Therapy of type 1 diabetes with CD4+CD25high CD127-regulatory T cells prolongs survival of pancreatic islets — Results of one year follow-up

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Abstract It is hypothesized that CD4+CD25+FoxP3+ regulatory T cells (Tregs) can prevent destruction of pancreatic islets protecting from type 1 diabetes (DM1).

Here we present results of one year follow-up of 12 DM1 children treated with autologous expanded *ex vivo* Tregs. Patients received either a single or double Tregs infusion up to the total dose of 30×10^6/kg.

No severe adverse effects were observed. The treatment did not impair post-immunization antibody responses. Tregs infusion was followed by increase in Tregs number in peripheral blood.

Most of the patients responded to the therapy with increase in C-peptide levels (8/12 and 4/6 after the first and the second dose, respectively). Tregs administration resulted also in lower requirement for exogenous insulin (8/12 treated patients *versus* 2/10 untreated controls in remission) with two children completely insulin independent at one year. Repetitive administration of Tregs is safe and can prolong survival of β-cells in DM1 (registration: ISRCTN06128462).

1. Introduction

The primary factors responsible for destruction of β cells in type 1 diabetes mellitus (DM1) still remain unknown. Initial histological changes observed in affected pancreatic islets are described as *insulitis* and are considered to be a manifestation of T cell dependent autoimmune attack [1]. According to the current knowledge one of the most important factors in etiology of DM1 is an impaired regulation of the immune system, thus autoaggressive effector T cells are not controlled by suppressive regulatory T cells [2]. Therefore, the majority of the current studies that aim to preserve β cells in DM1 are focused on suppression of autoreactive T cells and boosting activity of regulatory T cells [3].

Here we present a prospective, non-randomized safety and efficacy pilot study of cellular therapy of recent-onset DM1 with *ex vivo* expanded CD4$^+$CD25^{high}CD127-regulatory T cells (Tregs). Over a year ago, we applied Tregs to DM1 patients in an aim to restore regulation in the immune system and stop or at least delay progress of the disease. Our initial results with dose acceleration from 10×10^6 cells/kg of body weight (b.w.) to 20×10^6 cells/kg b.w. were promising [4]. However, data collected upon follow-up suggested that additional acceleration of the dose of Tregs may be beneficial for the patients. As our laboratory has a 6 year of uneventful experience with clinical therapy with *ex vivo* expanded Tregs, including the first-in-man clinical application of Tregs in graft *versus* host disease (GvHD) [5,6], we decided to administer the second accelerating dose of Tregs to 6 patients (amended approval of Bioethics Committee NKEBN/8/2010). Hence, the highest total dose of administered Tregs was 30×10^6 cells/kg of b.w.

In the present paper we show the results of the one year follow-up of the outcomes.

2. Materials and methods

2.1. Protocol and treatment

The study has been registered at the Current Controlled Trials database: http://www.controlled-trials.com/ISRCTN06128462 where the detailed study protocol is available.

Briefly, a cohort of 12 Caucasian children from Polish population with recently diagnosed DM1 was treated with *ex vivo* expanded autologous Tregs. The general health and metabolic status of treated individuals were followed and compared at 4 and 12 months after inclusion to the study with 10 non-treated control patients matched for age, sex and disease duration.

Patients were eligible to participate in the study if they fulfilled the following criteria: autoimmune DM1 diagnosed within 2 months and presence of at least one type of anti-islet autoantibody: anti-GAD, anti-IA2, IAA, ICA, age 5–18 years, fasting plasma C-peptide > 0.4 ng/ml, written informed consent signed by parents (and patients if above 16 years old), involvement of the patient and parents in the intensive diabetes management defined as self-monitoring of glucose values no less than three times a day and by the administration of insulin injections each day or insulin pump therapy, patient and parents mentally stable and able to comply with the procedures of the study protocol and appropriate venous access for blood drawing. The control group was recruited among patients who fulfilled the same criteria but due to inappropriate venous access were not qualified to the treated group.

