Decreased interleukin-35 levels and CD4$^+$EBI3$^+$ T cells in patients with type 1 diabetes and the effects of the antibody against CD20 (rituximab)

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Previous study has shown that immune dysfunction including the abnormal function of T regulatory (Treg) cells, deregulation of Th1/Th2 cytokine, and hyperfunction of inflammatory cytokines were considered to have a substantial influence on type 1 diabetes development [1]. Type 1 diabetes (T1D) occurs due to genetic predisposition, viral infection, immune dysfunction status, toxins, and dietary structure [2]. Despite the mysteries surrounding the onset and progression of type 1 diabetes, several studies have implicated cytokines in its initiation and development [3].

Interleukin-35 (IL-35) is a cytokine belonging to the IL-12 family. It is a heterodimeric cytokine comprising two covalently bound sub-units: Epstein-Barr virus-induced gene protein 3 (EBI3) and the p35 subunit of IL-12 [4]. Foxp3$^+$T regulatory (Treg) cells secrets EBI3, a specificity IL-35 subunit, which is a typical factor of Treg cells [5]. Several studies have implicated Tregs, as well as Treg-derived cytokines, in the occurrences of many autoimmune diseases, as well as type 1 diabetes.

IL-35 is effective in the suppression of Teff cell activity, which in turn inhibits the progression of both autoimmune and inflammatory diseases. Herein we aimed to examine the levels of IL-35 in the serum and the proportion of CD4+EBI3+T-cells in type 1 diabetes patients to reveal their interrelationship and immunopathological roles in type 1 diabetes, as well as their influence on the disease activity and manifestations.

Peripheral venous blood was collected from 31 type 1 diabetes patients (diagnosis according to International Society for Paediatric and Adolescent Diabetes (ISPAD) Consensus Guidelines 2014 criteria [6]) and 30 healthy volunteers who acted as controls. All patients did not receive treatment with humanised insulin, or they were just treated with insulin in a very short period of time. We excluded patients who had microvascular complications, inflammatory diseases, or coexisting autoimmune. We administered 4 doses (375 mg/m^2) of rituximab on days 1, 8, 15, and 22. We collected blood samples at baseline, as well as multiple time points (days 7, 14, 21, and 28). Table I displays the clinical features and demographics of the selected patients. The Ethics Committee of our hospital approved this study, as well as the informed consent forms.

We used specific ELISA kits (USCN Life Science, Inc.) to measure the serum IL-35 levels in the samples, an FACSCalibur instrument (Becton Dickinson) was employed to measure the levels of cytokine-secreting CD4$^+$EBI3$^+$T-cells in the samples via flow cytometry, and we recorded the

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common laboratory parameters in this study. To assess the impact of rituximab on the number of CD4+EBI3+T-cells and serum IL-35 levels *in vivo*, we received blood samples from the 31 recent-onset type 1 diabetes patients at baseline and various time points following rituximab (375 mg/m^2) treatment.

Data were analysed using SPSS, v.23.0. The results are expressed as the mean ± standard deviation (SD). Independent samples *t*-test was employed to assess the relationship between clinical and laboratory parameters of type 1 diabetes patients. The Pearson correlation coefficient was utilised for the correlation analysis. Repeated measures analysis of variance (ANOVA) was used to compare the parameters before and after the treatment. Values with $p < 0.05$ (two-tailed) were regarded as statistically significant.

There was a substantial reduction in the serum IL-35 levels in type 1 diabetes patients, relative to healthy controls (58.63 ±12.56 pg/ml vs. 104.53 ±16.92 pg/ml, $p < 0.01$). Based on the results, the levels of CD4+EBI3+T-cells were considerably lower in the type 1 diabetes patients relative to the normal controls (1.02 ±0.29% vs. 1.97 ±0.35%, $p < 0.01$).

According to the results, the two factors were inversely related to the CRP (C-reactive protein) levels ($r^2 = 0.23$ and 0.17, $p < 0.05$ for each). The levels of IL-35 in the serum were substantially lower in the type 1 diabetes patients who exhibited increased glycosylated haemoglobin (HbA_{1c}) and decreased C-peptide ($r^2 = 0.31$ and 0.26, $p < 0.05$ for each). Also, we observed a marked reduction in $CD4^+EBI3^+$T-cell counts in the type 1 diabetes patients with decreased C-peptide levels ($r^2 = 0.27$, $p < 0.05$). Positive diabetes autoantibodies were associated with decreased serum IL-35 levels, as well as $CD4^+EBI3^+$T-cell counts, as shown in Table II.

