Original Article

Regulatory T cells in children with recently diagnosed type 1 diabetes

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ABSTRACT

Background: Regulatory T cells have an important role in the control of immune reactivity against self antigens and probably play a role in pathogenesis of type 1 diabetes (T1D). We aimed to determine the frequency of regulatory T cells in recently diagnosed children with/T1D. **Materials and Methods:** 20 children with/T1D and 20 healthy children of matched age and sex as controls were enrolled in this study. All cases were subjected to a thorough history taking, full clinical examinations and investigations which include; insulin C peptide levels and flow cytometric detection of B-, T-lymphocytes and regulatory T cells. **Results:** Insulin C peptide level was significantly lower in children with/ T1D compared with controls. The percentages of B and T-lymphocytes were not significantly different between patients and controls. The percentages of CD4+CD25+High and CD4+CD25+High Foxp3+ cells both in total lymphocytes and in CD4+ lymphocytes were significantly decreased in patients than controls, while the percentages of total CD4+CD25+ and CD4+CD25+Intermediate both in total lymphocytes and in CD4+ lymphocytes were not significantly different between patients and controls. The geometric mean of fluorescence intensity (MFI) of Foxp3+ expression in CD4+CD25+High cells was significantly decreased in patients than controls. Positive correlations were observed between both age and insulin C peptide and frequency of CD4+CD25+High Foxp3. **Conclusion:** The percentage of regulatory T cells; CD4+CD25+High Foxp3 was decreased in children with recent T1D and may have a role in its pathogenesis. Their role as a prognostic signifi cance and their relation to various complications should be explored.

Key words: Insulin C peptide, regulatory T cell, type 1 diabetes

INTRODUCTION

Type 1 diabetes (T1D), is a chronic autoimmune disease and it is common in children The body's own immune system attacks the beta-cells in the islets of Langerhans of the pancreas, destroying or damaging them. It can lead to long-term complications including cardiovascular disease, blindness and kidney failure.[1] The damage of the beta-cells in the islets of Langerhans which caused by cytotoxic lymphocytes results in insulin deficiency and hyperglycemia. Environmental factors trigger/T1D in genetically susceptible individuals.[2] Autoreactive T-cells that recognize islet autoantigens have been identified and are thought to play a direct role in T1D immunopathogenesis.[3] The breakdown of beta cell-specific self-tolerance by T lymphocytes involves a number of dysregulated events intrinsic and extrinsic to T cells. The peripheral tolerance to self antigens is maintained through several regulatory mechanisms, including T regulatory cells. T regulatory cells are minor population of CD4+ T cells express high levels of CD25. It has an important role in the control of immune reactivity against self antigens, and probably plays a role in pathogenesis of T1D.[4,5] Different studies[1,6-9] had recently reported findings related to the frequency and function of regulatory T cells in T1D, but their results represents a somewhat conflicting body of findings. We aimed to estimate the frequency of regulatory T cells in recently diagnosed diabetes in children attending Assiut Children University Hospital, Egypt.

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Patients and Methods

Patients populations and sampling protocol

All aspects of this study were approved by the Assiut University Institutional Review Board. Patients and controls were enrolled after obtaining the informed consent from the parents. Twenty patients meeting the diagnostic criteria of T1D[10] were recruited consecutively at the Pediatric Clinical Endocrinology Unit, Children Hospital of Assiut University, Faculty of Medicine. In addition to twenty children with age and sex-matched, none of whom had either a personal or family history of diabetes or other autoimmune pathologies as control were included in the study.

Inclusion criteria
- Definite diagnosis of T1D according to the definition of the World Health Organization criteria[10] that defines this form of diabetes with permanent insulinopenia prone to ketoacidosis, result from a cellular-mediated autoimmune destruction of the beta cells of the pancreas.
- On insulin replacement therapy.
- Age range 2-16 years.
- Diabetic duration less than 12 weeks.

