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Combined therapy with CD4^{+}CD25highCD127^{-} T regulatory cells and anti-CD20 antibody in recent-onset type 1 diabetes is superior to monotherapy: Randomized phase I/II trial

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Abstract

Aims: Monotherapy with autologous expanded CD4^{+}CD25^{high}CD127^{-} T regulatory cells (Tregs) or rituximab has been documented to slow disease progression in patients with recent-onset type 1 diabetes mellitus (T1DM). Whether a combined therapy including both drugs would further benefit this patient population is unknown.

Materials and Methods: We conducted a three-arms clinical trial to explore the efficacy and safety of the combined treatment with Tregs and rituximab in paediatric patients with T1DM. The patients were allocated to three groups: Tregs only (n = 13), Tregs + rituximab (n = 12) and control (n = 11). The key primary efficacy analyses were C-peptide levels (mixed meal tolerance test) and the proportion of patients in remission at 12 and 24 months.

Results: At month 24, as compared with the control, both treatment groups remained superior in the area under the curve of C-peptide mixed meal tolerance test, whereas in the analysis of all visits only the combined therapy improved area under the curve at 12 and 24 months. The proportion of patients in remission was significantly higher in the combined group than in the control group at 3, 6, 9 and 21 months but not at 18 and 24 months. There was no significant difference between the Tregs only group and control group. Adverse events occurred in 80% patients, mostly in the combined group and Tregs only group. No adverse events led to the withdrawal of the intervention or death. All comparisons were performed with alpha level of 5%.

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Conclusions: Over 2 years, combined therapy with Tregs and rituximab was consistently superior to monotherapy in delaying T1DM progression in terms of C-peptide levels and the maintenance of remission.

KEYWORDS
cell therapy, immunotherapy, rituximab, T regulatory cells, type 1 diabetes

1 | INTRODUCTION

Various mechanisms underlying the pathogenesis of type 1 diabetes mellitus (T1DM), including islet destruction resulting from deficits in the number and suppressive activity of T regulatory cells (Tregs) and the presentation of autoantigens to T cells by B lymphocytes, have been targeted for clinical drug development.[1,2] Despite intensive research efforts against its increasing prevalence, no approved drug stops the progression of diabetes.[3-5]

Tregs are critical regulators of peripheral immune tolerance.[6-9] Our previous research showed that the administration of expanded autologous $CD4^{+}CD25^{high}CD127^{-}$ Tregs in paediatric patients suppressed the self-reactive effector T cells and thereby delayed pancreatic islet cell destruction.[10,11] We further found that early administration and repetitive doses of Tregs positively affected these suppressive effects.[12,13] Other studies reported the selective depletion of B lymphocytes by the anti-CD20 antibody rituximab preserved the function of islet beta cells in these patients.[14-17] Although the therapeutic effects of both monotherapies were clinically significant, they were transient and did not prevent the patients from eventually progressing to the advanced stage.[17-19] Here, we present results of the three-arms clinical trial in which combined therapy with Tregs and anti-CD20 rituximab was compared with the monotherapy with Tregs and the standard of care with exogenous insulin only.

2 | MATERIALS AND METHODS

2.1 | Study design

TregVac 2.0 (trial reg. ISRCTN37116985 and EudraCT 2014-004319-35) was a phase 1/2, prospective, three-arm, randomized (rituximab treatment; 1:1), open-label (regarding Tregs treatment, control group) and single-blinded (patient blinded, regarding anti-CD20 antibody rituximab treatment groups), multicentre clinical study performed in children and adolescents with recent-onset T1DM. There were two parallel groups and a treatment-free control arm (total N = 45, Tregs group n = 15, Tregs + anti-CD20 rituximab group n = 15, treatment-free control group n = 15). The inclusion and exclusion criteria are presented in Table 1. The total duration of the follow-up was 24 months. The data were analysed by ICRC-Weyer GmbH (Berlin, Germany) and all authors had full access to the data.

An independent institutional review board approved the protocols, all patients signed informed consent forms (bioethics permission no. NKBBN/374/2012-NKBBN/374-7/2014). The trial was performed in accordance with the principles of the Declaration of Helsinki.

2.2 | Patients

Patients fulfilling the recruitment criteria, who agreed to take part in the study and signed informed consent, were offered the treatment in one of the randomized interventional arms or to be included into the control group. The randomization was performed by personnel with no access to clinical staff, patients and the study database. Patients in the interventional arms were treated with two doses of Tregs (30×10^6 Tregs/kg bw; each at day 0 and month 3) and randomly assigned using an element of chance (coin) to receive four infusions of rituximab (375 mg/m^2 of body surface area) (n = 12) or placebo (n = 13) on days 14, 22, 29 and 36. There were 11 patients, without the intervention treated under the standard-of-care rules, who were included as a control group (Figure 1; Supplementary 1 Table A1). The recruitment period did not allow the recruitment of the planned number of patients but the sample size achieved an estimated threshold of statistical power (see below).

2.3 | Outcomes

The primary endpoints were C-peptide level [mixed meal tolerance test (MMTT) and fasting], exogenous total daily dose of insulin (TDD) per kg bw, the proportion of patients in clinical remission (TDD <0.5 IU/kg/day and glycated haemoglobin (HbA1c; <6.5%) at months 12 (week 52) and 24 (week 104) after the first dose, and the number of adverse events (AEs) within 12 and 24 months after the first dose. Data were available for all groups for these endpoints (instances with missing data are noted in the tables and figures).

