Protocol of the study

The study: "The therapy of type 1 diabetes with ex vivo expanded CD4+CD25+CD127- T regulatory cells (Tregs) and anti-CD20 monoclonal antibody- the randomized study"

Acronym: TregVac 2.0

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Sponsor: Medical University of Gdańsk, Skłodowskiej-Curie 3a, 80-210 Gdańsk

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Name of products: nr PR1: Tregs limfocytes product

Nr PR2 Rituximab

Consent of the Bioethical Committee (sponsor's protocol number): NKBBN/374/2012 with correction NKBBN/374-7/2014

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List of Abbreviations

AE - adverse effect
APC - Antigen presenting cells
APEDEC -Autoimmune PoliEndocrinopathy Candidiasis Ectodermal Dystrophy

APS I - Autoimmune Polyendocrinopathy Syndrome Type I

bw – body weight

C – Celsius

CD – cluster of differentiation

CRF – case report form

CTLA-4 - Cytotoxic T-Lymphocyte Antigen 4)

DM1 – Diabetes Mellitus Type 1

EKG – electrocardiogram

HBV – Hepatitis B virus

HCV – Hepatitis C virus

HIV – Human immunodeficiency virus

GMP – Good Manufacuring Practice

GvHD – Graft versus Host Disease

IFN – Interferon

IL – Interleukin

IPEX – Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome

Tregs lymphocytes- Naturally occurring regulatory T cells

CD3+CD4+CD25highCD127-Lin-FoxP3+

IVD - for *in vitro* diagnostics

kg – kilogram

LAL – Limulus Amebocyte Lysate

ml – mililiter

mln – million

NOD – non-obese diabetic

PBMC –Peripheral Blood Mononuclear Cells

PCR – Polymerase Chain Reaction

PWE – Multi-electrolyte fluid

SAE – Serious adverse effect

SOP – standard operating procedure

UV – ultraviolet radiation

2. Abstract

The randomised study to investigate the safety and efficacy of therapy of type 1 diabetes in children with ex vivo expanded CD4+CD25+CD127- T regulatory cells (Tregs) and anti-CD20 monoclonal antibody

Patients: 45 [randomization 1:1; Tregs group: 15 patients treated with Tregs; TregsCD20 group: 15 patients treated with Tregs and CD20 antibody; control group: 15 patients without treatment]

Inclusion of patients: 18 months

Observation time: 24 months (‘0’ Day is a day of administration of first Tregs dose)

Time of study: 42 months

Type of study: A prospective, randomised study (1/2 phase)

The objective of the project is to refine the innovative cell therapy in the treatment of type I diabetes in children and to verify its effectiveness by randomizing. The method is based on the immunosuppressive action of isolated and expanded CD4 + CD25 + T regulatory lymphocytes. These cells administered to the patient during the period of "prediabetes" could stop or at least slow the process of pancreatic islets destruction by autoaggressive T cells in the time preceding the onset of clinical symptoms of diabetes. In this study, we would like to enhance the effectiveness of therapy by spreading the dose of Tregs (at least two doses at ‘0’ time and +90 days to a total dose of 60 million Tregs per kg of body weight) and administration at ‘0’ time in one of the antibody treatment cycle groups anti-CD20 (rituximab), which in previous studies was described as the most effective among the tested innovative biological drugs (Pescovitz MD 2009). Our research center was the first in the world to use this immunosuppressive method in the treatment of type 1 diabetes with very promising effects: prolonging the total insulin-dependent time and then reducing the dose of insulin administered in the treated group (Marek-Trzonkowska N 2012; Marek-Trzonkowska N 2014). This study protocol is based on our previous experience.