Exclusion criteria
- Children with secondary diabetes mellitus (DM).
- Children with type 2 DM.
- Evidence of active infection requiring antibiotic therapy or other concurrent diseases.
- Other autoimmune disease.
- Age <2 years >16 years.

Methodology

All cases were subjected to:
- Full history including demographic factors: age, sex, residence, family history of diabetes.
- Full clinical examination.
- Complete blood count (Celltac E automated hematology analyzer, Tokyo, Japan).
- Serum insulin C-peptide levels were measured by radioimmunoassay using commercial kits (Diagnostic Systems Laboratories Inc, Webster, Texas). Fasting normal insulin C peptide=0.78-5.19 ng/ml.[11]
- Flow cytometric detection of regulatory T cells, B-lymphocytes and T-lymphocytes.

Flow cytometric detection of regulatory T cells, B-lymphocytes and T-lymphocytes

$CD4^+CD25^+Foxp3^+$ regulatory T cells in whole blood samples were enumerated using fluoroisothiocyanate (FITC)-conjugated forkhead box protein 3 (Foxp3) (e Bioscience, USA), phycoerythrin (PE) conjugated CD25 (IQ Product, The Netherland) and peridinium-chlorophyll-protein (Per-CP)-conjugated CD4 (Becton Dickinson, Bioscience, USA). Fifty μl of blood sample was incubated with 10 μl of CD4, CD25 for 15 minutes at room temperature in the dark. Following incubation, RBC lysis, washing with phosphate buffer saline (PBS), addition of fixed solution to fix the cells and incubation for 10 minutes were done. After incubation, cells were washed with PBS, and then permelized solution and 10 μl of Foxp3 were added and incubated for 30 minutes at room temperature. For detection of B- and T-lymphocytes, 50 μl of blood sample was stained with 10 μl of FITC-conjugated CD19 and PE-conjugated CD3 (Becton Dickinson Biosciences, USA). The tubes were incubated for 15 minutes at room temperature in the dark. RBC lysis was done. After one wash, the cells were resuspended in PBS. Flow cytometric analysis was done by FACSCalibur flow cytometry with CellQuest software (Becton Dickinson Biosciences, USA). An isotype-matched negative control was used with each sample. Forward and side scatter histogram was used to define the lymphocyte population (R1). Total $CD4^+CD25^+$, $CD4^+CD25^{+intermediate}$, $CD4^+CD25^{+High}$ (defined as the population of CD4 positive T cells whose CD25 expression exceeded the level of CD25 positivity seen in the CD4 negative T cells)[12,13] and $CD4^+CD25^{+High}$ $Foxp3^+$ regulatory T cells was evaluated as a percentage of total lymphocytes and of $CD4^+$ as shown in Figure 1. The expression of $Foxp3^+$ in $CD4^+CD25^{+intermediate}$ and in $CD4^+CD25^{+high}$ cells was expressed as geometric mean of fluorescence intensity (MFI).

Statistical analysis

Statistical package for social sciences (SPSS), version 16 was used for data analysis. All data were expressed as the mean± standard error of mean (SEM). Due to the small sample size and a propensity for outliers in some of the variables, Mann-Whitney analysis was used to detect the statistical significance differences between groups. A *P* value of ≤0.05 denoted the presence of a statistically significant difference.

Results

Some demographic and clinical data of diabetic children and controls were presented in Table 1.

There were no significant difference in white blood cells count, platelet count and hemoglobin concentration between diabetic patients and controls [Table 2]. The level of insulin C peptide was significantly lower in children with/T1D compared with controls with $P < 0.000$.

There were no significant difference in the percentages of T lymphocytes (CD3$^+$), B lymphocytes (CD19$^+$) and T helper cells (CD4$^+$) between patients than controls [Table 3]. The percentages of total CD4$^+$CD25$^+$ and CD4$^+$CD25$^{+Intermediate}$ in total lymphocytes were not significantly different between patients and controls. The percentages of CD4$^+$CD25^{+High} and CD4$^+$CD25^{+High} Foxp3$^+$ in total lymphocytes were significantly decreased in patients than controls. Similar results were observed when these cells were analyzed as a percentage of CD4$^+$ T cells.