The key secondary endpoints were: (a) occurrence and severity of AEs directly related to treatments; (b) AEs of special importance (AESIs); (c) HbA1c level at weeks 2 and 5 and months 3, 6, 9, 12, 18 and 24; and (d) proportion of insulin-independent patients (TDD = 0 IU/kg bw) at months 3, 6, 9, 12, 18 and 24. The detailed description of the endpoints is listed in Table 1.

TABLE 1 Criteria of inclusion and exclusion to the study and endpoints

Inclusion criteria

1. 8-16 years of age.
2. BMI in the range of the 25th-75th percentile (according to the OLAF project)
3. Fasting plasma C-peptide >0.7 ng/ml and in stimulation test the increase ≥100%.
4. The presence of at least one anti-islet autoantibody (ICA, IAA, GAD): a high titre of IAA or GAD (≥4 times the norm) or a low titre (2-4 times the norm) of at least two of these antibodies.
5. Ability to provide written informed consent by parents (and patients if >16 years old).
6. Involvement of the patients and parents in intensive diabetes management defined as self-monitoring of glucose values no less than three times/day and the administration of insulin.
7. Appropriate venous access for blood drawing.

Exclusion criteria

1. No agreement for participation in the study and no inform consent signed.
2. Other than autoimmune type 1 diabetes.
3. Age <8 and >16 years.
4. IgA deficiency or other genetic defect present.
5. BMI <25th or >75th percentile for a particular age.
6. Hypersensitivity to anti-CD20 antibody rituximab or other components of the preparation.
7. Presence or history of active infection, including hepatitis B, hepatitis C, HIV, tuberculosis or syphilis. Subjects with laboratory evidence of active infection were excluded even in the absence of clinical evidence of active infection.
8. Presence of active EBV virus infection (positive IgM).
9. Presence or history of active systemic fungal infection.
10. Any history of malignancy.
11. Anaemia, lymphopenia, neutropenia or thrombocytopenia below the lower limits of the reference range during the 6 weeks before study.
12. Known hypercoagulative state.
13. Medical treatment requiring chronic use of drugs other than insulin longer than 3 months.
14. Treatment with any antidiabetic medication other than insulin within 4 weeks of enrolment.
15. Diabetic retinopathy.
16. Arterial hypertension.
17. Presence or history of macroalbuminuria.
18. For female subjects older than 15 years: a positive pregnancy test or an unwillingness to use effective contraceptive measures for the duration of the study and 4 months after discontinuation, when appropriate.
19. For male subjects: intent to procreate during the duration of the study or within 4 months after discontinuation when appropriate.
20. Excessive anxiety of the patient or parents related to the procedures.
21. Any medical condition that, in the opinion of the investigator, will interfere with safe participation in the trial.
22. For parents and paediatric patients older than 15 years: known active alcohol or substance abuse.

Endpoints

Primary endpoints

- C-peptide level (fasted/post MMTT stimulation) at 2 years after first dose of Tregs.
- Daily insulin dose per kg of body weight (TDD) 2 years after the first dose of Tregs.
- Number of treated patients in remission 1 and 2 years after first dose of Tregs. The number of patients with TDD lower than 0.5 U/kg/day and HbA1c lower than 6.5%.
- Number of AEs reported 2 years (week 104) after the first dose of Tregs.

Secondary endpoints

- Assessment of the occurrence and severity of side effects directly related to Tregs (hypersensitivity reactions, injection-site thromboembolic events) and blood sampling (>2 g/dl drop in haemoglobin levels).
- Assessment of the occurrence and severity of effects directly related to anti-CD20 antibody rituximab administration (hypersensitivity reactions).
- Assessment of the occurrence and severity of side effects associated with administration of Tregs or anti-CD20 rituximab antibodies, primarily immunosuppressive effects: occurrence of infections of any aetiology and de novo tumours detected.
- Any serious AEs in two or more patients with confirmed association to the administration of therapy.
- These four secondary safety endpoints will be documented as AEs of special interest and related treatment-emergent AEs, where appropriate.
- C-peptide level (fasted) from all visits (weeks 2, 5, 12, 26, 39, 52, 65, 78, 92 and 104).
- C-peptide level (post-MMTT stimulation) from all visits (weeks 12, 26, 52, 78 and 104).
- Exogenous insulin dose per kg of body weight from all visits (weeks 2, 3, 4, 5, 12, 14, 26, 39, 52, 65, 78, 92 and 104).
- Proportion of insulin-independent patients [TDD = 0 UI/kg body weight (bw)] (weeks 52 and 104).
- Proportion of patients in remission (TDD ≤0.5 UI/kg bw and HbA1c <6.5%) (weeks 52 and 104).
- HbA1c level (%) from all visits (week 2, 5, 12, 26, 39, 52, 65, 78, 92 and 104) as glycaemic control (fasting average of 7 days).
- Amount and intensity of side effects of therapy (weeks 52 and 104).
- Peripheral blood lymphocyte immunophenotype from all visits (weeks 2, 5, 12, 14, 26, 39, 52, 65, 78, 92 and 104) with basic phenotype results.

