Administration of CD4$^+$CD25^{high}CD127$^-$ Regulatory T Cells Preserves β-Cell Function in Type 1 Diabetes in Children

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OBJECTIVE—Type 1 diabetes is a condition in which pancreatic islets are destroyed by self-reactive T cells. The process is facilitated by deficits in the number and suppressive activity of regulatory T cells (Tregs). Here, we show for the first time that the infusion of autologous Tregs prolongs remission in recently diagnosed type 1 diabetes in children.

RESEARCH DESIGN AND METHODS—We have administered Tregs in 10 type 1 diabetic children (aged 8–16 years) within 2 months since diagnosis. In total, 4 patients received 10×10^6 Tregs/kg body wt and the remaining 6 patients received 20×10^6 Tregs/kg body wt. The preparation consisted of sorted autologous CD3$^+$CD4$^+$CD25^{high}CD127$^-$ Tregs expanded under good manufacturing practice conditions.

RESULTS—No toxicity of the therapy was noted. A significant increase in the percentage of Tregs in the peripheral blood has been observed since the day of infusion. These patients were followed along with matched type 1 diabetic patients not treated with Tregs. Half a year after type 1 diabetes onset (4–5 months after Treg infusion), 8 patients treated with Tregs still required <0.5 UI/kg body wt of insulin daily, with 2 patients out of insulin completely, whereas the remission was over in the nontreated group. In addition, plasma C-peptide levels were significantly higher in the treated group as compared with those not treated.

CONCLUSIONS—This study shows that the administration of Tregs is safe and tolerable in children with recent-onset type 1 diabetes.

Type 1 diabetes is a rising problem around the world. For example, it is estimated that the morbidity in Poland doubles every 10 years (1). The condition develops as a result of autoimmune attack of self-reactive T cells that infiltrate pancreatic islets and destroy insulin-producing β-cells (2,3). The transfer of self-reactive T cells from diabetic animals induces insulitis and diabetes in previously healthy animals (4). This autoimmune process can be prevented by highly suppressive regulatory T cells (Tregs) (5,6). It has been found in several animal models that CD4$^+$CD25$^+$FoxP3$^+$ Tregs can stop the destruction of pancreatic islets and protect from autoimmune type 1 diabetes (7,8). In humans, mutation in the *foxp3* gene results in immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome, which is associated with lack of functional Tregs and subsequent autoimmune diabetes (9).

Tregs can regulate ongoing immune reactions and, therefore, can be used to suppress them. In 2009, we successfully administered expanded Tregs in patients with chronic graft-versus-host disease (10). The efficacy of such an approach recently has been confirmed in the prophylaxis of graft-versus-host disease (11,12). Here, we show the effects of adoptive transfer of Tregs in children with recently diagnosed type 1 diabetes.

RESEARCH DESIGN AND METHODS

In the current study, 10 children (aged 8–16 years, 6 girls and 4 boys) with type 1 diabetes, diagnosed in compliance with World Health Organization criteria (13), were treated with Tregs (Supplementary Fig. 1). The inclusion criteria were age 5–18 years; up to 2 months since type 1 diabetes diagnosis; the presence of anti-GAD65 antibody, islet cell antibody, and insulin autoantibody; fasting C-peptide levels >0.4 ng/mL in two consecutive measurements (a challenge test was not permitted by the ethics committee); BMI range of 25th–75th percentiles for a particular age; and adequate venous access. The following exclusion criteria were applied: any cytopenia or low hemoglobin levels; carriage of HLA-DQB1*0602 allele; positive test for hepatitis B virus, hepatitis C virus, HIV, *Treponema pallidum*, or other active infections; history of neoplasm; excessive anxiety related to the procedure; and chronic disease requiring pharmacological treatment. Up to 250 mL peripheral blood was drawn with anesthesiological assistance. For children with body weight <50 kg, the blood volume drawn was equal to 0.5% of body weight (see study protocol and patient consent form in the Supplementary Data online).