Tregs were isolated from the patients' peripheral blood (up to 250 ml was drawn per expansion) with HEPA-enclosed FACS sorter (Influx, BDBioscience, USA) using exchangeable sterile sample lines. An average post sort Tregs purity was ≈98% (range 97–100%). The expansion was performed under current Good Manufacturing Practice (cGMP) conditions according to our previously described protocol using beads coated with anti-CD3 and anti-CD28 antibodies (Ab), interleukin 2 (IL-2) and autologous serum [4–6]. The release criteria for the final Tregs product were: 1) FoxP3 expression above 90% [median (min.−max) = 91% (90−97)], 2) passed IFNγ suppression assay and 3) negative microbiological tests [4–6].

Initial study protocol was modified and approved by the Ethics Committee in order to accelerate the dose of administered Tregs. According to the amendment a second dose of Tregs could be injected to reach the total administered Tregs dose ≤ 30×10^6 of Tregs/kg b.w. Therefore, after additional written consent signed by the parents and/or the patients, 6 out of 12 treated children received the second Tregs dose with 6−9 month interval between the infusions. This maneuver was offered to the patients with good clinical and metabolic responses to the first dose of Tregs [fasting C-peptide >0.4 ng/ml and/or daily insulin dose (DDI) < 0.5 U/kg b.w.], who presented symptoms of the disease progression after ≥ 6 months of the follow-up.

Among 10 initially recruited patients, 2 were lost to follow-up, thus additional two patients were recruited and we present data of all 12 patients. The administered accelerated Tregs doses were as follow: 10×10^6 of Tregs/kg b.w. in a single infusion (3 patients), 20×10^6 of Tregs/kg b.w. in a single infusion (3 patients), and 30×10^6 of Tregs/kg b.w. in two infusions (6 patients).

The primary endpoints of the trial were safety and remission defined as DDI $\leq$ 0.5 UI/kg b.w. and fasting C-peptide > 0.5 ng/ml 1 year after recruitment.

2.2. Metabolic and immune responses

Fasting C-peptide levels were checked at all control points of the study. Glucagon stimulation testing was performed after 1 year follow-up in selected patients. The test was not performed at the inclusion to the study and not all the patients underwent the test, because according to the Ethics Committee the assay could be performed only with physician's indications. To perform the test fasting blood sample was drawn in order to establish baseline level of C-peptide. Then, patient received an intravenous glucagon injection (0.5 mg for the patient with b.w. $\leq$ 30 kg and 1.0 mg for the patient with b.w. > 30 kg) and 6 min after the stimulation another blood sample was drawn.

Immunization responses to hepatitis B and rubella viruses were measured with commercial ECLIA kits (Elecsys assays, Roche, Poland) according to the manufacturer's instructions. Patients were immunized according to the routine national immunization schedule independent of the trial.

Fasting glucose and HbA1c levels, as well as FoxP3+CD4+CD3+ Tregs phenotype were measured as we described in previous reports [4–6].

2.3. Statistical analysis

Data were computed with the software Statistica 10.0 (Statsoft, Poland). As indicated by distribution of the variables non-parametric tests were used. The analysis was carried out with Kruskal–Wallis ANOVA, U–Mann–Whitney test, Wilcoxon test and Spearman's rank correlation. P < 0.05 was considered statistically significant.

2.4. Study approval

The study was conducted according to the Declaration of Helsinki principles and was approved by the Ethics Committee of the Medical University of Gdańsk, Poland (NKEBN/8/2010 with amendments). The trial was registered at the Current Controlled Trials database: http://www.controlled-trials.com/ISRCTN06128462 (the trial number ISRCTN06128462). Written informed consent was received from parents of all the participants and the patients if above 16 years old.