The final result of the project will be **a completed protocol of treatment** of patients in the period of "prediabetes" and **observation of the effectiveness of this treatment.**

Stages of procedures:

1. Patient’s blood volume (age-adjusted) is collected in the hospital from which T regulatory lymphocytes of CD3(+)CD4(+)CD25(high)CD127(-)doublet(-)lin(-) are separated.
2. Due to low percentage of these cells in blood (around 100-200 thousand in 1 liter of peripheral blood) isolated cells can not be administered directly after isolation. In

order to increase their amount they are multiplied in a „high purity" laboratory meeting GMP requirements. This way within 15 to 20 days from 100 thousand cells over 1 billion of T regulatory cells are obtained, ready for administration to the patient.

3. Patients receive proliferated Tregs lymphocytes and in one of the groups they also receive a series of anti-CD20 antibody regimens at the minimum dose that was recommended in previous studies.
4. Procedure of Tregs administration in both groups is repeated on day +90.
5. All patient are monitored clinically and laboratory for 2 years after the first administration of regulatory lymphocytes.

3. State of the art

3.1 Diabetes Mellitus Type 1

Diabetes Mellitus Type 1 (DM1) is the autoimmune disorder that usually begins before the age of 35, and in most cases before the 18. DM1 is currently one of the most common chronic diseases of childhood. It has also become the main cause of loss of sight, end-stage renal failure, amputation and premature death in developed and developing countries. According to the International Diabetes Federation (IDF) there were 193 mln of patients in 2003 and this number had increased to 246 mln in 2007. However, according to the estimates of the World Health Organization (WHO), by 2030, 366 million people will develop diabetes. Among this number, 10-15% are cases of DM1A. In the United States, DM1A is diagnosed in 15/100,000 children, in Finland in 35/100,000 and in Japan less than 1/100000. In Poland, the incidence rate in DM1A shows regional differences and fluctuates in recent years from 5 / 100,000 in the Lublin macroregion and 6.18 / 100,000 in the ex- Rzeszów province to 9.2 / 100,000 in the Pomeranian province and the Lodz region, showing constant, systematic growth in subsequent years (even by 100% in 10 years for the Lodz region).

Nowadays, DM1 is believed to develop as a result of abnormal recognition of pancreatic β-cells by their own T-lymphocytes, which kill island cells leading to a gradual deficiency of insulin and loss of blood glucose control. This is usually manifested by excessive thirst and polyuria and weight loss, and in extreme cases by ketoacid coma.

Today, treatment of DM1 is primarily based on insulin substitution by exogenous insulin as an injection or in the form of an insulin pump. Both this method of treatment as well as disease-related complications mentioned above constitute a significant deterioration of the quality and shorten the life expectancy of patients. Unfortunately, today, there are no effective methods to stop the progression of the disease in its early period, when the pancreatic isles are still preserved and it would be possible to maintain the proper level of glucose while avoiding the supply of exogenous insulin. For this reason, any attempt to treat this disease at its early stage is particularly valuable.

3.2. T regulatory lymphocytes

Regulatory T cells (Tregs) is a special population of cells in the immune system due to the fact that they do not actively participate in combating infections, but their role is mainly to inhibit the excessive activation of other cells to protect their own tissues against autoaggression. Tregs have great therapeutic potential. Today, less severe disorders regarding the amount and function of Tregs have been described properly in most autoimmune and allergic diseases. Hence, skilfully used, they seem to be a hope for treatment many diseases that until now have not been successfully treated. The most known phenotype of these cells is CD3 + CD4 + CD25highCD127-FoxP3 +.

The role of Treg in diabetes has been proven in animal models and in humans. The lack of these cells or their inadequate function causes the development of autoimmune diseases, including DM1. This is most evident in patients with IPEX syndrome, which is caused by the mutation of the foxp3 gene, crucial for the Tregs function. In animals, it was shown that the administration of these cells to the NOD mice predisposed to development of diabetes prevents the onset of diabetes, and after the occurrence of *insulitis*, it inhibits the progression of changes in the pancreas. Treg reports have cemented the scientists' belief in the need for targeted immunosuppression using biological therapies. The surprisingly good effects of treating diabetes with anti-CD3 antibodies are also associated with the induction of Tregs. Our own experience by the TregVac study (1.0) ISRCTN06128462 (NKEBN / 8/2010) indicates that administration of Tregs is important for delaying the development of DM1 in children.