Tregs were prepared as previously described (10,14). In brief, the blood cells

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were sorted to $CD3^+CD4^+CD25^{high}CD127^-lin^-doublet^-$ Tregs using combined immunomagnetic/fluorescence-activated technique with the purity of ~100% (median [minimum–maximum] 98% [97–99]). As compared with our previous protocol, important improvements in cell processing were the application of the good manufacturing practice (GMP)-adapted fluorescence-activated cell sorter (FACS) equipped with single-use sample lines (Influx; BD Biosciences, San Jose, CA), which eliminated the risk of cross-contamination, and the application of GMP-grade CellGro medium (CellGenix, Breisgau, Germany) supplemented with 10% autologous serum, interleukin-2 (1,000 units/mL; Proleukin; Chiron, San Diego, CA), and clinical-grade anti-CD3/anti-CD28 beads (Invitrogen, Carlsbad, CA) in 1:1 ratio with cells. Tregs were cultured until the required number was achieved, but for no longer than 2 weeks (median [minimum–maximum]: 10 days [7–14]). Taking all these measures into consideration, we have significantly improved the stability and quality of the Tregs in the final product. The release criteria for Tregs preparation were FoxP3 expression >90% (93% [90–97]), passed interferon (IFN)-γ suppression assay, and negative microbiological tests (no genetic material of hepatitis B virus, hepatitis C virus, or HIV and no microbial contamination in the culture supernatants). Tregs for infusion were washed out completely, suspended in 250 mL 0.9% NaCl (Polfa, Starogard Gd., Poland), and transferred in slow infusion to the patient under anesthesiological assistance within 1 h of product release. The administered dose was either 20×10^6/kg body wt (n = 6) or when protocol required cessation of Tregs expansion at 2 weeks, 10×10^6/kg body wt (n = 4). As control, the other 10 type 1 diabetic patients and their respective matching characteristics were included in the follow-up but did not receive Tregs. The trial was neither blind nor randomized, and the comparison group did not undergo any interventions, such as initial blood drawing for cell processing or mock transfusions (comparison of clinical characteristics of the groups provided in Table 1). End points of the study were fasting C-peptide, HbA_{1c} levels, and insulin requirement, with special attention to daily insulin dose (DDI; 0.5 UI/kg body wt) as a borderline for remission in our protocol. The study was conducted according to the protocol approved by the Ethics Committee of the Medical University of Gdańsk (NKEBN/8/2010). Written informed consent was obtained before starting the procedures.

RESULTS—No serious infections, episodes of acute hyper- or hypoglycemia, or other adverse effects were observed after the administration of Tregs during the entire follow-up. In one of the patients, the infusion was coincident with influenza, which was diagnosed a day after the procedure (confirmed from nasopharyngeal specimen [bioNexia Influenza; bioMérieux, Marcy l'Etoile, France]). The administration of Tregs was followed by a significant decrease in the requirement of exogenous insulin and a decrease in HbA_{1c} levels in all the patients after 2 weeks (Fig. 1 and Supplementary Figs. 2 and 3). In addition, a significant increase in the percentage of Tregs in the peripheral blood were observed since the day of treatment (Wilcoxon test, P = 0.04) (Fig. 2 and Supplementary Figs. 2 and 3).

Significant differences between the study group and the comparison group were noted for the first time half a year after the diagnosis of type 1 diabetes (4–5 months after Tregs administration). Of the 10 patients treated with Tregs, 8 were still in clinical remission, defined as DDI <0.5 UI/kg body wt (DDI median [minimum–maximum] 0.22 UI/kg body wt [0–0.60]), with 2 patients out of insulin completely.