The levels of IL-35 in the serum, as well as double-positive T cells in the 31 type 1 diabetes patients, increased gradually at day 7, 14, 21, and 28 after the four 375 mg/m^2 doses of rituximab treatment relative to those before treatment. Serum IL-35 levels (pg/ml): Baseline vs. Day7 vs. Day14 vs. Day21 vs. Day28 = (58.63 ±12.56) vs. (59.72 ±14.10) vs. (66.35 ±13.82) vs. (70.83 ±16.31) vs. (87.44 ±15.87)*; CD4+EBI3+T-cells (%): Baseline vs. Day7 vs. Day14 vs. Day21 vs. Day28 = (1.02 ±0.29) vs. (1.14 ±0.36) vs. (1.26 ±0.30) vs. (1.57 ±0.39)* vs. (1.86 ±0.47)**; *vs. baseline $p < 0.05$, **vs. baseline $p < 0.01$.

Several lines of evidence have linked type 1 diabetes to chronic inflammation [7, 8]. Neverthe-

Table I. General clinical characteristics and laboratory indicators of the type 1 diabetes patients and healthy controls

Categories	Type 1 diabetes patients	Healthy controls
Case number (*N*)	31	30
Age [years]	25.24 ±2.32	26.73 ±2.75
Male : female	14 : 17	15 : 15
Duration [months]	8.76 ±4.23	–
BMI [kg/m^2]	21.43 ±3.55	22.02 ±3.28
FBS [mg/dl]	191.43 ±89.35**	87.22 ±9.63
Chol [mg/dl]	167.83 ±38.81	165.11 ±19.27
TG [mg/dl]	86.82 ±31.34*	101.45 ±35.93
HDL [mg/dl]	51.45 ±19.94	49.34 ±9.54
LDL [mg/dl]	81.43 ±18.73	79.32 ±10.72
HbA_{1c} (%)	8.93 ±4.71**	5.16 ±0.19
CRP [mg/l]	3.45 ±1.75*	0.76 ±0.14
C-peptide levels (fasting) [ng/ml]	0.54 ±0.32**	3.29 ±0.83
GADA (+/–)	9/25	0/31
ICA (+/–)	8/23	0/31
IAA (+/–)	10/21	0/31

**vs. Healthy controls, p < 0.05, **vs. healthy controls, p < 0.01. BMI – body mass index, FBS – fasting blood glucose, Chol – cholesterol, TG – triglyceride, HDL – high-density lipoprotein, LDL – low-density lipoprotein. GADA – glutamic acid decarboxylase antibody, ICA – islet cell antibody, IAA – insulin autoantibody.*

Table II. Associations of the serum IL-35 level and the percentage of $CD4^+EBI3^+$T-cells with the laboratory parameters of the type 1 diabetes patients

Variable	±	Number	Serum IL-35 level [pg/ml]	*P*-value	$CD4^+EBI3^+$T-cells (%)	*P*-value
GADA (+)	+	9	70.93 ±13.28	0.04	1.06 ±0.13	0.04
	–	25	91.89 ±16.64		1.68 ±0.15	
ICA (+)	+	8	69.69 ±10.74	0.03	0.93 ±0.15	0.03
	–	23	89.81 ±12.37		1.61 ±0.18	
IAA (+)	+	10	75.29 ±9.47	0.03	1.02 ±0.16	0.02
	–	21	92.74 ±17.93		1.79 ±0.32	
Renal involvement	+	11	86.71 ±10.95	0.89	1.41 ±0.21	0.83
	–	20	88.95 ±12.22		1.62 ±0.24	

less, there have been few studies evaluating the function of anti-inflammatory cytokine in type 1 diabetes in humans. IL-35 is a novel anti-inflammatory cytokine that is secreted by Treg cells and which directly inhibits the proliferation of T cells [9]. Considering that IL-35 expression in β-cells could protect NOD mice against autoimmune diabetes, IL-35 is thought to have a direct function in type 1 diabetes progression [10]. These results imply that IL-35 might facilitate the occurrence of type 1 diabetes.