3.3 Anti-CD20 antibody (Rituximab)

Rituximab (anti-CD20 Ig) is a cytotoxic anti-CD20 antibody directed against B lymphocytes. In the present study, the idea of administering anti-CD20 antibodies, is related to the function of these cells in the antigens presentation in the immune response. This phenomenon is important in the early stages of response to own antigens during the initiation and propagation of autoimmune diseases as so-called ‘epitope spread’. Disintegrating own tissues are a source of ever new antigens that effectively presented by B lymphocytes with effector T-lymphocytes cause the autoimmune reaction. Therefore, the elimination of B cells can cause significant inhibition of the development of autoimmune reactions. The selective removal of B lymphocytes by the anti-CD20 antibody (rituximab) has indeed been described as a way of prolonging insulin dependence in patients with newly diagnosed DM1.

3.4 The combination therapy

Both, the TregVac study with Tregs and the rituximab study, proved the importance of these drugs in preventing the pancreatic islets destruction. Nevertheless, in both studies it was also shown that the use of monotherapy caused only a transient effect and patients eventually developed diabetes. For this reason, in this study we want to influence two independent mechanisms of DM1 development. For the regulatory/suppressive activity of Tregs, we want to add B lymphocytes depletion to limit the presentation of pancreatic autoantigens, and thus

their impact on the propagation of autoimmune disease. This combination therapy is intended to stop or significantly slow down the autoimmune process, and consequently prolong significantly insulin dependence and remission in DM1.

4 The objective of the study

4.1 The aim

The primary objective of the study is to assess the safety and efficacy of the treatment in individual patients groups treated with Tregs or the combination of Tregs and rituximab.

4.2. Primary Endpoints:

- Number of adverse effects reported 2 years after the first dose of Tregs
- C-peptide level (fasted/post MMTT stimulation and after glucagon test) 2 years after first dose of Tregs
- Daily insulin dose per kg of body weight (DDI) 2 years after the first dose of Tregs
- Number of treated patiens in remission 1 and 2 years after first dose of Tregs – the numer of patients with daily insulin dose lower than 0.5U/kg/day and HbA1c lower than 6.5%

The key secondary endpoints:

- ✓ Day of Tregs administration:
    - Assessment of the occurence and severity of side effects directly related to Tregs (hypersensitivity reactions, congestion) and blood sampling (>2g/dL drop in hemoglobin levels)
- ✓ Day of anti-CD20 antibody administration (after every dose):
    - Assessment of the occurence and severity of effects directly related to anti-CD20 antibody administration (hypersensitivity reactions)
- ✓ 14 days after administration of Tregs lymphocytes
- ✓ 60 ± 30 days after the administration of the first dose of Tregs
- ✓ 90 ± 30 days after the administration of the first dose of Tregs [second dose point]
- ✓ 180 ± 30 days after the administration of the first dose of Tregs

- ✓ 270 ± 30 days after the administration of the first dose of Tregs
- ✓ 360 ± 30 days after the administration of the first dose of Tregs
- ✓ 450 ± 30 days after the administration of the first dose of Tregs
- ✓ 540 ± 30 days after the administration of the first dose of Tregs
- ✓ 630 ± 30 days after the administration of the first dose of Tregs
- ✓ 720 ± 30 days after the administration of the first dose of Tregs

- Assessment of the occurence and severity of side effects associated with administration of Tregs or anti-CD20 antibodies, primarily immunosuppressive effects: occurence of any etiology and de novo tumors detected

Cessation of the Trial Criteria:

- Any severe adverse effect in two or more patients with confirmed association to the administration of therapy

Efficacy Endpoints:

- ✓ 60 ± 30 days after the first dose of Tregs
- ✓ 90 ± 30 days after the first dose of Tregs [second dose point]
- ✓ 180 ± 30 days after the first dose of Tregs
- ✓ 270 ± 30 days after the first dose of Tregs
- ✓ 360 ± 30 days after the first dose of Tregs
- ✓ 450 ± 30 days after the first dose of Tregs
- ✓ 540 ± 30 days after the first dose of Tregs
- ✓ 630 ± 30 days after the first dose of Tregs
- ✓ 720 ± 30 days after the first dose of Tregs

Defined as measurement of the following parameters:

- C-peptide level (fasted/post MMTT stimulation and in glukagon test)
- Insulin dose per kg of body weight
- The proportion of insulin-independent subjects
- The proportion of subjects with DDI ≤ 0.5UI/kg b.w.
- HbA1C level

- Glycemic control (fasting average of 7 days)
- The amount and intensity of side effects of therapy
- Quality of Life Questionnaire (QOL)
- Peripheral blood lymphocyte immunophenotype

5. The study

5.1 Inclusion and Exclusion Criteria

Inclusion Criteria

1. 8 to 16 years of age.
2. BMI the range of 25-75 percentiles (according to OLAF)
3. Fasting plasma C-peptide more than 0.7ng/mL and in stimulation test the increase ≥ 100%
4. The presence of anti-islet autoantibody (ICA, IAA, GAD) - high titer one of the antibody (≥ 4 times the norm, not applicable ICA) or low titer two or three antibodies (2-4 times the norm)
5. Ability to provide written informed consent by parents (and patients if above 16years old)
6. Involvement of the patients and parents in the intensive diabetes management defined as self monitoring of glucose values no less than three times/ day and by 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 singed
2. Other than autoimmune type 1 diabetes
3. Age below 8 and above 16 years
4. IgA deficiency or other genetic defect present
5. BMI < 25 or >75 percentiles for a particular age.
6. Hypersensitivity to anti-CD20 or other components of the preparation
7. Presence or history of active infection including hepatitis B, hepatitis C, HIV, tuberculosis (TB) or syphilis. Subjects with laboratory evidence of active infection are 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. Anemia, 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 then 3 months
14. Treatment with any anti-diabetic 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 positive pregnancy test, 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 children older than 15 years: known active alcohol or substance abuse.

5.2. Randomization and control group

Patient will receive a sequential number (1 to 45) and be randomized to one of the groups (Tregs or Tregs CD20) prior to blood collection for expansion. Due to the specificity of study, the randomization will be performed by the team involved in the expansion of Tregs. For the same reason, the person responsible for randomization will not have access to the clinical and laboratory results collected by the team responsible for the clinical part of the study until the end of the study. The control group will be patients with no intervention, e.g.
- refuse of blood donation or treatment with the proposed scheme but agreement to routine testing;
- exluded from blood drawing due to inappropriate venous access.

Patients who start treatment but for various reasons will be disqualified will be an observation group.

Patients will be qualified for the following groups:

The group	Treatment
TregsCD20 (n=15)	Tregs infusion at time "0" + 4 doses of anti-CD20 antibody; second infusion of Tregs during "+ 90 ± 30 days"
Tregs (n=15)	Tregs infusion during "0" 4 doses of anti-CD20 antibody; second infusion of Tregs during "90 ± 30 days"
NoTregs (n=15)	No intervention (refuse of blood donation or treatment with the proposed scheme but agreement to routine testing, exluded from blood drawing due to inappropriate venous access)
The observation group	Patients enrolled in the study, but disqualified due to failure to meet the

	criteria for individual groups

5.3. Preparation, storage and administration of the tested preparations

5.3.1a. Preparation of the Tregs lymphocyte preparation

Blood for the expansion of Tregs is collected at the Department of Pediatric Diabetology at the University Clinical Center. The patient included in the study is admitted to the Department on a routine basis 1 day before the procedure. In order to properly prepare the patient should be hydrated, recommending drinking 1-2 liters of liquids 1-3 hours prior to blood collection. The patient receives an infusion of PWE (500 - 1000 ml) the night before blood collection, and after, the blood loss is supplemented with 6% Volulyte in the volume of blood drawn. It is recommended, but not necessary, blood collection on an empty stomach. The blood is collected from a peripheral vein in a closed system to bags with an anticoagulant under the supervision of an anaesthesiologist. The maximum volume of blood is 250ml. After collecting, ALL bags are marked with the name of the patient, body weight, date, time of collection, and then blood is sent to the Laboratory of Preparations of the Provincial Blood Donation and Blood Treatment Center.

