analysis of serum cytokines in patients with T2DM, CT1DM and FT1DM. A. IL-12p70 concentration (pg/ml), B. IP-10 concentration (pg/ml), C. IFN-γ concentration (pg/ml) and D. IL-9 concentration (pg/ml). Data were shown as mean ± SEM. *P<0.05 compared with Normal.

cytokines associated with inflammation (TNF-α, IL-6, IL-1β, IL-8, MIP-1β) were significantly higher in FT1DM cases compared with those in classic type 1 DM cases (P<0.05) (**Figure 1**). The th1 cytokines (IL-12p70, IP-10, IFN-γ, and IL-9) were also higher in FT1DM cases (**Figure 2**). There were no obvious differences in cytokines status (IL-15, IL-17A, IL-10, MCP-1, MIP-1a, Fractalkine, and MCP-3) among controls, type 2 DM, classic type 1 DM and FT1DM cases (P>0.05) (**Figures 3** and **4**).

Discussion

The involvement of immune disorders and its complication in the process of T1DM gain more attention today [11]. In the present study, the levels of $CD4^+CD25Foxp3^+$ decreased in the periphery blood of patients with T1DM, whereas Th1 cells are increased, indicating that distinct $CD4^+$T cell subset polarization occurred in T1D patients. We noted that the imbalance of Th1/Treg cells present cytokines status (TNF-α,

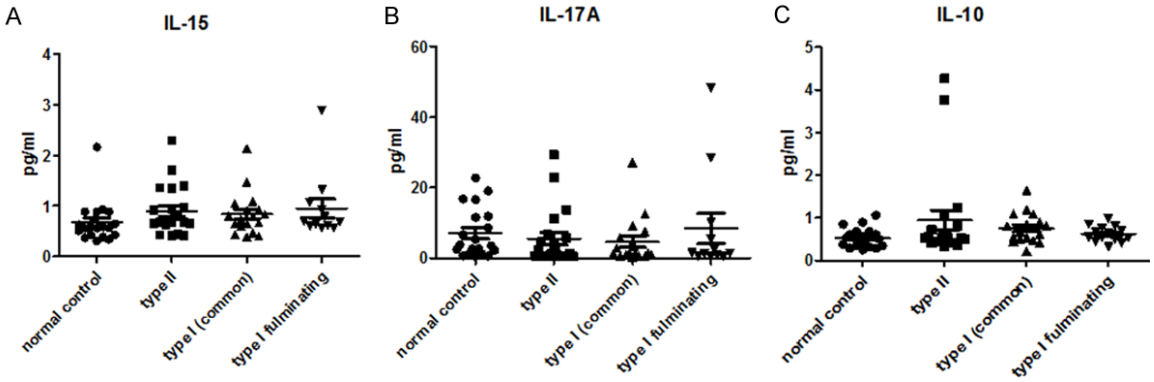

Figure 3. Detection analysis of serum cytokines in patients with T2DM, CT1DM and FT1DM. A. IL-15 concentration (pg/ml), B. IL-17A concentration (pg/ml), C. IL-15 concentration (pg/ml). Data were shown as mean ± SEM. *P<0.05 compared with Normal.