Abbreviations: AE, adverse events; BMI, body mass index; EBV, Epstein-Barr virus; GAD, glutamic acid decarboxylase antibodies; HIV, human immunodeficiency virus; IAA, insulin antibodies; ICA, islet cell antibodies; TDD, total daily dose of insulin; Tregs, regulatory T cells.

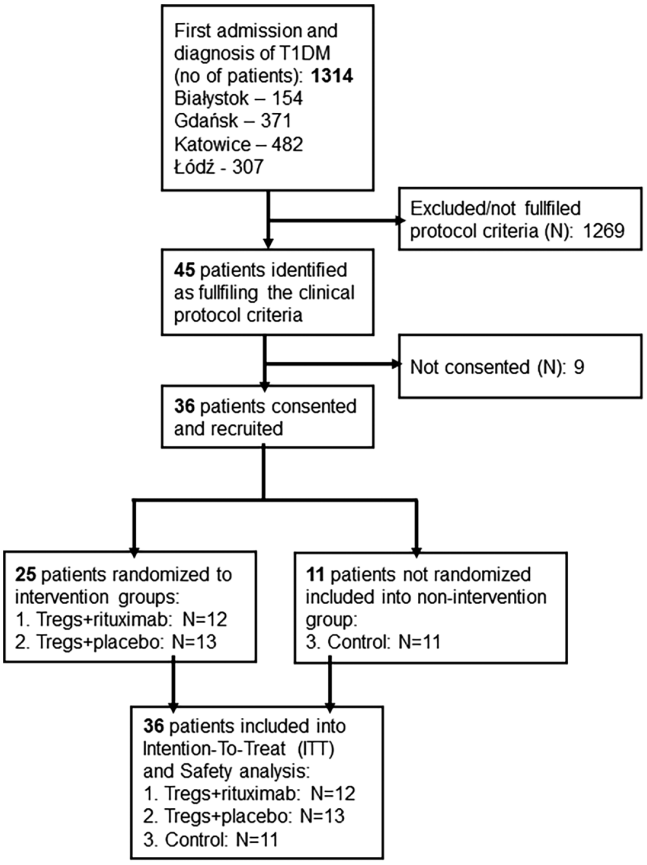

FIGURE 1 Consort diagram with patient flow. Tregs, regulatory T cells

2.3.1 | Mixed meal tolerance test

The fasting and stimulated blood glucose and C-peptide levels were assessed using the MMTT. Patients were asked to refrain from a meal and not to take short-acting insulin from 20:00 h before the test. Patients using the pump remained at the basal insulin level. Long-acting insulin and drinking water were permitted. The next morning, the level of fasting glucose was checked and the test was commenced when it was at the level of 70-180 mg/dl (3.89-10 mmol/L). Immediately after blood donation for fasting baseline/fasting levels of glucose and C-peptide, the patients were receiving a standard high protein mix (Boost, Nestle, USA) in a dose of 6 ml per kg body weight (but no more than 300 ml). At +15, +30, +60, +90, +120, +180, +210 and +240 min from the start of the meal, blood samples were taken to evaluate the stimulated glucose and C-peptide levels. The area under the curve (AUC) was used for the assessment of the endpoints.

2.4 | Statistical analysis

For the present study, a sample size of 13 patients per randomized treatment arm detected a 20% difference in the geometric mean ratio of AUC (0-240 min) of C-peptide, with a pairwise comparison following a non-inferiority one-side testing approach and an alpha value of 5% with a power estimate of >80% assuming a coefficient of variation of 0.2 if the real geometric mean ratio is 1. Assuming a drop-out rate of approximately 10%, it meant this sample size could be achieved by enrolling 15 participants per treatment group. Similar assumptions were applied for the secondary endpoints in an exploratory manner. Using the same assumptions, an exploration of the impact of not achieving the proposed number of patients per randomized treatment arm, the impact of a reduced sample size on the power of detection for a 20% difference in the geometric mean ratio between the treatment groups is limited and a power level of about 80% can be achieved if the coefficient of variation is ≤20% and the sample size is ≥11 subjects per treatment group (Table 2).

For the primary endpoints, treatment differences were estimated with analysis of covariance (ANCOVA) with 90% confidence intervals (CIs) calculated using a one-sided testing approach (alpha level = 5%). Logarithmized baseline values (day 0 or the last obtained before study intervention) were used as a continuous covariate, and age group (≤12 years), sex and treatment were used as fixed categorical effects. For C-peptide (MMTT and fasting), these values were back-transformed from the logarithmic scale to obtain intergroup geometric mean ratios and their 90% CIs for one-sided tests of superiority towards the control group. For TDD per kg bw, original (not log-transformed) values were used to provide LSmeans and their 90% CIs for group differences. Ratios for pairwise comparisons among the active treatment groups and one-sided non-inferiority tests were applied using a 20% threshold. All analyses were performed using the SAS software (version 9.3) on an exploratory basis without adjustments of multiplicity of tests.

3 | RESULTS

3.1 | Participants

At baseline, there were no statistically significant differences between randomized groups in demographic characteristics or the primary endpoint assessments such as AUC of C peptide, C-peptide (fasting) concentration and the variables defining clinical remission, such as daily insulin dose per kg bw (TDD) and HbA1c (all $p > .05$) (Table 2).

