Arch. Immunol. Ther. Exp. (2012) 60:307–313
DOI 10.1007/s00005-012-0177-y

ORIGINAL ARTICLE

Low Frequency of Regulatory T Cells in the Peripheral Blood of Children with Type 1 Diabetes Diagnosed under the Age of Five

Agnieszka Szypowska · Anna Stelmaszczyk-Emmel · Urszula Demkow · Włodzimierz Łuczyński

Received: 20 October 2011 / Accepted: 11 May 2012 / Published online: 9 June 2012

Abstract The highest annual increase in the incidence of type 1 diabetes (T1D) in children under the age of 5 years and aggressive process of β-cell destruction in this age group indicate the need to assess the immune system. The aim of this study was to evaluate regulatory T cells (Tregs) frequency in the peripheral blood of children <5 years of age with newly diagnosed T1D in comparison with diabetic children diagnosed at a later age and healthy controls. 40 children with newly diagnosed T1D (20 children <5 years of age and 20 older patients) and 40 age-matched controls were included in this study. Flow cytometric analysis of Tregs was performed using the following markers: CD4, CD25, CD127, FoxP3, IL-10, and TGF-β. Apoptosis was measured using anti-active caspase 3 monoclonal antibody. Fasting C-peptide and HbA1c were monitored as well. We showed that T1D children <5 years had lower C-peptide concentration than diabetic children ≥5 years of age (0.32 vs. 0.80 ng/ml, respectively, $p = 0.0005$). There was lower frequency of $CD4^+CD25^{high}CD127^{low}FoxP3^+$ Tregs in T1D children <5 years than ≥5 years of age (0.87 vs. 1.56 %, respectively, $p = 0.017$). Diabetic children <5 years had lower $CD4^+CD25^{high}CD127^{low}FoxP3^+$, $CD4^+CD25^{high}$IL-10, and $CD4^+CD25^{high}$TGF-β Tregs compared to age-matched controls. There was no difference in Tregs apoptosis between the examined groups. This study highlights the distinctiveness of diabetes in children <5 years of age. Understanding the differences of immune system activity in the young diabetic children would open the way to identify children at risk for T1D and enables the use of novel forms of intervention.

Keywords Tregs · FoxP3 · IL-10 · TGF-β

A. Szypowska (✉)
Department of Pediatrics, Medical University of Warsaw, Dzialdowska 1, 01-184 Warsaw, Poland
e-mail: agnieszka.szypowska@gmail.com

A. Stelmaszczyk-Emmel · U. Demkow
Department of Laboratory Diagnostics and Clinical Immunology of Developmental Age, Medical University of Warsaw, Warsaw, Poland

W. Łuczyński
Department of Pediatrics Endocrinology, Diabetology with Cardiology Division, Medical University of Białystok, Białystok, Poland

Introduction

Recently, the highest annual increase in the incidence of type 1 diabetes (T1D) is observed in children below 5 years of age. The results of the EURODIAB study showed that the overall annual increase in type 1 diabetes in children under 15 years old was 3.9 % (Patterson et al. 2009). However, in children with diabetes recognized before 5 years the annual increase in T1D was estimated at 5.4 % (4.8–6.1). It is predicted that by 2020 the number of diabetic children under 5 years will have doubled. Moreover, there are data suggesting that the clinical course of diabetes in children under 5 years of age is different (Komulainen et al. 1996; Abdul-Rasoul et al. 2006). These studies showed that the process of β-cell destruction is more aggressive in children with early-onset diabetes than in patients diagnosed at a later age.

Type 1 diabetes is caused by a progressive autoimmune destruction of the pancreatic β-cells. The autoimmune process usually begins years before the first clinical symptoms become apparent. It is not entirely clear as to why β cell destruction is so aggressive in the youngest of children. A growing interest has recently been directed

towards a population of regulatory T cells (Tregs) which plays a crucial role in the maintenance of homeostasis and self-tolerance through their inhibitory impact on autoreactive effector T cells (Sojka et al. 2008). The suppressive capacity of Tregs is not only mainly contact-dependent but also based on the production of immunosuppressive cytokines including interleukin (IL)-10 and transforming growth factor (TGF)-β. Tregs have been characterized by a high expression of CD25, low expression of IL-7 receptor (CD127) and a lineage-specific transcription factor, more intimately associated with Tregs, transcription factor forkhead box P3 (FoxP3) (Liu et al. 2006). Abnormalities of Tregs, either in cell number or in function, are associated with the initiation and progression of type 1 diabetes (Pop et al. 2005).