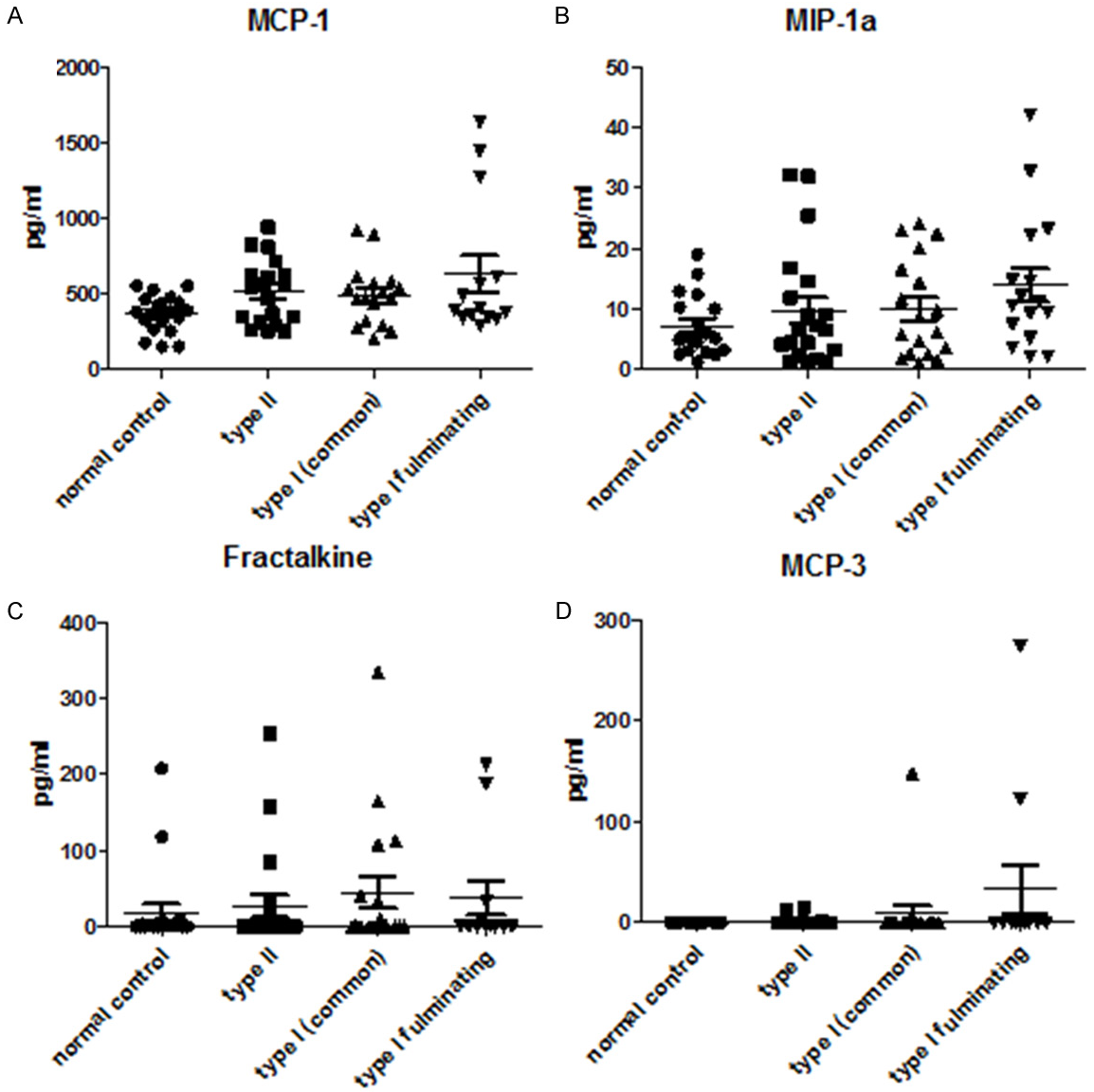

Figure 4. Detection analysis of serum cytokines in patients with Normal, T2DM, CT1DM and FT1DM. A. MCP-1 concentration (pg/ml), B. MIP-1a concentration (pg/ml), C. Fractalkine concentration (pg/ml) and D. MCP-3 concentration (pg/ml). Data were shown as mean ± SEM. *P<0.05 compared with Normal.