3. Theory/calculation

From their discovery, Tregs are known as a subset responsible for dominant tolerance in the immune system and therefore involved in the protection from autoimmune diseases including DM1. Hence, Tregs are considered as candidates for cellular therapy in a wide range of diseases with immune background [2,7]. It is however difficult due to complex phenotype of these cells, low percentage of Tregs in the periphery and loss of their activity during *ex vivo* expansion [6]. Only recently, several methods of efficient sorting and expansion have been developed and translated to the clinical trials [2,7]. Our hospital tested them in first-in-man study in

the treatment of graft-*versus*-host disease [5]. This study ensured us that the method is safe and can be applied in children with recent onset DM1. Although initial results of our DM1 trial proved short-term efficacy of the therapy [4], current paper reports primary endpoints of the trial at one year. Importantly, we show here for the first time that repetitive administration of Tregs is safe in children as there are no severe adverse effects of the therapy during the follow-up. Although encouraging, these long-term results suggest necessity of further work on timing and dose of administered Tregs in order to improve the outcome.

4. Results

4.1. Safety

The procedure was not associated with serious adverse events. The next day after the first Tregs infusion one patient developed laboratory-confirmed influenza (the Tregs administration took place during a peak of epidemic season; Tregs dose = 20×10^6 Tregs/kg b.w.), while another patient presented symptoms of a mild gastroenteritis of unknown origin (Tregs dose = 30×10^6 Tregs/kg b.w; laboratory tests excluded rotaviruses and adenoviruses). Both infections resolved within few days. Another patient (Tregs dose = 30×10^6 Tregs/kg b.w.) reported exacerbations of chronic sinusitis that resolved with standard treatment. Serological responses to vaccination against rubella and hepatitis B virus (HBV) were not affected by the therapy during the follow-up period in all children (Fig. 1). There was one patient, who was vaccinated the first time against HBV during the study, out of the study protocol (see maximum point at 6 month check point in Fig. 1). The patient responded correctly to the vaccine and kept the protective titer of the antibodies to the end of the follow-up.

4.2. Efficacy

One year after inclusion to the study, 8 out of 12 patients (66%), were still meeting the criteria of clinical remission (DDI $\leq$ 0.5 UI/kg b.w.). Moreover, two of them still remained insulin independent at that time. Both insulin-independent patients and three other patients in remission received in total 30×10^6 of Tregs per kg b.w. in two separate infusions. Other two patients in remission received 20×10^6 of Tregs per kg b.w. (single infusions) and the last one received 10×10^6 of Tregs per kg b.w. (single infusion). The remission in all cases was associated with fasting C-peptide levels > 0.5 ng/ml. At the same time, all individuals in untreated control group were insulin-dependent and only two of them met the remission criteria defined as above (Figs. 2 and 3). In addition, as compared to the non-treated control individuals, insulin doses (DDI/kg b.w.) were significantly lower in Tregs treated individuals at 4-months (intermediate efficacy point) and one year after commencing the trial (primary endpoint) [U–Mann–Whitney test, control vs treated: p = 0.04 and p = 0.02, respectively], while fasting C-peptide levels were significantly higher in Tregs treated individuals [U–Mann–Whitney test, control vs treated, 4-months: p = 0.01 and 1-year: p = 0.01].

A **Immunization level**
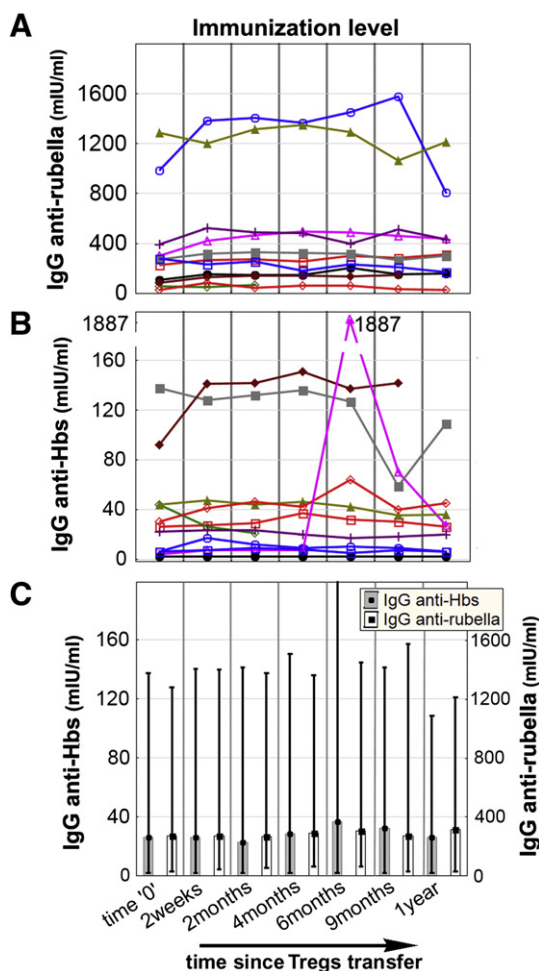
Figure 1 Response to the immunization during the follow-up. The figure depicts levels of antibodies after immunization against rubella and hepatitis B (HBV) viruses in patients treated with Tregs. These vaccines were given to participants according to the national vaccination schedule. Levels of anti-rubella (A) and anti-HBV (B) antibodies in the individual patients, as well, as medians (min.−max.) (C) are given.