3.2 | Efficacy

3.2.1 | C-peptide (mixed meal tolerance test)

At 24 months, C-peptide AUC (MMTT 0-240 min, AUC_{240}) results showed that both Tregs + anti-CD20 rituximab (treatment ratio 1.770, 90% CI 1.018-3.078) and Tregs + placebo (1.893, 90% CI 1.062-3.372) were statistically significantly superior to the control group. However, in the subsequent non-inferiority comparison of Tregs + placebo/Tregs + anti-CD20 rituximab, the difference was not significant (1.069, 90% CI 0.601-1.902) (see Figure 2 and in the Supplementary 1 Table A2, Figure A1).

TABLE 2 Baseline demographic and clinical characteristics of patients (intention-to-treat population)[a]

Characteristic	Tregs + placebo (N = 13)	Tregs + rituximab (N = 12)	Control (N = 11)	*p* value
Male sex, n (%)	7 (52.8)	5 (41.7%)	5 (45.5)	.61
Age, years	13.3 ± 1.5	12.9 ± 1.2	12.1 ± 2.2	.68
Body mass index, kg/m^2	19.57 ± 1.8	18.1 ± 1.8	18.4 ± 1.4	.45
Body mass index, Z-score	−0.24 ± 0.46	−0.01 ± 0.43	0.04 ± 0.42	.19
Ethnicity, n (%)				
White	13 (100.0)	12 (100.0)	11 (100.0)	
Months since diagnosis	6.5 ± 4.2	6.0 ± 4.2	5.0 ± 3.2	.48
Insulin (TDD per kg of body weight)	0.3 ± 0.3	0.2 ± 0.2	0.3 ± 0.3	.71
C-peptide				
Fasting C-peptide (μg/L)	1.1 ± 0.4	1.1 ± 0.3	0.98 ± 0.2	.45
Stimulated C-peptide AUC_{240} (h*μg/L)	10.1 ± 2.4	11.0 ± 3.7	9.8 ± 2.1	.38
Glycated haemoglobin (%)	6.3 ± 1.1	6.6 ± 1.2	6.6 ± 0.8	.72
Glucose (mg/dl) (mean from fasting from 7 days before the visit)	103.1 ± 9.7	109.5 ± 13.5	103.9 ± 13.3	.55
Autoantibodies				
Glutamic acid decarboxylase (IU/ml)	856.6 ± 936.5	381.0 ± 594.6	744.2 ± 768.1	.74
Insulin autoantibody (IU/ml)	8.7 ± 8.6	5.3 ± 5.5	5.0 ± 5.1	.38
Islet cell antibody (titre)	109.2 ± 180.5	125.0 ± 185.0	50.9 ± 47.6	.25

Abbreviations: AUC, area under the curve; n, number included in the analysis; TDD, total daily dose of insulin; Treg, regulatory T cells. Baseline was defined as the last value of assessment prior to first drug administration. *p*-values are based on one-way ANOVA F-statistics for continuous and Kruskal-Wallis statistics for multilevel categorical data.

[a]Data are means ± SD.

On the other side, when the secondary endpoint AUC of C-peptide (MMTT, 0-240 min) from all visits throughout the trial was assessed, the levels in the Tregs + anti-CD20 rituximab group were statistically significantly superior to the control group at 12, 18 and 24 months, while the Tregs + placebo group was not superior to the control group at any time point, and the comparison of the two treatment groups showed that there was no statistically significant difference between the combined therapy and the monotherapy (see Figure 2 and Supplementary 1 Table A3, Figure A2).

3.2.2 | C-Peptide (fasting)

At 24 months, the combined treatment was statistically significantly superior to the control (2.268, 90% CI 1.264-4.069), the monotherapy was not (1.253, 90% CI 0.692-2.270). This difference in the results of the comparisons of the two active treatments versus the control group is further underlined by the comparison for non-inferiority of Tregs alone versus the combined therapy, which proved inferiority of the monotherapy (0.553, 90% CI 0.309-0.989) (see Figure 2 and Table A4, Figure A3).

Taking into account secondary endpoint "all visits" [RMANCOVA analysis of AUC of C-Peptide (MMTT), 0-240-min, all visits], the results showed that both Tregs + anti-CD20 rituximab and Tregs + placebo were superior to the control at 3, 6, 12, 15, and 18 months (see Figure 2 and Supplementary 1. Table A5, Figure A4). The Tregs + anti-CD20 rituximab group but not the Tregs + placebo group was statistically significantly superior than the control group at 21 and 24 months. The combination therapy appeared to have superior results than the monotherapy at months 18 and 24 and this observation was supported by the finding of statistically significant inferiority of the monotherapy when compared with the combination therapy.

3.2.3 | Clinical remission

There was no differences between the groups in the remission rate (defined as TDD <0.5 UI/kg/day and HbA1c <6.5%), when it was analysed separately at 12 and 24 months only (all $p > .05$) (Figure 3). However, when all visits were taken into account there was a statistically significantly higher proportion of patients in remission in the Tregs + anti-CD20 rituximab group than in the control group at 3, 6, 9 and 21 months but not at 18 or 24 months. There was no statistically significant difference between the Tregs + placebo group and the control group at any time point, and the proportion of patients in remission was statistically significantly higher in the Tregs + anti-CD20 rituximab group than in the Tregs + placebo group at 6 months (Supplementary 1 Table A6). The evaluation when uncensored analysis was performed and the remission was recognized until the last time point when the patients were fulfilling the criteria revealed that about half of the patients from each interventional group were in remission at 24-month follow-up (Figure 3).