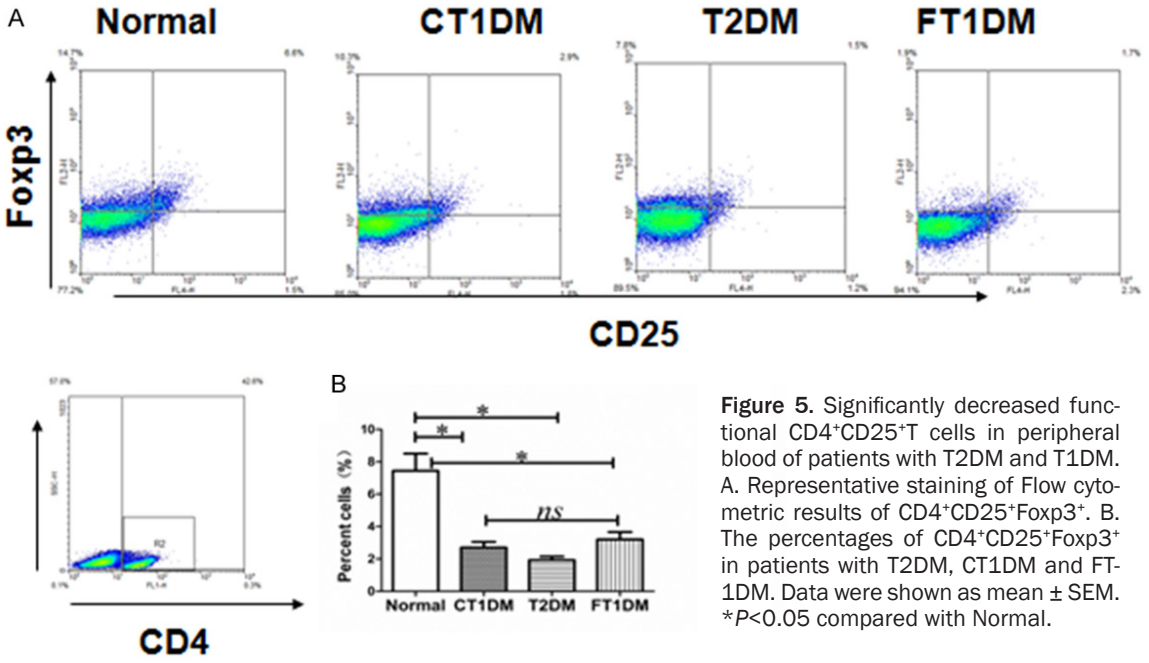

Figure 5. Significantly decreased functional $CD4^+CD25^+$T cells in peripheral blood of patients with T2DM and T1DM. A. Representative staining of Flow cytometric results of $CD4^+CD25^+Foxp3^+$. B. The percentages of $CD4^+CD25^+Foxp3^+$ in patients with T2DM, CT1DM and FT-1DM. Data were shown as mean ± SEM. *P<0.05 compared with Normal.