We compared serum levels of IL-35 between type 1 diabetes patients and their matching normal controls to determine whether IL-35 was abnormally expressed in the peripheral blood of patients with type 1 diabetes. Based on our results, IL-35 was considerably downregulated in type 1 diabetes patients relative compared to the healthy controls, implying that lower levels of IL-35 could play a crucial function in mediating type 1 diabetes. IL-35 suppresses the functioning of Teff cells and inhibits Th17 cell differentiation. Also, it is vital in modulating Treg cell activity [11]. Tregs assist in maintaining self-tolerance; they are scarce in most autoimmune disorders like type 1 diabetes [12].

IL-35 consists of EBI3 and p35; EBI3-p35 heterodimer might be a crucial modulator of immunity. Being the specificity subunit of IL-35, EBI3 can trigger the release of IL-17, IL-22, and RORγt [13]. However, the p35 gene has no obvious effect on autoimmune disease. Herein, we evaluated the expression of EBI3 in $CD4^+$T cells via flow cytometry. There was a considerable reduction in the $CD4^+EBI3^+$T-cell counts in active type 1 diabetes patients relative to healthy controls and inactive type 1 diabetes patients, indicating that $CD4^+EBI3^+$T-cells could function in the occurrence and progression of type 1 diabetes.

Herein, we demonstrated that the amount CD4+EBI3+T-cells, as well as the serum IL-35 levels, were negatively related to C-reactive protein (CRP), an essential chronic inflammation biomarker. Besides CRP, a negative correlation between IL-35 serum levels and glycated hemoglobin (HbA_{1c}) was observed. As a metabolic control indicator, HbA_{1c} levels provide a mean blood glucose index for a period of 3 to 4 months. Also, we observed a strong link in the serum IL-35 levels versus the C-peptide levels in type 1 diabetes. A similar correlation was found for the $CD4^+EBI3^+$T-cell counts. Additionally, positive diabetes autoantibodies that can predict progression to diabetes in adults was associated with decreased serum IL-35 levels. Similarly, $CD4^+EBI3^+$T-cell counts were prominently decreased in type 1 diabetes patients with the positive autoantibodies group, implying that the number of CD4+EBI3+T-cells and the serum levels of IL-35 are related to the severity of the disease.

More recently, studies show that B cells may be setting the T cells off by presenting them with antigens that stimulate the immune system. Rituximab is an antibody that depletes B cells and thus is effective against autoimmune disease. Administration of rituximab can deplete B cells and restore normal immune response. In this study, the effects of rituximab on immune response in type 1 diabetes patients were investigated. A few studies have observed no reduction in the levels of cytokines within the experimental periods, which could indicate that rituximab targets B cells. Also, given that the elevated cytokines are macrophage plus T-cell products, B-cell reduction therapy did not affect the cytokine levels. Interestingly, we observed a gradual increase in $CD4^+EBI3^+$ T-cell counts and the levels of IL-35 protein in type 1 diabetes patients following the administration of rituximab, implying that rituximab might modulate IL-35 expression and the amount of EBI3-producing $CD4^+$T-cells in type 1 diabetes patients. These observations raise two possible reasons. First, according to Pescovitz *et al.* [14], the B lymphocytes recover after rituximab treatment in type 1 diabetes patients, some evidence also indicates that B cells, as well as certain subsets of B cells, regulate immune responses [15] and ultimately influence the production of cytokines. Second, B cells contributed to the production of $CD4^+EBI3^+$ T-cells and IL-35 maybe through the activation by macrophages and T-cells indirectly, these data imply that IL-35 and EBI3-producing $CD4^+$T-cells might be crucial in preventing the onset of type 1 diabetes.

As far as we know, we are the first to reveal the relationship between serum IL-35 levels, as well as CD4+EBI3+T-cell counts and the occurrence of type 1 diabetes, implying that the two factors might play a crucial role in TID pathogenesis. Also, we revealed that rituximab administration can boost the levels of IL-35 in the serum, as well as the amount of CD4+EBI3+T-cells, implying that these two factors might play crucial roles in active type 1 diabetes patients. There are also some shortcomings in this paper: (i) the sample size was small; and (ii) the demographic and clinical characteristics of the patients and grading of disease activity may influence the results. The above shortcomings will be the focus of our work in the future.

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Han Ouyang and Jian Wen contributed equally to this study.

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Conflict of interest

The authors declare no conflict of interest.

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