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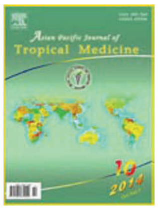

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Role of immune dysfunction in pathogenesis of type 1 diabetes mellitus in children

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ABSTRACT

Objective: To investigate the function of cytokines, chemokines, and regulatory T cells (Tregs) in the pathogenesis of type 1 diabetes mellitus (T1DM) in children. **Methods:** A total of 35 children with T1DM and 30 healthy controls were enrolled in this study. Levels of serum cytokines (IL–1α, IL–6, IL–10, IL–12, and TNF–α) and chemokines (MIP–1α, MIP–1β and MCP–1) were detected by enzyme–linked immunosorbent assay. Peripheral blood mononuclear cells (PBMCs) were isolated and culture supernatant of phytohaemagglutinin (PHA)–stimulated PBMCs was subjected to ELISA for levels of cytokines (IL–1α, IL–6, IL–10, IL–12 and TNF–α) in T1DM and control group. Furthermore, flow cytometry was used to determine the percentage of Tregs in PBMCs of two groups. **Results:** Levels of serum cytokines including IL–1α, IL–6, IL–10 and TNF–α as well as chemokines, such as MIP–1α and MIP–1β in children with T1DM children were significantly higher than those in healthy controls ($P<0.05$, respectively). PBMCs with PHA stimulation in T1DM group secreted more IL–1α and TNF–α ($P<0.05$, respectively), but less IL–10 ($P<0.05$), as compared with control group. Furthermore, the proportion of CD4^{+}, CD25^{+}, Foxp3^{+}, Tregs in PBMCs isolated from children with T1DM was obviously lower than those in healthy controls ($P<0.05$). **Conclusions:** Immune dysfunction, with upregulation of inflammatory factors such as IL–1α, IL–6, TNF–α and MIP–1α, downregulation of IL–10 and Tregs, plays an important role in the pathogenesis of T1DM in children.

1. Introduction

The incidence of diabetes is approximately 0.02% in children younger than 14 years old, about 70 000 children are diagnosed as diabetes each year[1], and the prevalence is increasing per year[2]. In western country, above 90% diabetes in children is type 1 diabetes mellitus (T1DM)[3]. T1DM in children is a T cell–mediated autoimmune disease. Recently, immune dysfunction including deregulation of Th1/Th2 cytokine network[4], abnormal number or function of regulatory T cells (Tregs)[5] and hyperfunction of inflammatory cytokines[6] were involved in initiation and development of T1DM.

The pathogenesis of T1DM is complex, including genetic predisposition, the status of immune dysfunction, viruses, toxins and diet[7]. Recent researches commonly consider that T1DM is an autoimmune disease mainly caused by T cell attacking pancreatic β–cell, and inflammatory cells, cytokines and chemokines participate in these inflammatory reactions[8]. However, how these inflammatory cells or molecules promoting initiation and development of T1DM is controversial. In this study, we systemically analyzed the status of cytokines, chemokines and immune cells in children with T1DM, in order to provide a new insight for prevention and treatment of T1DM.

2. Materials and methods

2.1. Clinical data and reagents

A total of 35 children without fever, acute infection, stress and chronic disease including 16 males and 19

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females [range: 3-14 years; mean: (8.92±4.47) years], who were newly diagnosed T1DM according to the diagnostic criteria established by WHO in 1999[9] during March 2011 to March 2014, were selected as T1DM group. All the patients did not receive insulin and immunosuppressant therapy before. Another 30 healthy children with normal blood, liver function and blood glucose tests including 13 males and 17 females [range: 3-15 years; mean: (9.21±4.73) years], who received regular checkups during the same period, were selected as control group. All the healthy children did not have family history of diabetes and/or autoimmune disease. The age and sex between T1DM and control group is comparable. Samples were collected and used after obtaining informed consent. The Fujian Medical University Ethics Committee approved all protocols according to the Helsinki Declaration (as revised in Edinburgh 2000).