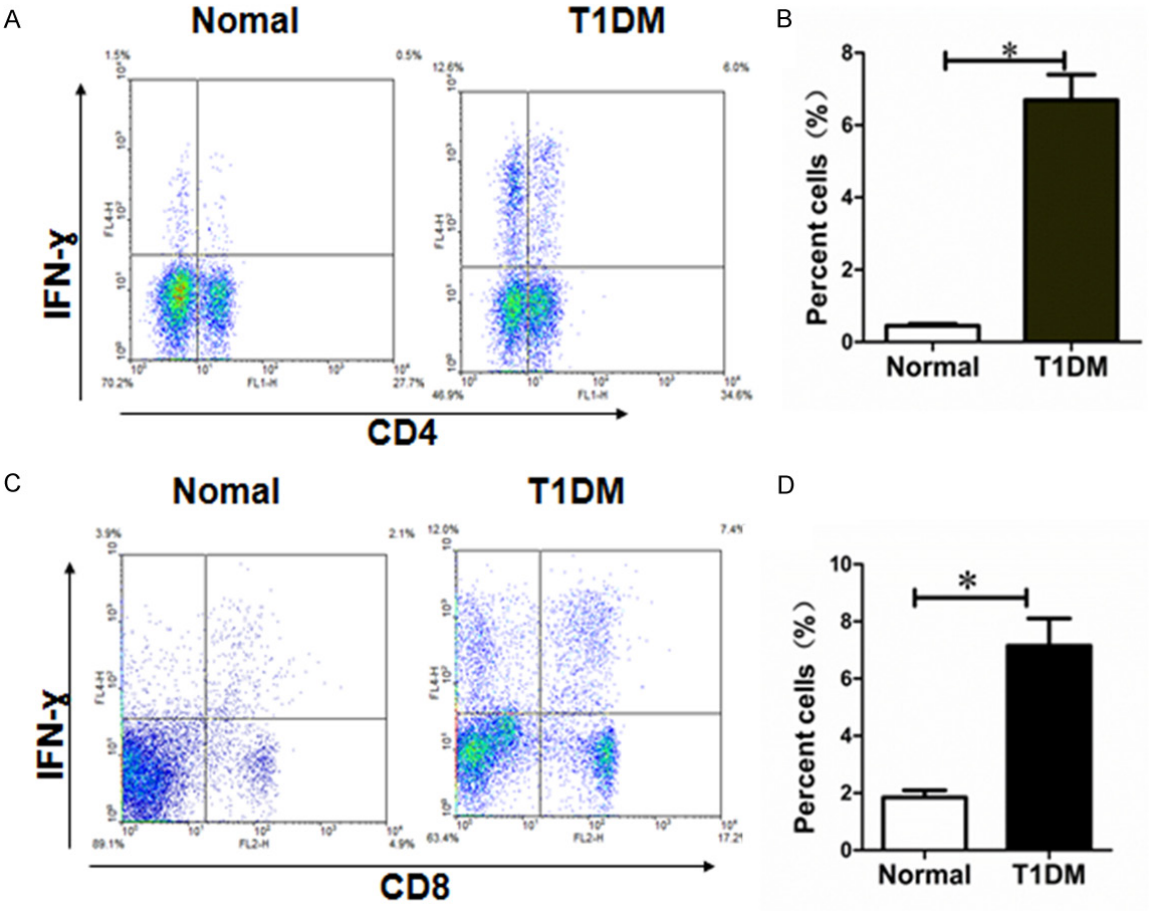

Figure 6. Increscent of $CD4^+IFN\text{-}\gamma^+$ and $CD8^+IFN\text{-}\gamma^+$ cells in peripheral blood of patients with T1DM. A, C. Representative staining of Flow cytometric results of $CD4^+CD25^+IFN\text{-}\gamma^+$. B, D. The percentages of $CD4^+CD25^+Foxp3^+$ in patients with T1DM. Data were shown as mean ± SEM. *P<0.05 compared with Normal.