4.3. Factors affecting efficacy

The best responders were children with short disease duration and high fasting C-peptide levels (≥ 0.7 ng/ml) at the inclusion. Both infusions of Tregs were associated with the increase in Tregs levels in peripheral blood [Wilcoxon tests, first infusion: $p = 0 \cdot 01$; second infusion $p = 0 \cdot 02$; 8/12 and 4/6 responders after the first and the second dose, respectively]. However, the peaks of Tregs immediately after the second infusion were lower than those after the first infusion.

Dose escalation up to 30×10^6 of Tregs/kg b.w. achieved with supplemental infusion of Tregs seemed to improve the efficacy of the therapy as metabolic outcomes at one year of the follow-up were the best in the group who received two doses of Tregs (Fig. 4). One year after initiation of the study fasting and stimulated C-peptide levels were the highest in individuals treated with two doses of Tregs, intermediate in those who received one dose of Tregs and the lowest in the control untreated group (Kruskal−Wallis ANOVA; $p = 0.01$

and $p = 0.02$ for fasting and stimulated C-peptide concentration, respectively). Interestingly, this was associated with metabolic control. HbA1c levels were the lowest in patients treated with two doses of Tregs, intermediate in those treated with one dose and the highest in untreated individuals (Kruskal−Wallis ANOVA; $p = 0 \cdot 01$).

In addition, when data of the treated and untreated patients were analyzed together after 4 month and one year follow-up, we observed that C-peptide levels correlated with percentage of Tregs in peripheral blood (Fig. 5) [Spearman's rank correlation; after 4 months: $R = 0.47$, $p = 0.04$; after 1 year $R = 0.55$, $p = 0.01$].

There was also a trend suggesting that the increase in percentage of Tregs in peripheral blood just after the Tregs infusion might have an impact on DDI later on (Fig. 6) [Spearman's rank correlation, after the first Tregs dose: $R = -0.57$, $p = 0.05$ and after the second Tregs dose: $R = -0.66$ $p = 0.15$]. In addition percentage of Tregs was in negative correlation with DDI when data of all the patients were analyzed at a 1 year control point (Fig. 6) [Spearman's rank correlation; $R = -0.44$, $p = 0.05$].

5. Discussion

Presented study confirmed safety of the Tregs therapy. Neither single nor double doses were associated with severe adverse effects. In addition, 66% of DM1 patients treated with *ex vivo* expanded autologous Tregs remained in remission during one year follow-up.

In terms of new treatments of DM1, safety is the priority as the disease is not associated with imminent death. Therefore, the risk and benefit ratio should be as low as possible. In addition, DM1 affects mainly the most vulnerably patients — children. Hence, any new method of the treatment should be devoid of severe side effects and cannot affect the patient's quality of life. In this regard, our results fulfill these criteria as no serious adverse effects were noted. Although three patients developed mild infections, they all resolved without sequels. In addition, there is no evidence that Tregs application was responsible for the onset of these infections. Noteworthy, the therapy did not impair immunity as the levels of post-immunization protective antibodies were not affected.