To our best knowledge, there are no prior studies evaluating Tregs population in diabetic children under the age of 5. We speculated that in this age group the pronounced autoimmune process may be due to a lower percentage of regulatory T lymphocytes and a lower production of immunosuppressive cytokines. We performed this study to test this hypothesis.

The aim of the study was to assess the frequency of $CD4^+CD25^{high}CD127^{low}FoxP3^+$ regulatory T cells and $CD4^+CD25^{high}$ cells with IL-10 or TGF-β expression as well as evaluate Tregs apoptosis in the peripheral blood of children under 5 years of age with newly diagnosed T1D and compare the results with those of both children diagnosed at a later age and with those of age-matched healthy subjects.

Materials and Methods

Peripheral blood samples were obtained from 80 Caucasian children: 40 with newly recognized T1D treated in the Department of Pediatrics at the Medical University of Warsaw and 40 age-matched healthy controls. All subjects were divided into age-dependent groups. There was no difference in age between both study groups and corresponding control groups. The characteristic of the patients is shown in Table 1. T1D was diagnosed according to the ISPAD criteria (Craig et al. 2009). All patients with newly recognized diabetes had the presenting symptoms of hyperglycemia and a random serum glucose test of over 11.1 mmol/l. Intensive insulin therapy was given to all diabetic children. Inclusion criteria for the control group were a fasting blood glucose of <5.5 mmol/l and no personal or familial history of T1D. Patients with signs of other autoimmune, chronic, inflammatory or neoplasmatic disease were also excluded. The study was approved by the local ethics committee and informed consent was obtained from all parents and participants over 16 years old. In diabetic patients, peripheral blood samples were collected in the second week of hospitalization after obtaining metabolic compensation. β-cell function was measured by a fasting C-peptide (Greenbaum et al. 2008). C-peptide immunoradiometric assay (IRMA-C-PEP) was used for the determination of C-peptide serum. The equipment employed was manufactured by CIS Bio international and GIF-SUR-YVETTE CEDEX (France), normal range 1.06–3.53 ng/ml.

Flow Cytometry Analysis

Whole blood was collected on K-EDTA and immediately subjected to cellular staining. Mononuclear cells were isolated using a standard Ficoll-Histopaque®-1077 (Sigma Aldrich Co, St. Louis, USA) gradient centrifugation (Sigma Diagnostic Instruction Manual). Flow cytometric analyses were performed using the following monoclonal antibodies purchased from Becton–Dickinson (USA): anti-CD4 (PE-Cy-5), anti-CD25 (PE-Cy7), anti-CD127 (PE), and anti-FoxP3 (Alexa Flour 488). $CD4^+CD25^{high}$ cells were analyzed for interleukin expression using IL-10 (PE) and TGF-β (PE) purchased from R&D (USA). Appropriate isotype control antibodies were used. Intracellular staining for the assessment of FoxP3, IL-10, and TGF-β was performed according to the manufacturer's instructions (Human FoxP3 Buffer Set from Beckton Dickinson, IntraPrep from Beckman-Coulter (Poland), respectively). The percentage of apoptotic cells was measured within $CD4^+CD25^{high}FoxP3^+$ cells. Intracellular staining for the assessment of FoxP3 (Alexa Fluor 488) and active caspase-3 (PE) level was performed according to the manufacturer's instructions (Human FoxP3 Buffer Set from Beckton Dickinson). Samples were analyzed within 12 h on a Cytomics FC500 flow cytometer (Beckman Coulter, Poland). A minimum of 10^5 events was acquired for each analysis. The percentages of positive cells were calculated.

Table 1 Characteristic of children included into analysis

Patients	Number	Gender F/M	Age (years)	HbA1c (%)
T1D <5 years	20	7/13	2.8 ± 1.1 (0.9–4.9)	11.8 ± 1.8
T1D ≥5 years	20	11/9	12.9 ± 3.4 (5–16.5)	11.9 ± 1.8
Control <5 years	20	9/11	2.6 ± 1.0 (1.3–4.6)	–
Control ≥5 years	20	10/10	10.7 ± 3.7 (5.3–15.9)	–

To determine absolute cell counts, a small volume of blood was analyzed for complete blood count with a differential when available. Absolute counts were determined by multiplying the frequency of positive cells determined in the cytometric analysis by the number of lymphocytes $\times 10^3/\mu l$ as determined by complete blood count. No differences were seen between the study groups in any immunological parameters of the peripheral blood mononuclear cells (data not shown). The gating strategy for Tregs is shown in Fig. 1.