Lymphocytes separation medium were from Axis-Shield Co. (Dundee, UK). RPMI-1640 medium, fetal bovine serum (FBS) and penicillin/streptomycin antibiotic were purchased from Invitrogen Co. (Carlsbad, CA, USA). Phytohaemagglutinin (PHA) was obtained from Sigma Co. (St-Louis, MO, USA). IL-1α, IL-6, IL-10, IL-12 and TNF-α cytokines testing kits were purchased from Jingmei bio engineering Co., Ltd. (Beijing, China). MIP-1α, MIP-1β and MCP-1 chemokines testing kits were from Senxiong biotech Co., Ltd. (Shanghai, China). Mouse anti-human CD4-FITC, Foxp3-PE and CD25-percp fluorescent antibodies as well as FACS Calibur flow cytometer were purchased from BD Co. (Franklin Lakes, NJ, USA). Foxp3 staining kit was obtained from eBioscience Co. (San Diego, CA, USA).

2.2. Blood sample collection and PBMCs isolation

10 mL anticoagulant venous blood and 5 mL coagulant venous blood were aseptically collected. The coagulant venous blood was placed at room temperature for 30 min, and then serum was obtained after centrifugation and frozen at -80 ℃. The anticoagulant venous blood was mixed with equal RPMI-1640 medium, and then mixture was slowly added into lymphocytes separation medium. PBMCs were collected by centrifugation at 2 200 r/min for 20 min. Cells were washed with PBS for three times and resuspended in RPMI-1640 medium containing 10% FBS and 1% penicillin/streptomycin antibiotic.

2.3. Tregs staining

2×10^6 PBMCs were incubated with 1 μL mouse anti-human CD4-FITC and 1 μL CD25-Percp fluorescent antibody at room temperature for 30 min. After 1 h incubation with 0.3 mL fixation/permeabilization buffer, PBMCs were washed for twice and incubated with 1 μL Foxp3-PE fluorescent antibody at room temperature for 1 h. Then cells were resuspended in 200 μL PBS and subjected to FACS Callibur flow cytometer for Tregs counting.

2.4. Determination of cytokines and chemokins

Levels of serum cytokines (IL-1α, IL-6, IL-10, IL-12 and TNF-α) and chemokins (MIP-1α, MIP-1β and MCP-1) were detected using ELISA kits according to the manufacturer's guidelines. 2×10^6 PBMCs that treated with PHA (5 μg/mL) were seeded into 24-well plates and cultured in a humidified 5% CO_2 incubator at 37 ℃ for 24 h. Culture supernatants were collected and analyzed for levels of cytokines (IL-1α, IL-6, IL-10, IL-12 and TNF-α).

2.5. Statistical analysis

All date are presented as the mean±SD. The SPSS statistical package for Windows Version 13 (SPSS, Chicago, IL, USA) was used for a two-tailed Student's *t* test. $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Levels of serum cytokines and chemokines in children with T1DM

To determine the status of immune function in children with T1DM, we tested levels of serum cytokines and chemokines by ELISA in 35 samples of peripheral blood who were newly diagnosed as T1DM. A cohort of 30 healthy children, who received regular checkups, was selected as control group. In these cases, we found that serum levels of IL-1α, IL-6, IL-10 and TNF-α in child with T1DM were significantly higher than those in healthy control ($P<0.05$, respectively, Table 1). But it was comparable between T1DM and control group in serum level of IL-12 (Table 1). Furthermore, we demonstrated that serum levels of MIP-1α and MIP-1β in T1DM group were prominently higher as compared with control group ($P<0.05$, respectively, Table 2). As shown in Table 2, the difference of serum MCP-1 level between two groups was not significant.

Table 1

Levels of serum cytokines in the peripheral blood from T1DM and healthy children.

Group	IL-1α (pg/mL)	IL-6 (pg/mlL)	IL-10 (pg/mL)	IL-12 (pg/mL)	TNF-α (pg/mL)
Control	214.13±129.05	139.43±54.28	99.47±43.11	178.90±60.25	423.84±251.49
T1DM	368.94±246.37	216.45±184.90	245.06±90.52	190.68±81.39	610.89±324.71
t	3.095	2.200	2.522	0.654	2.563
P	0.003*	0.032*	0.014*	0.516	0.013*

*Statistically significant.