Tregs do not seem to be a 'magic bullet', which reverses autoimmune diseases after a single infusion. The optimal dose and time point for the Tregs application have to be determined [2,4,5,7]. It has been found in animal models that adoptive transfer of Tregs can prevent destruction of pancreatic islets and fully protect from the development of autoimmune diabetes [8]. Nevertheless, several differences between animal and human studies have to be taken into account. First of all in animal models the relative doses of Tregs are much higher than we applied in man. For example 7×10^6 Tregs/mouse [8] is $\approx 166 \times 10^6$/kg b.w. (if we assume that mouse b.w. = 60 g). In addition, the follow-up in animal models is usually terminated after few weeks (no observations after 1 year). Another important issue is the time of adminis-tration after Tregs isolation. In animal models Tregs are usually administered immediately after isolation, as several animals can be scarified in aim to obtain required number of Tregs for the transfer to the treated animal. We have previously found that suppressive activity of these cells can be lost upon too long
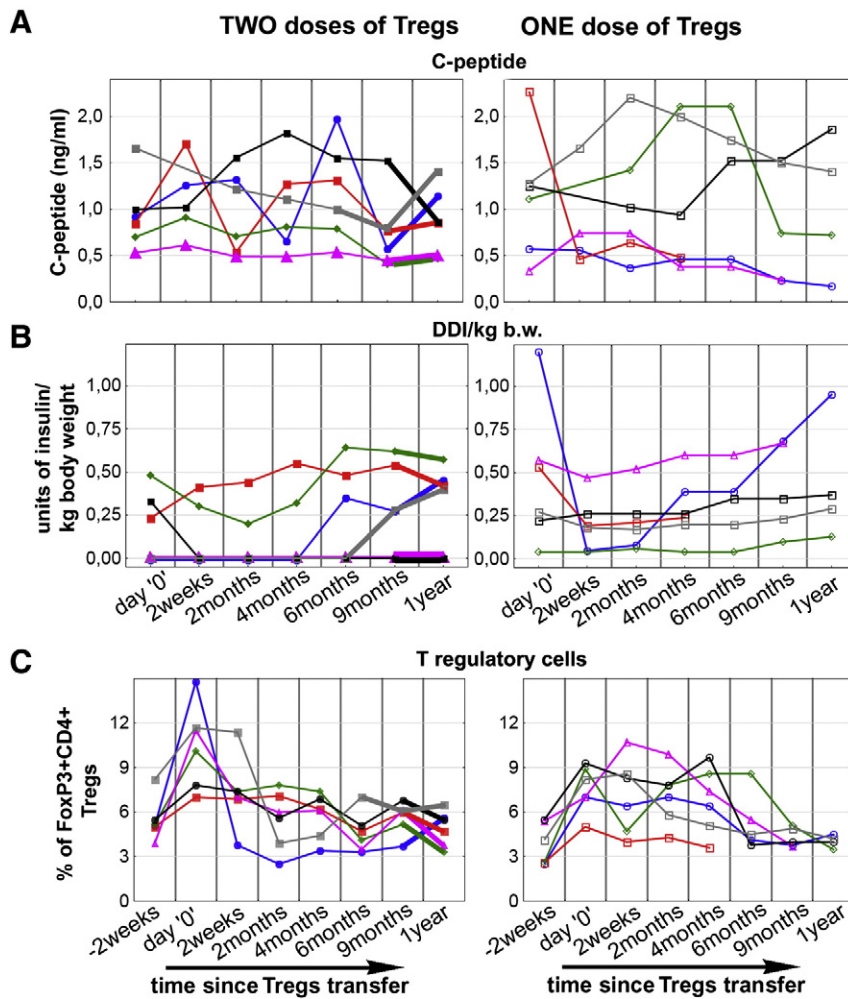
Figure 2 Individual results of C-peptide, daily doses of insulin/kg b.w. and the percentage of Tregs in the treated group. The figure depicts C-peptide levels (A), daily doses of insulin/kg b.w. (DDI/kg b.w.) (B) and the percentage of Tregs (C) in each treated individual at the particular control points. Left column presents data of the patients treated with two doses of Tregs. Bolded parts of the lines present values after the second dose. Right column shows results of the individuals treated with one dose of Tregs. The same symbol and color are assigned to the particular patient throughout the charts.