Statistical Analysis

The results were analyzed in Statistica 8.0 for Windows (StatSoft, Poland) and GraphPad Prism 5.0 (GraphPad Software, USA). The assumption that data were sampled

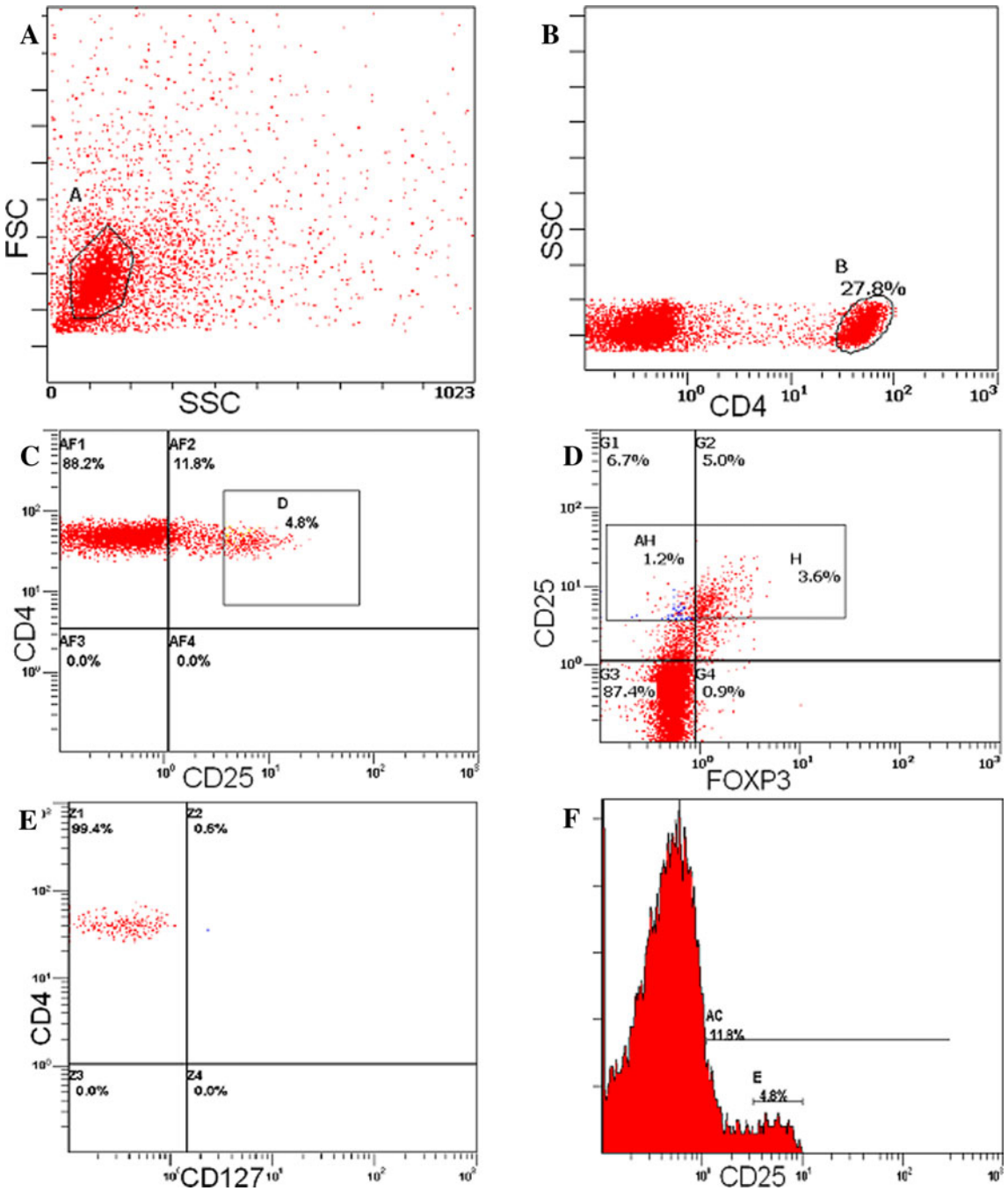

Fig. 1 Gating strategy for Treg cells. Sample gated on lymphocytes according to forward and side scatter (FSC/SSC) (**a**), *plots* showing gate for $CD4^+$ T cells (**b**) and $CD4^+CD25^{high}$ cells (**c**), $FoxP3^+$ expression in the gate H(D), low CD127 expression on cells from the gate H (**e**), a *fluorescence histogram* shows the gating strategy of $CD4^+CD25^{high}$ cells from the gate D (**f**)

from populations that follow Gaussian distributions was tested using the Kolmogorov and Smirnov methods. The comparisons between groups were made using the Student *t* test (unpaired, 2-tailed) or, in the case of non-parametric data, by the Mann–Whitney *U* test. Results are presented as mean values with standard deviations (SD) or for non-parametric data as median values and 25–75th percentile. The degree of association between two variables was calculated by Spearman rank correlation coefficient (*r*). *p* values less than 0.05 were considered as significant.