The MFI of Foxp3$^+$ expression in CD4$^+$CD25^{+High} Foxp3 cells was significantly decreased in patients than controls, while MFI of Foxp3$^+$ expression in CD4$^+$CD25$^{+Intermediate}$ cells was not significantly different between patients and controls [Table 3].

The frequency of CD4$^+$CD25^{+High} Foxp3 was positively correlated with age of the patients (r = 0.585, $P < 0.000$), and the level of insulin C peptide (r = 682, $P < 0.000$) [Figures 2 and 3].

Table 1: Some demographic and clinical data of diabetic children and control

	Patients (*n*=20)	Controls (*n*=20)
Age	8.51±0.89	8.84±0.93
Sex (male/female)	9/11	8/12
Duration of diabetes (weeks)	10.13±1.52	-
Diabetic ketoacidosis (n)	1.2±0.21	-
Insulin dose (unit/kg)	0.7	-

DISCUSSION

Type 1 diabetes is a well-known autoimmune disease; however there are still some processes in its pathogenesis to be elucidated. T regulatory cells are essential for maintaining peripheral tolerance, preventing autoimmune diseases and limiting chronic inflammatory diseases. These cells modulate the intensity and quality of immune reactions through attenuation of the cytolytic activities of reactive immune cells.[14]

In this study, CD4$^+$CD25^{+High} Foxp3$^+$ cells were considered as regulatory T cells, as the suppressive capacity of regulatory T cells in humans seems to be confined to CD4$^+$CD25$^+$cells with the highest expression of CD25 (CD4$^+$CD25^{High}, whereas CD4$^+$T cell with intermediate expression of CD25 might also contain recently activated T cells and effector T-cells without regulatory function.[15-17] Also, the identification of Foxp3 as a regulatory lineage specific factor provided a useful phenotypic and optimal marker for regulatory T cells,[18-20] and the suppressive phenotype and the development of regulatory function depend on the expression of Foxp3.[21-24] Indeed, recent results indicate that Foxp3 behaves as a master regulator of the regulatory T cells phenotype.

We found the frequency of CD4$^+$CD25^{+High} and CD4$^+$CD25^{+High} Foxp3$^+$ both in total lymphocytes and in CD4$^+$cells were significantly decreased in diabetic patients

Table 2: Some laboratory characteristics of diabetic patients and controls

	Patients (*n*=20)	Controls (*n*=20)	*P* value
Blood glucose concentration (mg/dl)	223±2.66	87.76±8.98	0.002
Insulin C peptide (ng/ml)	0.67±0.07	2.41±0.21	0.000
Platelets (109/L)	187.69±15.62	226.04±18.54	0.089
WBCs (109/L)	5.97±0.34	5.51±0.30	0. 357
Hemoglobin (gm/dl)	10.79±0.34	11.47±0.31	0.360

Mann-Whitney test: Data represented as means±SEM, *P*≤0.05 is significant, WBCs: White blood cells