stimulation *in vitro* [6]. As in rodent studies long term culture of Tregs is avoided, cells do not lose their immunosuppressive potential. These differences in protocols may explain the success of animal studies with Tregs. Therefore, the dose and time of the culture seem to be crucial for the efficacy of Tregs therapy. There was a trend in statistics in our study suggesting that the increase in percentage of Tregs in peripheral blood immediately after Tregs administration was associated with insulin requirement later on (DDI). The higher the increase in Tregs number was, the lower DDI administered had to be. Admittedly, these data did not reach statistical significance, but future studies on more numerous cohorts will verify this observation. Nevertheless, further escalation of Tregs dose in human may theoretically increase the risk for opportunistic infections or neoplasm as it is observed for other forms of immunosuppression [9].

Another important factor affecting the outcome of our therapy seems to be the initial serum level of C-peptide which reflects β-cell mass. In general, the higher initial C-peptide,

the longer patient remains in remission. Not surprisingly this is consistent with other DM1 trials focused on immunotherapy [10].

Despite most of the treated patients remained in remission during 1 year follow-up, a slow progression of DM1 and decrease in Tregs numbers (come back to the baseline values) were observed with time. These data may suggest that *ex vivo* expanded Tregs have short lifetime after administration as they underwent multiple rounds of divisions *in vitro*. In addition, there is an ongoing discussion regarding Tregs stability [11–13] and some scientists claim that Tregs may convert into proinflammatory Th17 cells. Nevertheless, this seems to be the less probable scenario in our study, as the treated patients are characterized by better clinical outcomes than non-treated individuals for the whole follow-up, even after decrease in Tregs frequency. Another explanation for the decrease in Tregs number in treated patients is homing to peripheral tissues. Animal studies suggest that Tregs accumulate in peripheral lymph nodes and islets and thus delay
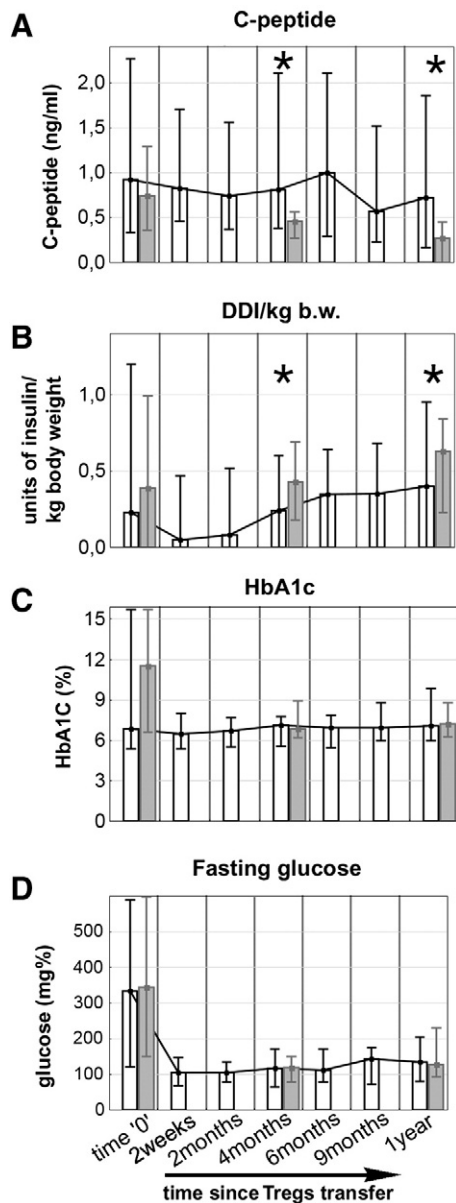
Figure 3 C-peptide, daily doses of insulin/kg b.w., HbA1C and fasting glucose in studied groups. The figure depicts levels of C-peptide (A), daily doses of insulin/kg b.w. (DDI/kg b.w.) (B), HbA1C (C) and fasting glucose (D) in type 1 diabetes children treated with Tregs during the follow-up (white columns). The values for the matched control patients not treated with Tregs are shown as gray columns at time '0', +4 months and +1 year. Asterisks show statistically significant differences between treated and not treated control individuals. Values are shown as medians (min.–max.).