Results

Fasting C-Peptide

There was a significantly lower fasting C-peptide level in diabetic children below 5 years than in subjects with diabetes presenting over 5 years of age; median value 0.32 ng/ml (0.27–0.62) vs. 0.80 ng/ml (0.52–1.07), respectively; $p = 0.0005$ (Fig. 2). There was significant correlation between the age of diabetic children and their fasting C-peptide level $r = 0.64$, 95 % CI [0.39 to 0.8], $p < 0.0001$. There was no correlation between fasting C-peptide levels and the frequency of the following Tregs: $CD4^+CD25^{high}CD127^{low}FoxP3^+$, ($r = 0.01$, 95 % CI [−0.31 to 0.34]; $p = 0.932$); $CD4^+CD25^{high}$ with IL-10 expression ($r = –0.15$, 95 % CI [−0.45 to 0.18]; $p = 0.357$), and $CD4^+CD25^{high}$ with TGF-β expression ($r = 0.27$, 95 % CI [−0.06 to 0.55]; $p = 0.109$).

Flow Cytometry Analysis

The frequency of $CD4^+CD25^{high}CD127^{low}FoxP3^+$ Tregs was lower in T1D children below the age of 5 compared with diabetic children over 5 years of age 0.87 % (0.6–1.43) vs. 1.56 % (0.84–2.42), respectively ($p = 0.017$). The percentage of such cells was lower in T1D children below 5 years than levels shown in the age-matched healthy controls; median values were 0.87 % (0.6–1.43) vs. 4.53 % (3.56–5.71), respectively ($p < 0.0001$). The frequency of this subpopulation of Tregs was also lower in T1D children over 5 years of age (1.56 % [0.84–2.42]) when compared to the age-matched control subjects (3.44 % [2.24–4.72]); $p = 0.0009$. There was no difference in Tregs between the control group over 5 years of age and those younger than 5 years; median values of 3.44 % (2.24–4.72) vs. 4.53 % (3.56–5.71), respectively; $p = 0.058$ (Fig. 3). The correlation between the age of diabetic children and the percentage of $CD4^+CD25^{high}CD127^{low}FoxP3^+$ Tregs was close to statistical significance ($r = 0.321$, 95 % CI [0.001–0.58]; $p = 0.05$). No correlation was noticed between the age of healthy children and the percentage of $CD4^+CD25^{high}CD127^{low}FoxP3^+$ cells ($r = 0.22$, 95 % CI [−0.11 to 0.50]; $p = 0.189$).

The frequency of $CD4^+CD25^{high}$ cells with IL-10 expression was significantly lower in T1D children below the age of 5 than the age-matched healthy controls; median values of 0.87 % (0.23–1.68) vs. 1.92 % (0.56–3.06), respectively ($p = 0.036$). There was no significant difference in the percentage of these cells between children with diabetes diagnosed below and over the age of 5. Also, no difference was noted between T1D children below 5 years of age and age-matched healthy controls (Fig. 4). There was no correlation between age of diabetic children and percentage of $CD4^+CD25^{high}$IL-10 cells ($r = 0.11$ 95 % CI [−0.21 to 0.42]; $p = 0.492$). Also, no correlation was found between the age of healthy controls and the frequency of $CD4^+CD25^{high}$IL-10 cells ($r = –0.24$ 95 % CI [−0.51 to 0.08]; $p = 0.142$).

The frequency of $CD4^+CD25^{high}$ cells with TGF-β expression was significantly lower in T1D children below 5 years of age compared to age-matched healthy controls,

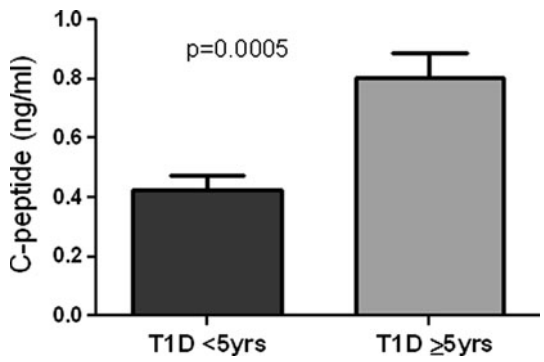

Fig. 2 The comparison between C-peptide levels in children with diabetes recognized before and after 5 years of age. Data shown as median values with 25–75 percentiles