Table 2

Levels of serum chemokines in the peripheral blood from T1DM and healthy children.

Group	MIP–1 α (pg/mL)	MIP–1 β (pg/mL)	MCP–1(pg/mL)
Control	78.34±28.61	47.31±10.73	43.01±11.82
T1DM	124.28±32.53	63.79±35.36	46.79±14.95
t	5.997	2.445	1.117
P	0.000*	0.017*	0.268

*Statistically significant.

3.2. Determination of cytokines secreted by PHA–stimulated PBMCs from children with T1DM

PBMCs were isolated from both T1DM and control group and stimulated with PHA for 24 h. Culture supernatants were subjected to ELISA for levels of IL–1 α , IL–6, IL–10, IL–12 and TNF– α . As shown in Table 3, levels of IL–1 α and TNF– α that secreted by PBMCs after PHA stimulation in T1DM group were significantly higher than those in control group ($P<0.05$, respectively). Conversely, level of IL–10 in T1DM group was obviously lower than that in control group ($P<0.05$). Furthermore, levels of IL–6 and IL–12 were comparable between T1DM and control group (Table 3).

3.3. Detection of Tregs in PBMCs from children with T1DM

Since Tregs plays an important role in suppressing activity of autoimmune T cells. To determine the percentage of Tregs in PBMCs, Tregs was labeled with CD25 and Foxp3 fluorescent antibodies and detected by flow cytometer. We found that the percentage of CD4+CD25+Foxp3+ Tregs in T1DM group was significantly lower than that in control group ($P<0.05$, Figure 1).

4. Discussion

IL–2, IL–12, IFN– γ and TNF– α belong to type Th1 cytokines, while IL–4, IL–5, IL–6 and IL–10 belong to type Th2 cytokines. Two kinds of cytokines mutual antagonize each other to maintain a balance[10]. Fisman EZ *et al*[11] reported that IL–1, IL–2 and IL–6 acted as promotive/inflammatory cytokines, while IL–4 and IL–10 played as protective/anti–inflammatory cytokines in diabetes. In this study, we found that serum levels of IL–1 α , IL–6, IL–10 and TNF– α in peripheral blood from children with T1DM were significantly higher than those in control group, but level of IL–12 was comparable. These data indicate that type Th1/Th2 or inflammatory/anti–inflammatory cytokines exhibit a status of excessive secretion in children with T1DM.

Chemokines MIP–1 α , MIP–1 β and MCP–1 belong to chemokine CC subfamily, which induce expression of adhesion molecules and co–stimulatory molecules on cell surface in T cells, NK cells, macrophages and monocytes. Furthermore, MIP–1 α , MIP–1 β and MCP–1 mediate chemotaxis of these cells and promote inflammatory cells secreting cytokines such as interleukin. MCP–1 or MIP–1 α –overexpressing pancreatic β cell resulted in T1DM in mice[12]. However MIP–1 β can prevent T1DM by changing inflammatory reaction in pancreatic islet[13]. We found that serum levels of MIP–1 α and MIP–1 β in peripheral blood from children with T1DM were significantly higher

Table 3

Detection of cytokines secreted by PBMCs after PHA stimulation.

Group	IL–1 α (pg/mL)	IL–6 (pg/mL)	IL–10 (pg/mL)	IL–12 (pg/mL)	TNF– α (pg/mL)
Control	535.28±201.61	404.57±187.39	511.56±253.49	812.07±341.82	290.42±116.83
T1DM	659.19±261.34	481.50±224.68	355.83±146.24	793.68±390.45	465.60±132.82
t	2.113	1.484	3.087	0.200	5.601
P	0.039*	0.143	0.003*	0.842	0.000*

*Statistically significant.