diabetes progression. We cannot verify this in human but this scenario seems to be probable only during the first months after Tregs administration, when slight decrease in Tregs number is associated with improved glycemic control, but not later (approximately 9 months after the first Tregs infusion), when their therapeutic effect wanes.

The drop in Tregs numbers in a long term might also result from Tregs exhaustion due to sustained activation during suppression of autoimmune response. The impact of the
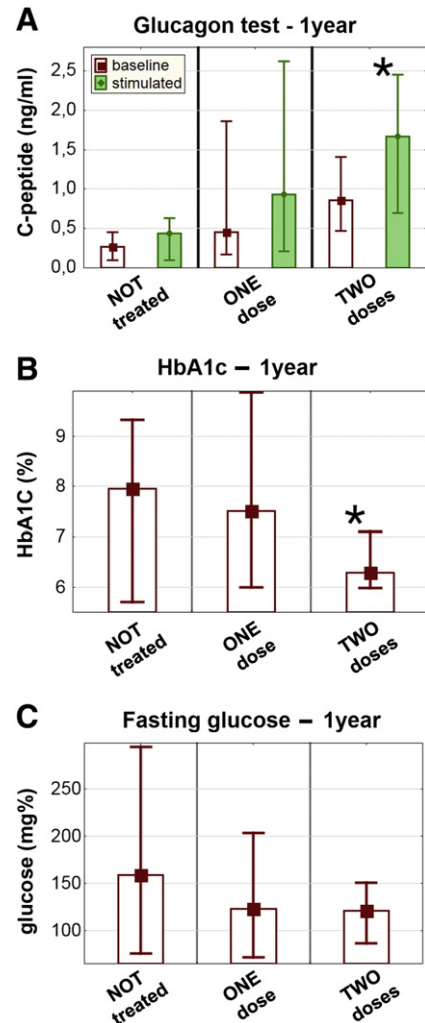
Figure 4 Metabolic control after 1 year follow-up. The figure shows results of the glucagon stimulation test performed 12–15 months after diagnosis of DM1 (one year after the first infusion of Tregs) in four patients treated with two doses of Tregs, three patients treated with one dose of Tregs and 10 patients from not treated control group (A). Levels of HbA1c (B) and fasting glucose (C, shown as median of 1 month records) were obtained from all the patients (10 patients treated with Tregs and 10 control individuals). Asterisks show statistically significant differences between the patients treated with two doses of Tregs and those treated with single dose and not treated. Data are shown as medians (min.–max.).

chronic disease on Tregs should not be neglected, as it was observed in several studies, including ours [14,15]. Therefore, the future studies on Tregs therapy in DM1 should focus on massive and fast Tregs expansion. In this way Tregs could be obtained from a single donation just after diagnosis of the disease, cryopreserved and administered in portions after thawing [16]. There are also alternative sources of Tregs, such as a third party umbilical blood [17] that should be taken into account.