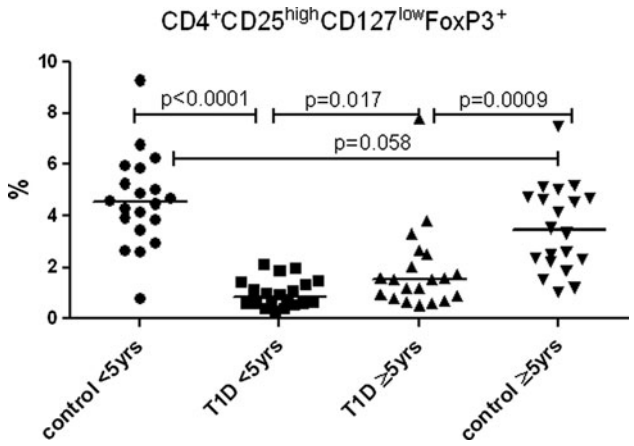

Fig. 3 Between groups comparisons in the frequency of $CD4^+CD25^{high}CD127^{low}FoxP3^+$ Tregs. Data are shown as median values

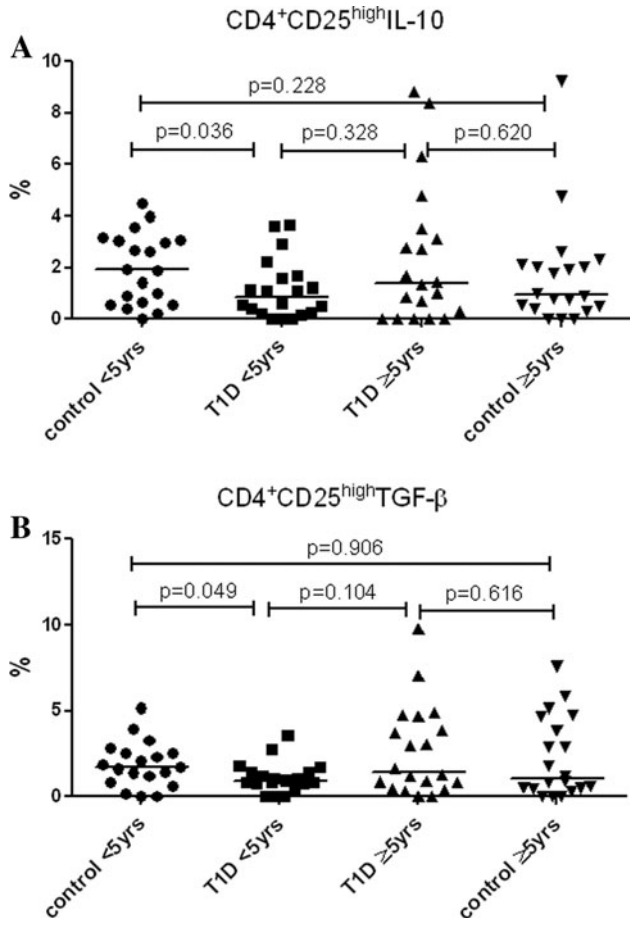

Fig. 4 Between groups comparisons in the frequency of $CD4^+CD25^{high}$ cells with IL-10 expression (**a**) and TGF-β expression (**b**). Data are shown as median values

median values of 0.93 % (0.76–1.46) vs. 1.74 % (0.87–2.52), respectively ($p = 0.049$). There was no significant difference in the percentage of these cells between children with diabetes diagnosed below and over the age of 5. Also, no difference was noted between T1D children under 5 years of age and age-matched healthy controls (Fig. 4). There was a correlation between the age of diabetic children and the percentage of $CD4^+CD25^{high}$TGF-β cells ($r = 0.393$ 95 % CI [0.08–0.63]; $p = 0.015$). No correlation was noticed between the age of healthy controls and percentage of $CD4^+CD25^{high}$TGF-β cells ($r = -0.07$ 95 % CI [−0.37 to 0.25]; $p = 0.658$).

There was no difference in the frequency of apoptotic $CD4^+CD25^{high}FoxP3^+$ cells between diabetic children under the age of 5 and age-matched healthy controls, median value of 0.30 % (0–5.58) vs. 0.23 % (0–0.75), respectively ($p = 0.383$). No difference in the frequency of apoptotic $CD4^+CD25^{high}FoxP3^+$ cells was noted between children with diabetes recognized over the age of 5 (median value 0 %; range 0–0.91) and age-matched healthy controls (median value 0 %; range 0–0.38; $p = 0.680$). There was no difference in the percentage of these cells between children with diabetes diagnosed under and over the age of 5. The inverse correlation between the C-peptide level and the percentage of apoptotic $CD4^+CD25^{high}$-$FoxP3^+$ cells ($r = -0.31$, 95 % CI [−0.58 to 0.02], $p = 0.060$) was close to statistical significance.