Table 3: Regulatory T cells in diabetic patients and controls

Percentage	Patients (20)	Control (20)	*P* value
Lymphocytes	50.39±3.13	55.71±3.19	0.279
CD3$^+$	54.30±1.33	55.46±1.66	0.671
CD 19$^+$	12.52±0.50	13.13±0.46	0.271
CD4$^+$	39.58±1.73	42.03±1.52	0.133
CD25$^+$/total lymphocytes	4.86±0.24	4.73±0.23	0.957
CD4$^+$CD25$^+$/CD4$^+$	13.91±0.68	14.97±0.59	0.189
CD4$^+$CD25$^{+Intermediate}$/total lymphocytes	4.03±0.15	4.47±0.29	0.133
CD4$^+$CD25^{Intermediate}/CD4$^+$	11.34±0.61	10.11±0.37	0.130
CD4$^+$CD25^{+High}/total lymphocytes	1.05±0.14	1.70±0.21	0.022
CD4$^+$CD25^{+High}/CD4$^+$	1.85±0.19	4.34±0.39	0.000
CD4$^+$CD25^{+High} Foxp3$^+$/total lymphocytes	0.36±0.57	0.84±0.10	0.000
CD4$^+$CD25^{+High} Foxp3$^+$/CD4$^+$	1.16±0.09	2.28±0.18	0.000
MFI of Foxp3$^+$ expression in CD4$^+$CD25$^{+Intermediate}$	29.05±2.49	37.09±3.50	0.072
MFI of Foxp3$^+$ expression in CD4$^+$CD25^{+High}	44.68±2.34	74.81±3.47	0.000

Mann-Whitney test: Data represented as means±SEM, *P*≤0.05 is significant, Foxp3: Forkhead box protein 3, MFI: Mean fluorescent intensity
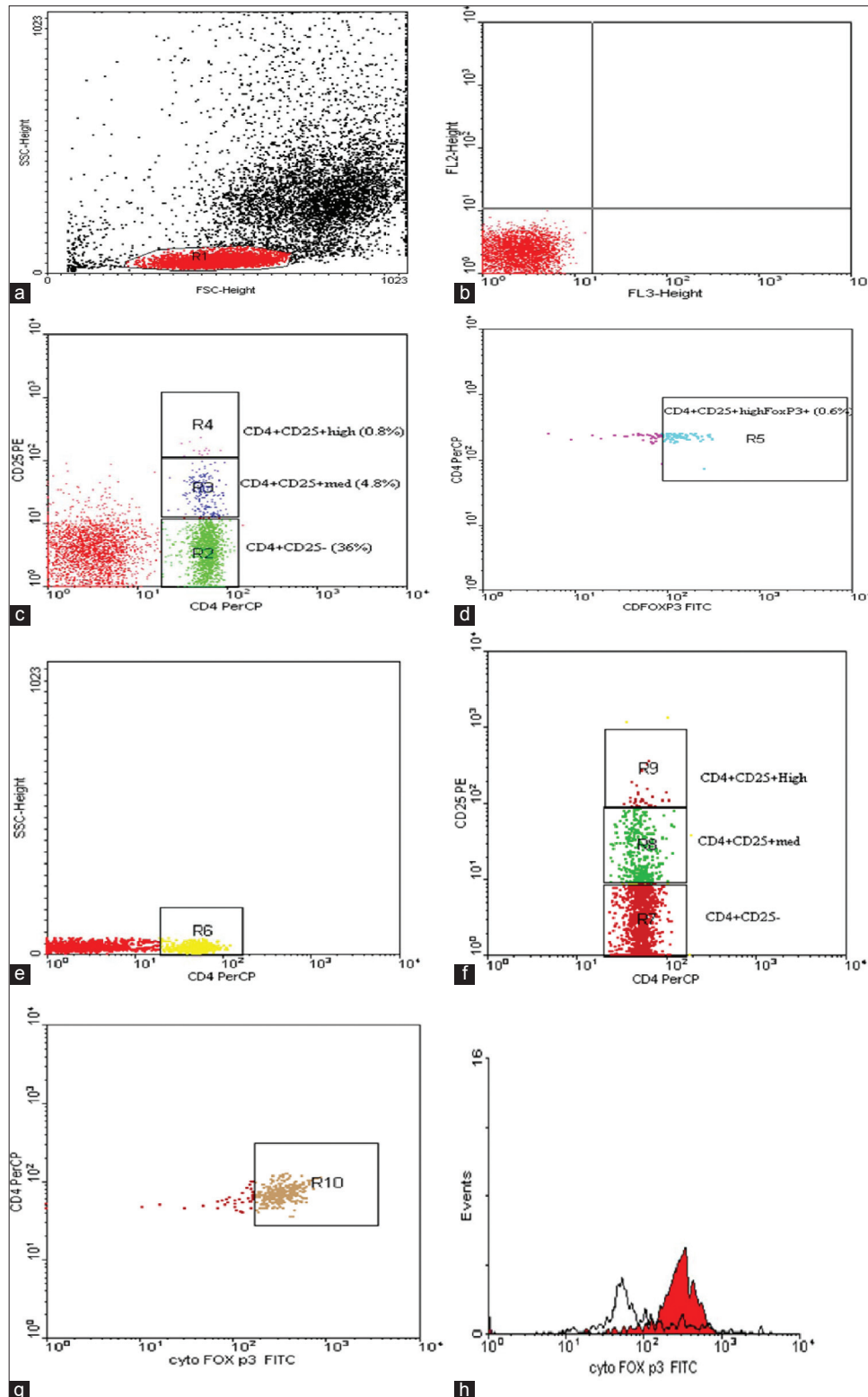
Figure 1: Flow cytometric detection of regulatory T cells. (a) Forward and side scatter histogram was used to define the lymphocytes population (R1). (b,c) The expression of CD4 and CD25 in total lymphocytes (R1) was detected, compared with the negative isotype control and and different gates were drown to define CD4+ CD25-cells (R2), CD4+CD25+intermediate (med) cells (R3), and CD4+CD25+High cells (R4). (d) The percentage of CD4+CD25+high FoxP3+cells (R5) in total lymphocytes was determined. (e-g) Show the analysis of regulatory T cells in CD4+ cells (R 6). CD4+ CD25-cells (R7), CD4+CD25+intermediate (med) cells (R8), and CD4+CD25+High cells (R9). (h) Show the expression as a geometric mean of fluorescence intensity (MFI) of FoxP3+ in CD4+CD25+high cells. The positivity was defined as fluorescence (red histogram) higher than that of the isotype control (open histogram)