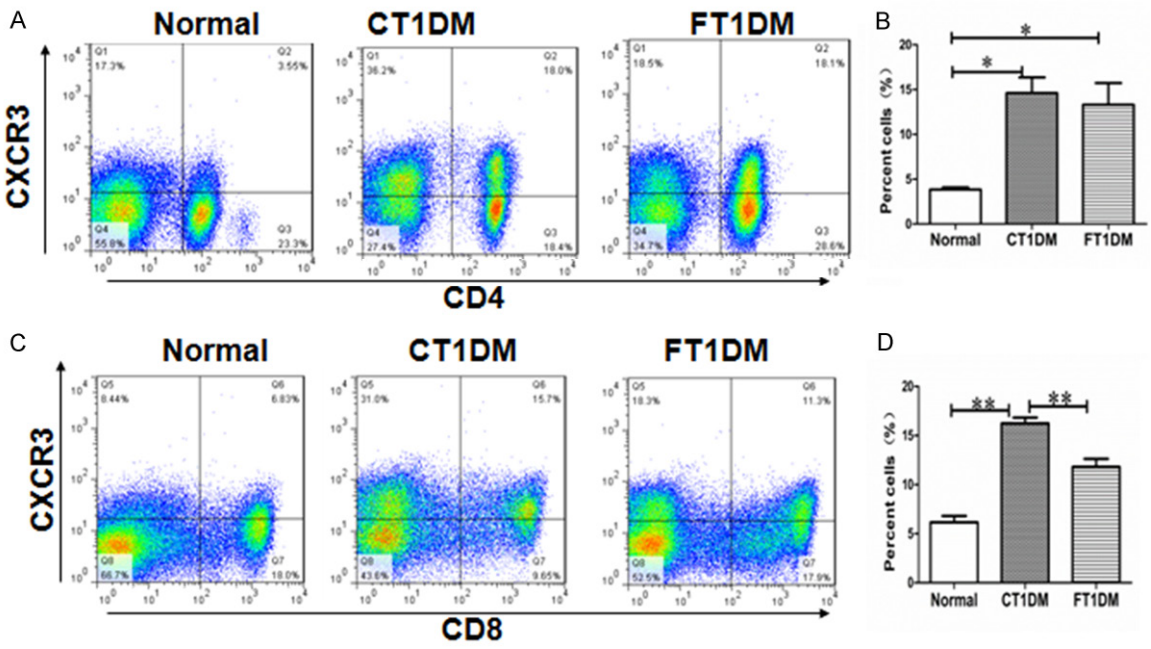

Figure 7. Increscent of $CD4^+$ and $CD8^+$ chemotaxis cells in peripheral blood of patients with T1DM. A. Representative staining of Flow cytometric results of $CD4^+CXCR3$. B. The percentages of $CD4^+CXCR3$ in patients with CT1DM and FT1DM. C. Representative staining of Flow cytometric results of $CD8^+CXCR3$. D. Representative staining of Flow cytometric results of $CD8^+CXCR3$. Data were shown as mean ± SEM. *P<0.05 compared with Normal.

IL-6, IL-1β, IL-8, MIP-1β, IL-12p70, IP-10, IFN-γ, and IL-9) was significantly higher in FT1DM cases compared with those in classic T1DM cases, which indicated that the increase of these inflammatory markers may predict the risk of FT1DM.

FT1DM is a rare condition, which often progresses rapidly to critical illness, even death. FT1DM is characterized by acute destruction of pancreatic beta cells [12-14]. Studies demonstrated that $CD4^+CD25^+$Tregs play a pivotal role in self-tolerance and resistance to autoimmune disease via its ability to down-regulate the function of innate and adaptive immune effector cells. Dysfunction of $CD4^+CD25^+$Tregs promotes the development of T1DM in animals and human beings [15, 16]. Kotani R et al suggested autoreactive T cells might contribute to the development of fulminant Type 1 diabetes [17].

Hence, timely diagnosis and medications of FT1DM is vital for the treatment of FT1DM. Regrettably, FT1DM cases often share common flu-like and abdominal symptoms with CT1DM cases; it is very difficult to diagnose FT1DM rapidly. In this sense, our findings were of great implications that the monitoring of status of these cytokines may be helpful for predicting the risk of FT1DM [18].

Several mechanisms may account for the association between FT1DM risk and the status of inflammatory markers. First, viral infection was closely associated with the risk of FT1DM; viral infection may induce the secretion of various cytokines, particularly inflammatory markers. On the other hand, immune reaction was increased during the course of FT1DM, which also can elevate the status of certain cytokines. Second, inflammation was closely associated with the progress of DM, such as the onset of some complications. FT1DM is likely to be with the rapid exacerbation of β cell function. Inflammation may be involved in this process. Finally, FT1DM is likely to lead to the dysregulation of internal environment, such as acidosis, which may also result in the increase of certain cytokines.

In the past, several studies were performed to investigate the possible risk factors associated with the susceptibility to FT1DM. O'Hara et al [19] reported that FT1DM was associated with Coxsackie virus A1 infection. Miyoshi et al [20] reported that nivolumab induces FT1DM. Gaudy et al [21] reported that anti-PD1 pembrolizumab can also induce exceptional FT1DM. Nishiumi et al [22] reported that FT1DM was closely associated with parvovirus B19 infection. All these previous findings supported the idea that FT1DM was closely associated with immune and inflammatory reaction, which was consistent with our findings.

Our findings are of great implications. However, several limitations should be considered in our study. First, the small number of participants limited the statistical power; future larger number of participants should be needed. Second, the association between the cytokine status and the biochemical indexes in DM should be studied, which may be helpful for the in-depth understanding of the inflammatory markers in FT1DM. Finally, the prognosis of DM cases should also be investigated, which may be helpful for understanding the association between the cytokines and DM prognosis. Hence, in a word, larger number, whole investigations should be performed in the future.

In conclusion, our investigation of present study showed that the dramatically decrease of $CD4^+CD25^+$Tregs and improvement of Th1 cells in FT1D patients may contribute to the enhanced immune activation and inflammation, as well as the subsequent complications. FT1DM is closely associated with the cytokines status (TNF-α, IL-6, IL-1β, IL-8, MIP-1β, IL-12p70, IP-10, IFN-γ, and IL-9), which may be associated with the progression of FT1DM. However, larger number of studies should be conducted to validate our findings.

Disclosure of conflict of interest

None.

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