Discussion

We noted an extremely low frequency of Tregs in children under the age of 5 with newly diagnosed type 1 diabetes. The frequency of Tregs was lower in the youngest children as compared to children with diabetes recognized at a later age and to age-matched healthy controls. Moreover, the correlation between the age of diabetic children and the percentage of $CD4^+CD25^{high}CD127^{low}FoxP3^+$ Tregs was close to statistical significance ($p = 0.05$).

Abnormalities in Tregs may significantly disturb the balance between activation and suppression of the immune system, contributing to destruction of the β cells of the pancreas (Vrabelova et al. 2008). Over the years, authors have been repeatedly drawn towards investigating different courses of diabetes in the youngest children. Young age is associated with a lower C-peptide concentration which reflects more aggressive autoimmune process and a lower absolute β-cell mass in this age group than the children with diabetes presenting at a later age. The strength of our study was the homogeneity of populations studied and the inclusion of a unique group of very young children. The average age in the group with diabetes diagnosed below 5 years of age was 2.8 years. We found a lower C-peptide concentration in the youngest diabetic children than in children with diabetes diagnosed at a later age. This correlates with the results of other authors. Previous studies showed that children below the age of 5 had a low partial remission (Bowden et al. 2008). Moreover, no remission was observed in children under the age of 3 (Abdul-Rasoul et al. 2006). Partial remission is common in patients with newly recognized diabetes (68.9 %), and this period is characterized by a reduced insulin requirement with good metabolic control (Bober et al. 2001). Lack of remission in the youngest children reflects a more progressive and rapid destruction of the β cells and a lower residual insulin level at disease onset. Unfortunately, it is not clear why the incidence of diabetes is so high and the process of β-cell destruction is so rapid in the youngest children. These observations indicate the need to assess the immune system of young diabetic children to shed light on the pathogenesis of this phenomenon.

$FoxP3^+$ Tregs have been implicated as a central control target in T1D progression and any defects in their development or function may represent a major predisposing factor for spontaneous autoimmunity in animals (Tritt et al.

2008; Chen and Liu 2009). Some human studies have shown significantly reduced numbers of Tregs in T1D patients (Kukreja et al. 2002; Łuczyński et al. 2009). In contrast, other studies have not observed any differences in the frequency of these cells between diabetic patients and control subjects (Brusko et al. 2005; Putnam et al. 2005). Minimal research has been conducted within the pediatric population. Current studies have noted a low frequency of Tregs in children with newly diagnosed T1D (Sanda et al. 2008; Łuczyński et al. 2009). Moreover, Sanda et al. (2008) found no adverse effects of hyperglycemia and severe metabolic stress on the expression of FoxP3 and CD127. In contrast, Brusko et al. (2005) who included both children and young adults did not find any differences in the frequency of Tregs in individuals with new-onset T1D compared to controls. Low frequency of Tregs was also confirmed in children with long-standing diabetes and poor glycemic control (Ryba et al. 2011). The authors demonstrated that diabetic children had a lower percentage of $CD4^+CD25^{high}$ and $CD4^+CD25^{high}$ cells with CD62L expression and a higher percentage of $CD4^+CD25^{high}$ cells expressing TNFR2 than healthy individuals. It was suggested that these abnormalities might be associated with inflammatory conditions. No published trials included very young children into analysis.

One of the reasons of a low frequency of Tregs observed in T1D patients might be Tregs apoptosis. Glisic-Milosavljevic et al. (2007b) found high levels of $CD4^+25^{high}$ T cell apoptosis in patients with newly diagnosed T1D and in unaffected relatives of diabetic patients, who were positive for multiple antibodies. In another study, Glisic-Milosavljevic et al. (2007a) showed that apoptosis of $CD4^+CD25^{high}$ T cells was high after diabetes onset and decreasing during the remission period. They found a negative correlation between Tregs apoptosis and total dose of insulin. However, high apoptosis was noted neither in long-standing diabetic patients nor in pediatric patients after 6 months following diagnosis. Thus, it was suggested that some differences in Tregs apoptosis might be observed in children with varying degrees of β-cell destruction. The inclusion of two groups of newly diagnosed diabetic children with statistically different C-peptide concentrations enabled us to compare the differences in Tregs apoptosis between participants with varying degrees of β-cell destruction. However, we did not find any differences in the apoptosis of Tregs in children with diabetes diagnosed before and after the age of 5. Moreover, similar to our previous report (Szypowska et al. 2010), no difference in Tregs apoptosis was noted between diabetic children and age-matched healthy controls. What is more, we did not observe a high level of apoptosis in newly recognized T1D patients, which was found in previous studies. In all examined groups, the median apoptosis level was low. However, the inverse correlation between the C-peptide level and the percentage of apoptotic Tregs was close to statistical significance ($p = 0.060$). This may suggest a link between Tregs apoptosis and β-cells destruction.