The low number of treated patients is a limitation of our study. Therefore, we show individual data of the patients. Nevertheless, the results of this trial are encouraging and the
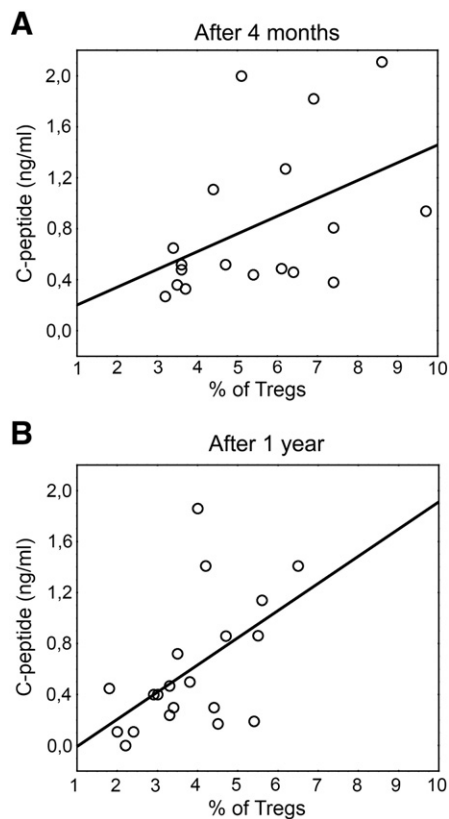
Figure 5 Correlation between % of Tregs and serum C-peptide level. The figure depicts correlation between % of Tregs in peripheral blood and C-peptide values of treated and control patients after 4 months (A; R = 0.47, p = 0.04; treated group n = 12; control group n = 6) and 1 year follow-up (B, R = 0.55, p = 0.01; treated group n = 10; control group n = 10).

diabetic patients. We have found that the administration of these cells to the children recently diagnosed with DM1 can spare the function of β cells and prolong the remission (66% patients in remission; 2 patients do not require insulin therapy 1 year after Tregs administration). Nevertheless, the therapeutic effect of Tregs wanes with time. Additional Tregs doses exerted beneficial but lower effects, than those observed after the first Tregs administration. There is need for further improvement of the therapy and verification of the inclusion criteria.

Acknowledgments

next phases of the study on a larger population are planned. There is only one similar study on Tregs therapy in adults with new onset DM1. The study is conducted in the USA but the results are not yet available [18].

In summary the results of our study confirmed safety of Tregs therapy in children with DM1. In addition, our observations indicate that Tregs are important to maintain the tolerance in

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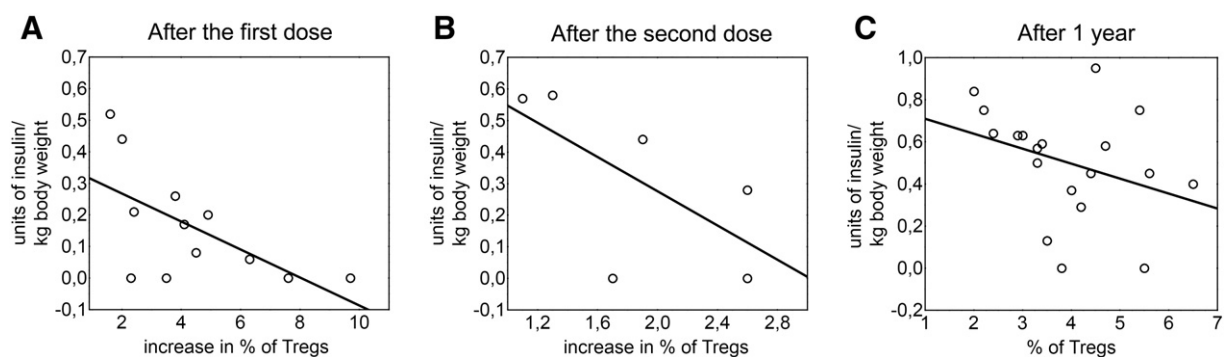
Figure 6 Correlation between % of Tregs and daily insulin dose. The figure depicts correlation between increase in % of Tregs after the first Treg dose and daily insulin dose (DDI) 3 months later (A; R = −0.57, p = 0.05, n = 12); correlation between increase in % of Tregs after the second Treg dose and DDI 3 months later (B; R = −0.66, p = 0.15, n = 6); and correlation between % of Tregs and DDI after 1 year follow-up in both treated (n = 10) and untreated (n = 10) groups (C; R = −0.44, p = 0.05).

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