Looking directly into the levels of TDD and HbA1c% throughout all visits, it was more clear that the combination therapy was superior

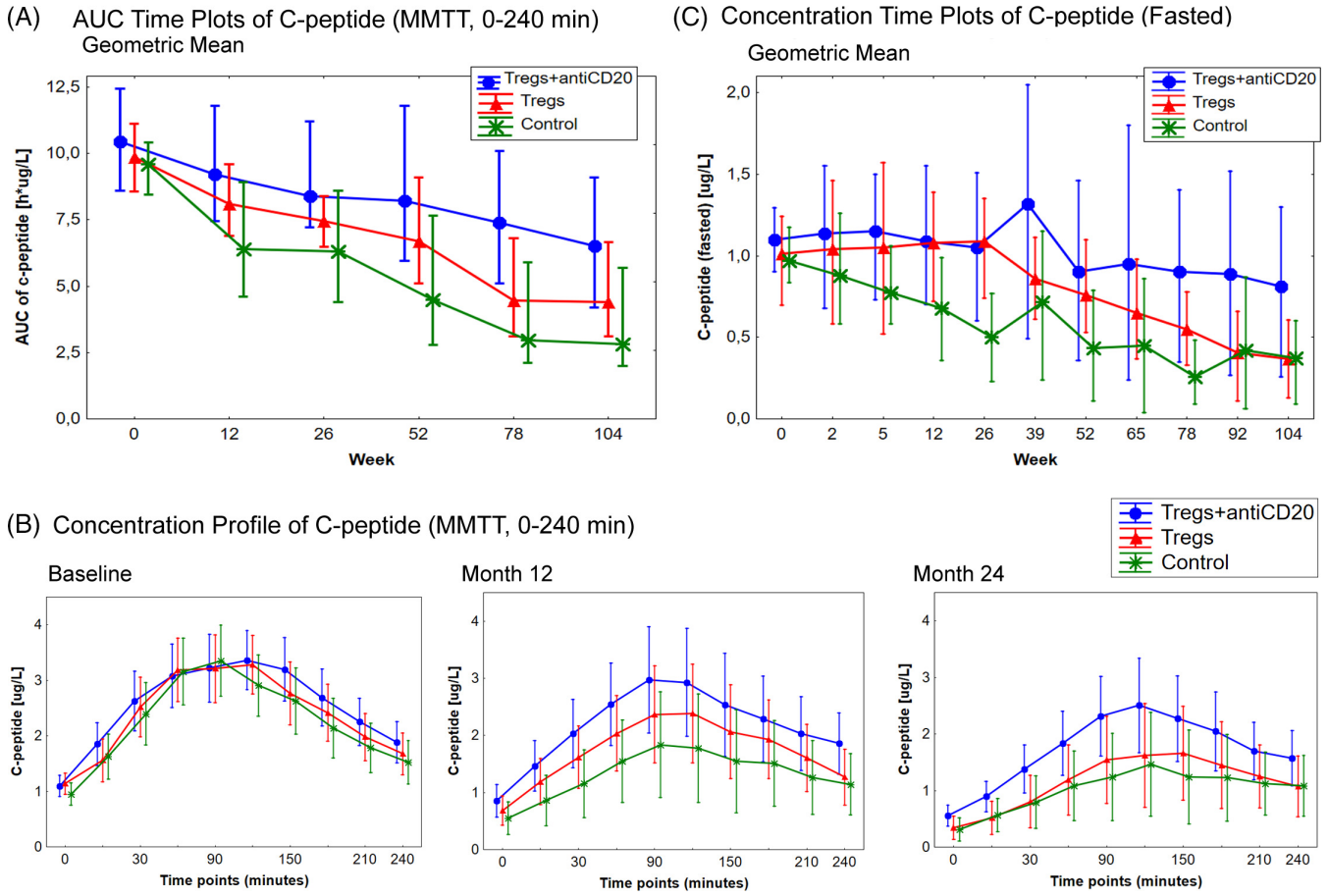

FIGURE 2 Area under curve (AUC) time plots and concentration profiles of C-peptide. A, Geometric means and 90% confidence intervals of the AUC time plots of the C-peptide [mixed meal tolerance test (MMTT), 0-240 min]. B, Concentration-time plots of C-peptide (MMTT, 0-240 min) levels and standard deviations at baseline, month 12 and month 24. C, Geometric means and 90% confidence intervals of the concentration-time plots of the C-peptide (fasted)

in keeping patients in this population in remission. RMANCOVA of HbA1c levels at months 3-24 visits showed that Tregs + anti-CD20 rituximab were statistically significantly lower than in the control group at months 3, 6, 9, 12, 21 and 24, while in the Tregs + placebo group HbA1c% was statistically significantly lower to the control only at the month 3 visit (see Figure 3 and Supplementary 1 Table A7, Figure A5). The point estimates for the treatment differences in TDD per kg bw showed that the Tregs + anti-CD20 rituximab group was statistically significantly superior to the control group (90% CI completely below 0) at month 6 and then from month 12 until month 24, while the Tregs + placebo group performed statistically significantly better than the control group only at month 15 and month 21 (see Figure 3 and Supplementary 1 Table A8, Figure A6).