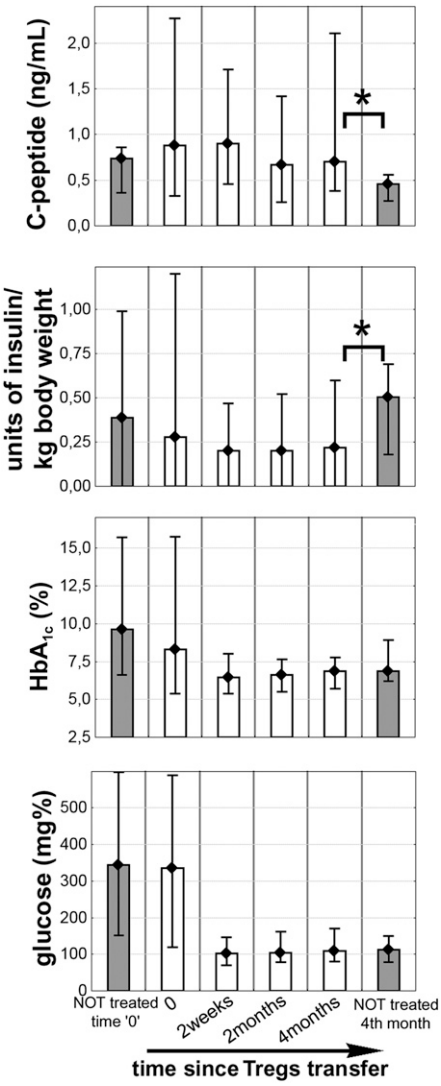

Figure 1—*C-peptide, DDI, HbA_{1c}, and fasting glucose in type 1 diabetic children treated with Tregs infusions* (n = *10) during follow-up. Values for matched comparison patients not treated with Tregs* (n = *10) are shown as gray columns. Values are given as median (minimum–maximum). Significant differences are marked with asterisks.*

Table 1—*Clinical characteristics of the patients*

	Treated (n = 10)	Not treated (n = 10)
Age (years)	12.2 (8–16)	11.8 (7–16)
BMI	16.9 (14.2–21.5)	16.9 (14.2–20.7)
Fasting glycemia at diagnosis (mg%)	354 (151–588)	354 (151–598)
Polydipsia at diagnosis	5	8
Polyuria at diagnosis	5	8
Loss of weight at diagnosis	4	3
pH at diagnosis (capillary blood)	7.39 (7.36–7.46)	7.39 (7.34–7.53)
pO_2 at diagnosis (capillary blood, mmHg)	69.3 (63.4–88.0)	69.0 (56.9–86.2)
pCO_2 at diagnosis (capillary blood, mmHg)	39.1 (28.0–41.8)	38.0 (24.0–41.0)
HCO_3 at diagnosis (capillary blood, mmHg)	23.85 (18.8–25.0)	23.3 (21.3–25.2)
Acid/base balance at diagnosis (BE, mEq/L)	−0.55 (−7.8 to 1.0)	−0.6 (−7.0 to 0.9)
SatO2 at diagnosis (capillary blood, %)	94.1 (90.2–97.3)	95.5 (92.4–98.0)
Anti-GAD65 antibody	10	10
Islet cell antibody	5	5
Insulin autoantibody	9	8

Data are median (minimum–maximum) or number of patients. BE, base excess; SatO2, oxygen saturation.

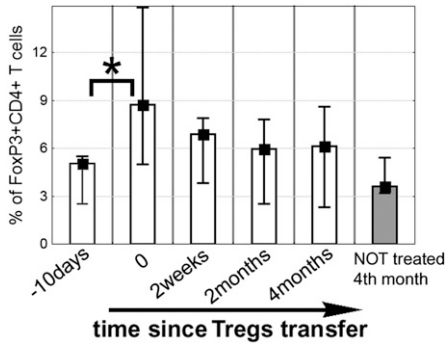

Figure 2—*Levels of $CD3^+CD4^+CD25^{high}CD127^-$ $FoxP3^+$ Tregs in type 1 diabetic children treated with Tregs infusions* (n = *10) during follow-up. Value for the −10-days point refers to the day of blood drawing for Tregs sorting. Gray column depicts values measured for the matched comparison patients not treated with Tregs (only six patients were available). Data are shown as medians (minimum–maximum). Significant differences are marked with asterisks.*

At the same time, remission was completed in 6 of 10 patients in the comparison group not treated with Tregs (DDI 0.52 UI/kg body wt [0.18–0.69]; Mann-Whitney *U* test, $P = 0.04$). In addition, plasma C-peptide levels were significantly higher in patients from the Tregs group as compared with the untreated group (0.75 [0.38–2.11] vs. 0.48 ng/mL [0.15–0.56]; Mann-Whitney *U* test, $P = 0.01$) (Fig. 1). No differences were seen between the two doses of Tregs used and, therefore, patients are presented as a single cohort.