Contact-dependent suppression of T cell proliferation is the main functional property of $FoxP3^+$ Treg. However, suppressive cytokines TGF-β and IL-10 have been implicated as active players in the effector function of Tregs. In the current study, children under 5 years of age had a lower frequency of $CD4^+CD25^{high}$ cells with TGF-β or IL-10 expression than the age-matched healthy controls. This difference was not observed between children with diabetes recognized at a later age and age-matched healthy controls. The results of animal (Perez et al. 2008; Cheatem et al. 2009) as well as human studies (Karges et al. 2006) highlight the role of cytokines IL-10 and TGF-β in the pathogenesis of T1D. Karges et al. (2006) concluded that IL-10-dependent regulatory $CD4^+$ T cell pathways are involved in β-cell recovery after the onset of hyperglycemia in autoimmune T1D. Lindley et al. (2005) noted that $CD4^+CD25^+$ T cells showed a reduced ability to inhibit T cell proliferation in adults with newly diagnosed T1D, compared to the control group. Cells from patients with newly diagnosed T1D were characterized by a proinflammatory phenotype with increased secretion of interferon α and decreased secretion of IL-10 compared with the control group. Brusko et al. (2005) demonstrated reduced secretion of TGF-β1 in cell culture of $CD4^+CD25^+$ Tregs in patients with diabetes compared with the control group, whereas there were no differences between groups in IL-10.

We noted that the difference in the percentage of Tregs between the controls of over and under 5 years of age was close to statistical significance ($p = 0.058$). This lack of significance could result from a small number of patients. This result of our study might suggest that the frequency of Tregs is higher in healthy children under the age of 5 than in older healthy subjects. Moreover, at diabetes diagnosis, the frequency of Tregs was lower in the youngest diabetic children than in children with diabetes diagnosed at the age of over 5 years. It can be hypothesized that the reduction of the relatively high number of Tregs observed in children under the age of 5 might significantly disturb the balance between activation and suppression of the immune system and could influence the rate of β-cell destruction. The incidence of type 1 diabetes in the youngest children has been increasing so dramatically that other studies are warranted to assess new markers of autoimmunity. Perhaps a low frequency of Tregs is characteristic of the youngest child with a predisposition for diabetes. Further studies are needed to investigate this hypothesis.

The limitation of our study is the lack of any functional data regarding the activity of Tregs. Unfortunately, studies involving children <5 years of age with newly diagnosed

T1D are difficult to perform because of a very small number of young patients in diabetological centers, compounding the difficulty of obtaining the material required for analysis. The age of the children under investigation prohibits, for ethical reasons, the collection of more blood than clinically relevant. This excludes a broader diagnostic panel.

In conclusion, this study highlights the distinctiveness of diabetes in children under the age of 5. The youngest children had a low secretion of C-peptide and an extremely low frequency of FoxP3 Tregs as well as a low frequency of suppressive cytokines IL-10 and TGF-β. The Tregs apoptosis level was low in all examined groups.

Further, studies investigating the immune regulation in the youngest children are of great importance. Understanding the differences of immune system activity in young diabetic children may open the way to identify children at risk for T1D and enable the use of novel forms of intervention, e.g., Tregs vaccination.

References

Abdul-Rasoul M, Habib H, Al-Khouly M (2006) 'The honeymoon phase' in children with type 1 diabetes mellitus: frequency, duration, and influential factors. Pediatr Diabetes 7:101–107

Bober E, Dundar B, Buyukgebiz A (2001) Partial remission phase and metabolic control in type 1 diabetes mellitus in children and adolescents. J Pediatr Endocrinol Metab 14:435–441

Bowden SA, Duck MM, Hoffman RP (2008) Young children (<5 yr) and adolescents (>12 yr) with type 1 diabetes mellitus have low rate of partial remission: diabetic ketoacidosis is an important risk factor. Pediatr Diabetes 9(Pt 1):197–201

Brusko TM, Wasserfall CH, Clare-Salzler MJ et al (2005) Functional defects and the influence of age on the frequency of $CD4^+CD25^+$T-cells in type 1 diabetes. Diabetes 54:1407–1414

Cheatem D, Ganesh BB, Gangi E et al (2009) Modulation of dendritic cells using granulocyte-macrophage colony-stimulating factor (GM-CSF) delays type 1 diabetes by enhancing $CD4^+CD25^+$regulatory T cell function. Clin Immunol 131:260–270