3.3 | Safety

3.3.1 | Adverse events

No deaths or AEs leading to withdrawal of the study drug occurred (Table 3). Across all groups, of 156 total AEs reported in 31 patients (86.1% of all patients), the most frequently reported AEs were 10 events categorized as respiratory tract infection, eight events categorized as abdominal pain, seven events of iron deficiency, and six events of headache. Among these events, the following were reported as related to study treatment: infections in five patients, abdominal pain in four patients, iron deficiency in one patient and headache in two patients (Supplementary 1 Table A9). Among the AESIs defined as AEs related with blood collection and administration of the Tregs, Treg product contamination, AEs related with the immunosuppressive activity of Tregs, and AEs related with administration of rituximab antibody (anti-CD20) (Supplementary 1 Table A10) the most common were various infections and infestations. In total, 11 such episodes occurred in seven patients from the Tregs + CD20 rituximab group (58.3% of this patient group), compared with nine events in six patients from the Tregs + placebo group (46.2% of this patient group) and five events in four control patients (36.4% of this patient group). Most of the reported infections were of mild severity, but two events (one each of upper respiratory tract infection and influenza) both in the same patient in the Tregs + CD20 rituximab group (8.3% of this patient group) as well as two events (one each of herpes zoster and mumps) in two patients of the Tregs + placebo group (15.4% of this patient group) were of moderate severity. In the Tregs + CD20 rituximab group, eight of the reported infections occurring in six

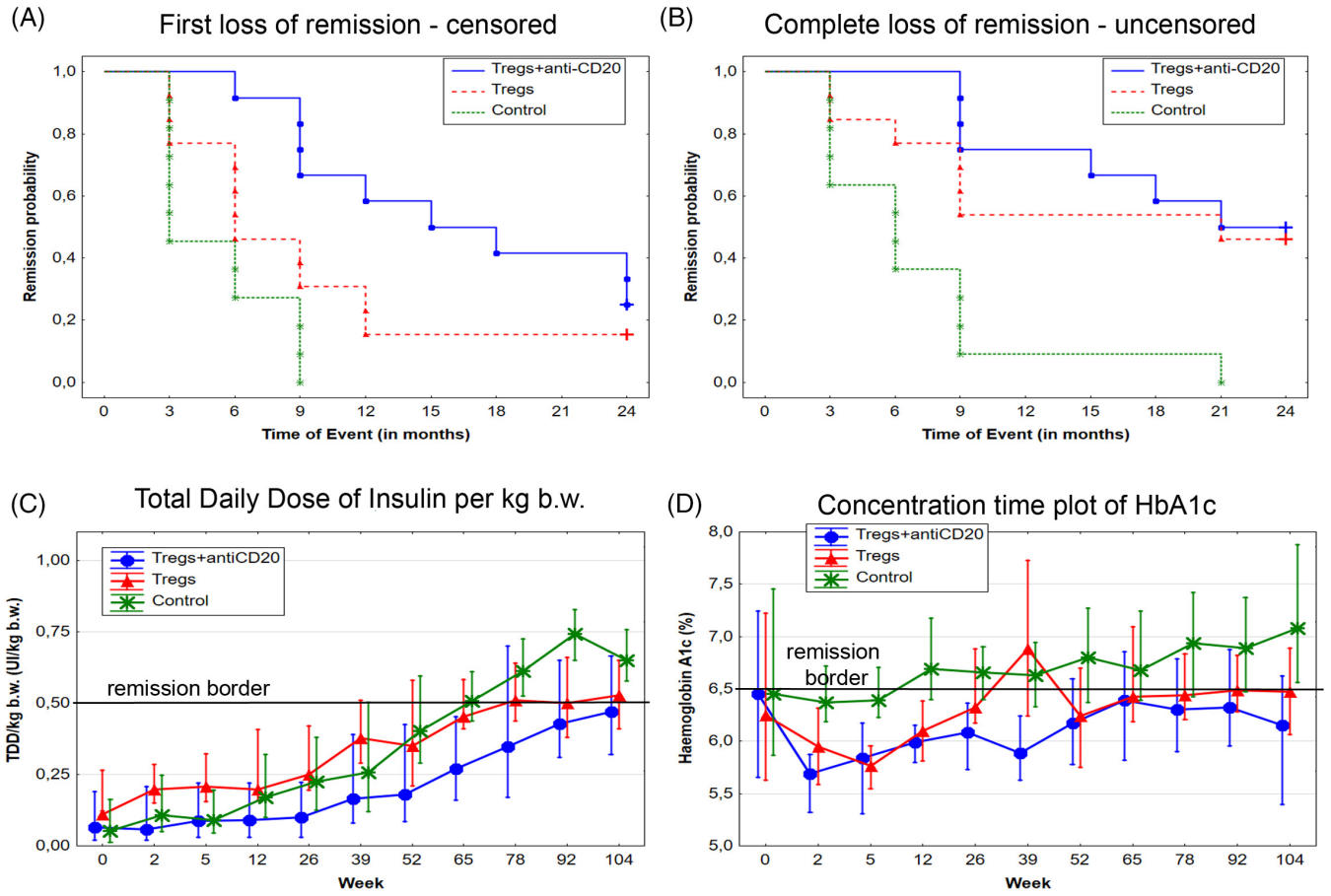

FIGURE 3 Loss of remission, total daily insulin dose and concentration-time plot of glycated haemoglobin (HbA1c). A, Kaplan-Meier plots of time to first loss of remission (censored) and, B, uncensored Kaplan-Meier plots of time to loss of remission. C, Geometric mean concentration-time plot with 90% confidence intervals of total daily insulin dose per kg body weight. D, Geometric mean concentration-time plot with 90% confidence intervals of HbA1c. TDD, total daily dose; Tregs, regulatory T cells