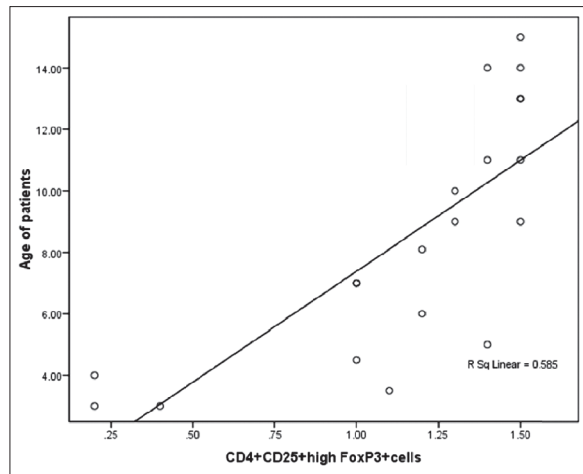**Figure 2:** Correlations between the frequency of CD4+CD25+HighFoxp3 and age of diabetic patients

Figure 3: Correlations between the frequency of CD4+CD25+HighFoxp3 and level of insulin C peptide

than controls while the frequency of total CD4+CD25+and CD4+CD25+Intermediate both in total lymphocytes and in CD4+cells were not significantly different in patients and controls. This decline in the frequency of CD4+CD25+High and CD4+ CD25+High Foxp3+ T cells in our patients could implies that the deficiency of regulatory T cells may has a role in the pathogenesis of type 1 diabetes. In accordance with our results, Luczyński *et al.*,[6] found a statistically significant decrease of T regulatory cells in children with newly diagnosed/T1D. Ryba *et al.*,[7] also reported lower percentage of regulatory T cells in children with/T1D. Luczyński *et al.*,[1] reported that percentage of CD4+CD25+High was decreased in diabetic patients than controls, the same as our study, while the percentages of CD4+CD25highCD127dim/− were very low and did not differ between T1D and control children and this difference could be due to the of different markers of regulatory T cells they used, CD127dim/− and not foxp3 as our study.