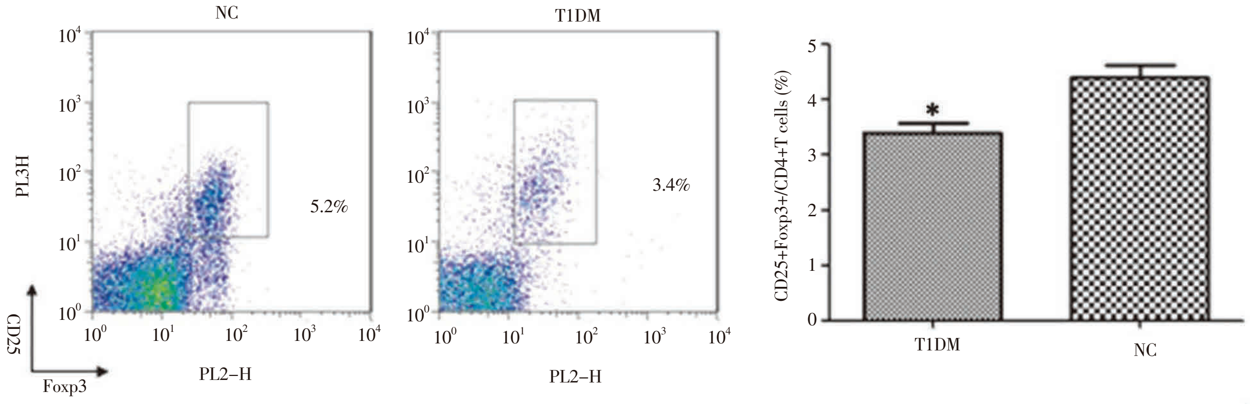

Figure 1. Detection of Tregs in PBMCs from T1DM and healthy children (control).

The percentage of Tregs in PBMCs from T1DM was significantly lower than that in control group. $n = 3$; * $P< 0.05$ by *t* test.

than those in control group, suggesting both inflammatory and anti-inflammatory factors were upregulated. But the comparable level of MCP-1 between T1DM and control group is not consistent with previous study[14], which may be related to the status of disease in children.

In order to investigate whether Th2-Th1 transition of T cells happens in children with T1DM, we stimulated PBMCs with PHA and detected levels of cytokines secreted by cells. We found that levels of IL-1 α and TNF-α secreted by PBMCs from T1DM group was obviously higher than control group, but level of IL-10 was significantly lower as compared with control, suggesting Th1/Th2 regulatory network was dysfunction in children with T1DM. Type Th1 cytokines secretion increased and type Th2 cytokines secretion decreased has been reported in children T1DM, which was consistent with our results[15]. We hypothesize that type Th1 inflammatory cytokines were excessively secreted in children with T1DM, which led to islet cell damage and progression of disease. While type Th2 anti-inflammatory cytokines were elevated under regulation of internal environment and homeostasis. Most cytokines were upregulated in peripheral blood, but type Th2 anti-inflammatory cytokine IL-10 was downregulated.

It has been reported that autoimmune T cell is found in healthy person, which can recognize autoantigen such as insulin[16]. But these cells are in an immunosuppressive status and will not attack pancreatic islet. Tregs play critical role in inhibiting these autoimmune T cell, and Tregs knockout lead to autoimmune disease in mice[17]. In this study, we selected Tregs from CD4$^+$ T cell using CD25 and Foxp3 staining. Our date indicated that the percentage of Tregs in PBMCs from children with T1DM was significantly lower as compared with that in control group, which was consistent with prior study[18]. These date suggest that reduction of Tregs may promote initiation and progression of T1DM.

In conclusion, elevated levels of serum cytokines and chemokines are observed in children with T1DM. PHA-stimulated PBMCs, which are isolated from T1DM patients, secrete more pro-inflammatory cytokines and less anti-inflammatory cytokines. Further, the percentage of Tregs in PBMCs obtained from T1DM patients is prominent lower than healthy controls. All these data suggest that immune dysfunction plays key role in the pathogenesis of T1DM in children.

Conflict of interest statement

We declare that we have no conflict of interest.

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