patients (50.0% of this patient group) were reported as causally related to study treatment, including both events of moderate severity. In the Tregs + placebo group, five of the reported infections in four patients (30.8% of this patient group) were reported as causally related to study treatment, also including both events of moderate activity (7.7% of this patient group). Taking into account the immunosuppressive nature of the treatment, there were AEs that needed special surveillance, such as infections [25 episodes noted in 17 patients (47.2%)] and cancer (not found).

4 | DISCUSSION

We found that the combined therapy with Tregs and rituximab consistently performed statistically significantly better than monotherapy with Tregs in controlling recent-onset T1DM in paediatric patients regarding C-peptide levels and remission. Both stimulated and fasting C-peptide were consistently higher in patients treated with the combined therapy. The combined therapy also had a significantly higher percentage of patients in remission than was found for either monotherapy and showed a clear trend towards later loss of insulin independence.

Most importantly, this study clearly shows the synergetic effects of Tregs and rituximab in controlling recent-onset T1DM. The benefits of monotherapy with Tregs were also confirmed by our data, in line with our previous pilot of the Tregs trial, validating the notion that autologous Tregs exert a suppressive effect on self-reactive effector T cells in paediatric patients.[10,19,20] The synergistic effects observed in the combined therapy group might be explained by superimposition of the depression of self-reactive T cells (induced by Tregs) and the depletion of CD20+ B cells, possibly those presenting autoantigens. While the former is a dominant mechanism of immune tolerance, the latter prevents the autoimmune epitope spread. These are separate mechanisms but both counteract autoimmunity. In addition, there are reports that endogenic Tregs are activated by rituximab administration, as indicated by the increased mRNA expression of Foxp3, GITR and CTLA-4 observed in these patients.[21-23] The state of immunosuppression imposed by B-cell depletion with rituximab is transient as B cell regenerate vigorously within few months. More important is probably rearrangement of B-cell subsets towards more tolerogenic phenotype with constant reduction of autoimmune B-cell clones, B cells presenting autoantigens, lower proinflammatory profile and higher percentage of B regulatory cells.[24-26] When combined with the infusion of Tregs, the treatment affects both arms of adoptive immunity

TABLE 3 Adverse events (AEs) and serious adverse events (safety population)[a]

Category	Tregs (N = 13) n (%), E	Tregs + anti-CD20 rituximab (N = 12) n (%), E	Control (N = 11) n (%), E	Total (N = 36) n (%), E
Any AE	9 (69.2), 28	12 (100), 76	10 (90.9), 52	31 (86.1),156
AE by relationship				
Any related AE	8 (61.5), 10	12 (100), 59	0	20 (55.6), 69
Any unrelated AE	5 (38.5), 18	7 (58.3), 17	10 (90.9), 52	22 (61.1), 87
AEs by severity				
Mild	8 (61.5), 18	12 (100), 66	9 (81.8), 48	29 (80.6),132
Moderate	4 (30.8), 10	2 (16.7), 10	2 (18.2), 4	8 (22.2), 24
Severe	0	0	0	0
System/organ class				
Infections and infestations[b]	6 (46.2), 9	7 (58.3), 11	4 (36.4), 5	17 (47.2), 25
Metabolism and nutrition disorders	2 (15.4), 3	3 (25.0), 8	6 (54.5), 11	11 (30.6), 22
Hyperglycaemia	0	2 (16.7), 2	2 (18.2), 2	4 (11.1), 4
Hypoglycaemia	1 (7.7), 1	1 (8.3), 2	2 (18.2), 2	4 (11.1), 5
Diabetes mellitus inadequate control	1 (7.7), 1	1 (8.3), 1	1 (9.1), 1	3 (8.3), 3
Hyperinsulinism	0	1 (8.3), 1	0	1 (2.8), 1
Ketoacidosis	1 (7.7), 1	0	0	1 (2.8), 1
Blood and lymphatic system disorders	0	4 (33.3), 4	3 (27.3), 3	7 (19.4), 7
Gastrointestinal disorders	1 (7.7), 5	4 (33.3), 14	1 (9.1), 1	6 (16.7), 20
Administration site conditions	2 (15.4), 2	4 (33.3), 5	0	6 (16.7), 7
Nervous system disorders	1 (7.7), 1	2 (16.7), 5	3 (27.3), 3	6 (16.7), 9
Surgical and medical procedures	2 (15.4), 2	1 (8.3), 1	3 (27.3), 3	6 (16.7), 6
Psychiatric disorders	1 (7.7), 4	2 (16.7), 7	2 (18.2), 6	5 (13.9), 17
Respiratory, thoracic and mediastinal disorders	0	3 (25.0), 5	0	3 (8.3), 5
Skin and subcutaneous tissue disorders	0	2 (16.7), 5	1 (9.1), 4	3 (8.3), 9
Cardiac disorders	0	2 (16.7), 3	0	2 (5.6), 3
Injury, poisoning and procedural complications	0	1 (8.3), 1	1 (9.1), 1	2 (5.6), 2
Social circumstances	0	2 (16.7), 2	0	2 (5.6), 2
Eye disorders	1 (7.7), 1	0	0	1 (2.8), 1
Musculoskeletal and connective tissue disorders	0	1 (8.3), 1	0	1 (2.8), 1
Product issues	0	1 (8.3), 1	0	1 (2.8), 1
Renal and urinary disorders	0	0	1 (9.1), 1	1 (2.8), 1
Vascular disorders	0	1 (8.3), 1	0	1 (2.8), 1