Brusko *et al.*[9] Putnam *et al.*[25] and Lindley *et al.*,[26] reported that there is no difference in the level of regulatory T cells between patients with/T1D and healthy controls. However, in these studies, the patients were adult[25] or have long lasting diabetes.[26] In Brusko *et al.*,[9] their patients and controls are older than our patients, and their controls from considerably older than their patients.

Glisic-Milosavljevic *et al.*,[27] reported that there is a higher level of ongoing apoptosis in CD4+CD25+High T cells in recent-onset T1D subjects and in subjects at high risk for the disease. On the contrary, in long-standing/T1D and/T2D subjects, CD4+CD25+high T cell apoptosis is at the same level as in control subjects. This high level of CD4+CD25+High T-cell apoptosis could explain the decrease of regulatory T cells in our patients.
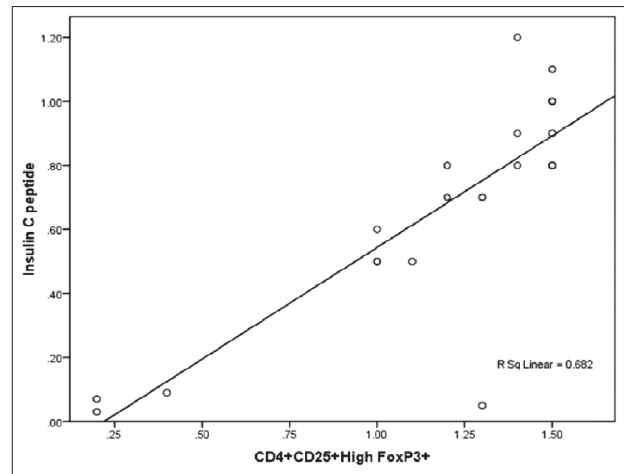
Foxp3, is a critical molecular switch for the genetic programming of natural regulatory T cell development and function.[24,28] In this study; the MFI of foxp3 expression in CD4+CD25High cells were 44.68 ± 2.34 and 74.81 ± 3.47 in patients and controls respectively, while the MFI of foxP3 expression in CD4+CD25intermediate were 29.05 ± 2.49 and 37.09 ± 3.50 in patients and controls respectively. These results are consistent with those of Qian *et al.*[29]

The expression of Foxp3+ in CD4+CD25+HighFoxp3 cells was significantly decreased in diabetic patients than controls, while its expression in CD4+CD25+Intermediate cells was not significantly different between patients and controls. Lawson *et al.* reported that there was no difference in Foxp3 expression on CD4+CD25High T cells in patients with/T1D, but in contrast to our study the patients had long standing diabetes, and both patients and controls were adult subjects.[30]

In the present study, CD4+CD25+HighFoxp3 was positively correlated with age of diabetic children. Brusko *et al.*,[31] reported in their study that increasing age was associated with an increase in total CD4+CD25+ frequency.

In the present study, Insulin C peptide level was significantly lower in children with/T1D compared with control. In addition, the frequency of CD4+CD25+High Foxp3 was positively and significanty correlated with the level of insulin C peptide. Insulin C-peptide level is the most reliable factor in evaluation of the endogenous insulin secretion in patients with/T1D. Autoimmune destruction of the beta cells of pancreas results in deficiency of both insulin and insulin C-peptide.[32]

CONCLUSIONS

This study concluded that children with/T1D have lower percentages of T regulatory cells in the peripheral blood which correlated positively with age of patients and the level of insulin C peptide.

Limitations of the study

1. The percentages of regulatory T cells were assessed in the peripheral blood but not at the site of affection (pancreas and/or draining lymph nodes).
2. The distinction of regulatory T cells by a flow cytometry including-high expression of CD25 antigen is very subjective and can result in different findings from different laboratories.

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