Chen C, Liu CP (2009) Regulatory function of a novel population of mouse autoantigen-specific Foxp3 regulatory T cells depends on IFN-gamma, NO, and contact with target cells. PLoS ONE 4:e7863

Craig M, Hattersley A, Donaghue K (2009) Definition, epidemiology, diagnosis and classification of diabetes in children and adolescents. Pediatr Diabetes 10(suppl 12):3–12

Glisic-Milosavljevic S, Wang T, Koppen M et al (2007a) Dynamic changes in $CD4^+CD25^{+high}$ T cell apoptosis after the diagnosis of type 1 diabetes. Clin Exp Immunol 150:75–82

Glisic-Milosavljevic S, Waukau J, Jailwala P et al (2007b) At risk and recent-onset type 1 diabetic subjects have increased apoptosis in the $CD4^+CD25^+$high T cell fraction. PLoS ONE 2:e146

Greenbaum CJ, Mandrup-Poulsen T, McGee PF et al (2008) Mixed-meal tolerance test versus glucagon stimulation test for the assessment of β-cell function in therapeutic trials in type 1 diabetes. Diabetes Care 31:1966–1971

Karges B, Durinovic-Belló I, Heinze E et al (2006) Immunological mechanisms associated with long-term remission of human type 1 diabetes. Diabetes Metab Res Rev 22:184–189

Komulainen J, Lounamaa R, Knip M et al (1996) Ketoacidosis at the diagnosis of type 1 (insulin dependent) diabetes mellitus is related to poor residual cell function. Arch Dis Child 75:410–415

Kukreja A, Cost G, Marker J et al (2002) Multiple immuno-regulatory defects in type-1 diabetes. J Clin Invest 109:131–140

Lindley S, Dayan CM, Bishop A et al (2005) Defective suppressor function in CD4(+)CD25(+) T-cells from patients with type 1 diabetes. Diabetes 54:92–99

Liu W, Putnam AL, Xu-Yu Z et al (2006) CD127 expression inversely correlates with FoxP3 and suppressive function of human $CD4^+$Treg cells. J Exp Med 203:1701–1711

Łuczyński W, Wawrusiewicz-Kurylonek N, Stasiak-Barmuta A et al (2009) Diminished expression of ICOS, GITR and CTLA-4 at the mRNA level in T regulatory cells of children with newly diagnosed type 1 diabetes. Acta Biochim Pol 56:361–370

Patterson CC, Dahlquist GG, Gyürüs E et al (2009) Incidence trends for childhood type 1 diabetes in Europe during 1989–2003 and predicted new cases 2005–20: a multicentre prospective registration study. Lancet 373:2027–2033

Perez N, Karumuthil-Melethil S, Li R et al (2008) Preferential costimulation by CD80 results in IL-10-dependent TGF-beta1 (+)-adaptive regulatory T cell generation. J Immunol 180: 6566–6576

Pop SM, Wong CP, Culton DA et al (2005) Single cell analysis shows decreasing FoxP3 and TGFbeta1 coexpressing $CD4^+CD25^+$regulatory T cells during autoimmune diabetes. J Exp Med 201:1333–1346

Putnam AL, Vendrame F, Dotta F et al (2005) $CD4^+CD25^{high}$ regulatory T cells in human autoimmune diabetes. J Autoimmun 24:55–62

Ryba M, Rybarczyk-Kapturska K, Zorena K et al (2011) Lower frequency of CD62L and higher frequency of TNFR2 Tregs are associated with inflammatory conditions in type 1 diabetic patients. Mediators Inflamm 2011:645643

Sanda S, Bart O, Roep BO et al (2008) Islet antigen specific $IL\text{-}10^+$ immune responses but not $CD4^+CD25^+FoxP3^+$ cells at diagnosis predict glycemic control in type 1 diabetes. Clin Immunol 127:138–143

Sojka DK, Huang YH, Fowell DJ (2008) Mechanisms of regulatory T-cell suppression—a diverse arsenal for a moving target. Immunology 124:13–22

Szypowska A, Stelmaszczyk-Emmel A, Demkow U et al (2010) Evaluation of T regulatory cell apoptosis in children with newly recognized type 1 diabetes mellitus. Eur J Med Res 15(suppl 2):198–201

Tritt M, Sgouroudis E, d'Hennezel E et al (2008) Functional waning of naturally occurring $CD4^+$regulatory T-cells contributes to the onset of autoimmune diabetes. Diabetes 57:113–123

Vrabelova Z, Hrotekova Z, Hladikova Z et al (2008) CD127– and $FoxP3^+$ expression on $CD25^+CD4^+$ T regulatory cells upon specific diabetogeneic stimulation in high-risk relatives of type 1 diabetes mellitus patients. Scand J Immunol 67:404–410