Note: %, (n/N) × 100, where N is the number of patients in each group.
System Organ Class and Preferred Term according to MedDRA dictionary Version 23.0.
Abbreviations: E, number of events; n, number of patients having an adverse event; N, number of patients at risk; Tregs, regulatory T cells.
[a]AEs that needed surveillance.
[b]None of the complications was significantly more frequent in one of the groups (all $p > .05$).

and reprogrammes this immunity towards tolerance more efficiently than Tregs alone.

While this therapy cannot keep the patients insulin-independent for life, it is the best we can offer for now. It has to be highlighted that T1DM is still an unmet medical need and there is no approved disease-modifying therapy available. The efficacy of the treatment is strongly limited by the stage of the disease when our intervention takes place. When first symptoms occur, only 20-30% of the islets remain alive and there is a need for exogenous insulin.[2-5,27] The islets do not regenerate and the patient is 'on the verge' of symptomatic progression. Unfortunately, autoimmunity is killing the remaining islets and pushes the patients towards exogenous insulin dependence. The therapies like ours counteract this process and protect the remaining islets secreting insulin. This marginal secretion is not enough to make these patients insulin-independent but it is still enough to fine-tune the level of blood glucose together with exogenously administered insulin. This fine-tuning by endogenous insulin is much more precise than the control of glycaemia by any medical device and therefore the

patients with preserved high levels of C-peptide secreting insulin are characterized by better metabolic control and delayed onset of the diabetes-related complications for many years.[28-30] Taking into account that the onset of the disease occurs mainly in childhood, each year with the proper control of glycaemia is a gain of delayed onset of the complications in later life, increased lifespan and better quality of life for these patients. We believe that this is the main benefit of this treatment and it should be administered as early as possible in the disease course. Potentially, it would be possible to administer it also in patients with presymptomatic T1DM and save them from onset of the disease for years. The application of Tregs without or with rituximab is safe in paediatric patients. Although AEs were common in all three groups, there were no deaths or AEs leading to withdrawal of both study drugs. AESIs of moderate severity were rarely reported but were in all cases considered related to study intervention. These data are in line with previous studies of monotherapy with either Tregs[19,20] or rituximab[14,31] in T1DM. Notably, the combined therapy did not increase AE frequency compared with either the Tregs or rituximab monotherapy. Importantly, as Tregs may affect patients' general immune competence and thus increase the risk for infections and the development of malignancies, monitoring of these AEs is critical.[18,32] Our data show that Tregs applied without or with rituximab yielded a frequency of AEs comparable with that of the control group, further supporting the general safety of treatment with Tregs with or without rituximab in these cohorts.

Strengths of this investigation include in-depth analyses of the efficacy and safety of the combined therapy based on data obtained in the clinical trial with a broad variety of robust evaluation methods. Limitations include the lack of availability of some data, notably historical nature of rituximab only reference. To make up this limitation, we retrieved from NIDDK repository raw data from the TN-05 study, which tested rituximab monotherapy[14] and compared this database with our results. With all the caution for this comparison, the combined treatment was significantly superior to the monotherapy with rituximab. Other limitations of our trial include the relatively low number of patients recruited, partial blinding and partial randomization. Although there are reasons for these limitations, it dictates a caution for the interpretation of the results.

Our 2-year clinical trial showed that the combined therapy with Tregs and rituximab was generally consistently superior to monotherapy with Tregs in controlling recent-onset T1DM in paediatric patients in terms of MMTT and fasting C-peptide levels, TDD, HbA1c, remission and insulin independence. In the future, a multicentre phase III trial with a high number of patients will probably confirm this conclusion.

AUTHOR CONTRIBUTION

MZ and MŻ contributed to the manufacturing of Tregs preparation, clinical and laboratory data collection, analysis, interpretation, and writing and reviewing of the report; DIG and MG contributed to the manufacturing of Tregs preparation and laboratory data collection and interpretation; MH, AJ-C, HK and AWD contributed to clinical assessment and data collection; JS contributed to lab assays, data collection and interpretation; RO, WM, PJCh, AB, AS, JS, NMT and MM contributed to the study design, protocol writing, data collection and interpretation and reviewed the report; PT was a supervisor of the study who contributed to the study design, protocol writing, cell preparation, data collection, analysis, interpretation writing and, reviewing of the report, and is the guarantor of the study.

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CONFLICT OF INTEREST

NMT, MM and PT are co-inventors of patents related to presented content and stakeholders of POLTREG venture. Medical University of Gdańsk received payment for the license to presented content. The other authors declare that they have no competing interests.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/dom.14723.

DATA AVAILABILITY STATEMENT

The full report and supplementary materials to this article will be attached to the study web registration page: https://www.isrctn.com/search?q=ISRCTN37116985.

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SUPPORTING INFORMATION

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