CONCLUSIONS—As previously described, Tregs from diabetic patients can be effectively expanded (15). However, there were doubts whether autologous Tregs could be effective in the treatment of type 1 diabetes. It is notable that a fraction of IFN-γ^+FoxP3^+ Tregs with reduced suppressive efficiency have been described in diabetic patients (16). We could also detect such cells, albeit in a very low proportion in our type 1 diabetic children (~1% of Tregs; data not shown), and they have never affected the results of the suppression assay in vitro. Hence, it might be possible that the majority of defects in type 1 diabetes are related to self-reactive effector T cells, which might be difficult to control by Tregs (17,18). Such activated effector T cells specific for self-islets have been documented recently in humans (19). Fortunately, in NOD mice, the transfer of Tregs could suppress the activity of self-reactive effector T cells and stop diabetes (20). Our study confirms a similar effect of such a Tregs transfer in the human setting.

The dose of Tregs administered in our study has a biological meaning because it was able to significantly increase the level of $FoxP3^+$ Tregs in peripheral blood. There were several issues that made our protocol efficacious. In all published trials, immunomagnetic isolation of Tregs was applied, resulting in a maximum of ~60% $FoxP3^+$ T cells in the product, but that figure could be <20% (11,12). So, at least 40% of cells in the product administered to the patients were not Tregs from the beginning. This made it difficult to assess the exact level of administered cells with immunosuppressive activity. With FACS sorting, we reliably achieved ~100% $CD3^+CD4^+CD25^{high}CD127^{low}FoxP3^+$ T cells, and in children, >90% of those cells were still $FoxP3^+$ Tregs after expansion. Although FACS sorting is considered a non-GMP procedure, its safety with new sorters is comparable with that of clinical-grade immunomagnetic sorting. In fact, novel single-use fluidic lines preventing cross-contamination and performing the procedure in clean room facilities, together with the resulting purity, make FACS sorting superior to immunomagnetic sorting. Another problem somehow related to the dose assessment is a quality check of the ready-to-administer expanded Tregs product. Proposed in the literature, suppression of the proliferation assay could not be included in release criteria because the result was available 5 days after commencing the assay. In this time period, the suppression of expanded Tregs could change dramatically. Our IFN-γ suppression assay could be included in release criteria, since the results were available within hours, and suppressive activity of the product was confirmed prior to the transfer of Tregs to patients.

There are several limitations of the study involving number of patients and timing. In addition, it is probable that the fast initial decrease in the level of HbA_{1c} was very much related to the improvement of the clinical status of the patients and medical interventions other than Tregs infusion. Nevertheless, even at this early 4-month point of our study, when we compare our patients with carefully matched untreated subjects, we already see the difference in the insulin dose and C-peptide levels. Further follow-up, which is under way, will confirm whether this is a sustained remission or only a delay in the destruction of islets.

In our opinion, maneuvers increasing the number of Tregs are safe and tolerable and may potentially control type 1 diabetes. As this study shows, in children with recently diagnosed type 1 diabetes, some improvement in the functioning of β-cells after the infusion of expanded autologous Tregs is a proof of this concept. These results should encourage further investigations involving our novel approach.

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N.M.-T., M.M., and P.T. contributed to the study design, protocol writing, cell preparation, data collection, analysis, interpretation, and writing and reviewing of the manuscript. A.D. contributed to cell separation and data collection. M.G. and I.T. contributed to data collection and interpretation. J.J. and M.A.W. contributed to data collection. P.W. contributed to data analysis and interpretation and reviewed the report. W.M. contributed to data collection, analysis, and interpretation and reviewed the manuscript. A.B. and J.M. contributed to data collection and interpretation and reviewed the manuscript. All authors have approved the manuscript for submission. P.T. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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