5.3.1b. Storage of the Tregs product

Tregs product can not be stored. After delivery to the Department must be given within two hours.

5.3.1c. Preparation, administration and dosage of Tregs product

The Tregs preparation is delivered in a transport container in a sterile closed syringe in the form of a cell suspension in 20ml of 0.9% sodium chloride. The number of cells is converted to the patient's body weight (up to 30 million Tregs / kg b.w.). **After removing the preparation from the transport container, check the compliance of the patient's name with the name on the preparation label.** The product should be prepared at room temperature under the principles of asepsis. The contents of the syringe should be injected into a sterile, pyrogen-free 250ml or 500ml transfusion container containing 9 mg / ml (0.9%) sodium chloride solution. To mix the solution, gently swirl the container to avoid foaming the contents. Ensure sterility of the prepared solution. The aseptic rules should be followed because the product does not contain antimicrobial preservatives or bacteriostatic substances. Before administration, visually inspect the infused medicinal product for free from any discoloration.

The Tregs product should be administered as a slow intravenous infusion (approximately 10 minutes) through an infusion line designed for this purpose (line with a blood transfusion filter). Do not administer the product as an intravenous injection or bolus. After completion of the infusion, the line should be rinsed with 100 ml of saline to administer the entire dose. Then the patient should remain under medical observation for at least the next 6 hours to prevent the effects of possible allergic reactions.

One patient may receive between 10 and 30 million Tregs per kg body weight. The total dose

in the whole study can not exceed 60 million Tregs lymphocytes per kg body weight (the next dose must be matched to the amount of lymphocytes already obtained).

5.3.2A. Preparation, administration and dosage of rituximab

The required volume of the product should be withdrawn from the vial using aseptic technique and reconstituted to the calculated concentration of 1 to 4 mg / ml rituximab in a sterile, pyrogen-free transfusion container, in a solution for injection of 9 mg / ml (0.9%) sodium chloride or in solution for injection of 5% D-glucose in water. To mix the solution, gently swirl the container to avoid foaming the contents. Ensure sterility of the prepared solution. The aseptic rules should be followed because the product does not contain antimicrobial preservatives or bacteriostatic substances. Before administration of parenteral medications, it should be visually checked that the medicinal product prepared for infusion contains no precipitates and does not change color.
The prepared rituximab solution should be administered as an intravenous infusion via an infusion line designed for this purpose. Do not administer the product as an intravenous injection or bolus. Any unused medicinal product or waste material should be disposed of in accordance with local requirements.

Patients will receive a single course of treatment with rituximab: four injections, each at a dose of 375 mg / m2 on the following study days: +14, +22, +29 and +36 (scheme: 1-8-15-22). This is a pattern proven in the works of the Type 1 Diabetes Trialnet AntiCD20 Study Group, which tested this drug successfully in patients with DM1 (Pescovitz MD 2009). Administration of the drug will be performed in the operating room of the Department of Pediatric Diabetology under medical / anesthesiological care. EKG monitoring is recommended during the infusion. The drug will be administered as a slow intravenous infusion. The initial infusion rate recommended by the manufacturer is 50 mg / hour; after the first 30 minutes, the infusion rate can be increased by 50 mg / hour; every 30 minutes to a maximum speed of 400 mg / hour. In order to reduce adverse effects associated with drug administration, patients will be premedicated with antipyretic / analgesic (eg paracetamol 0.5g 30min before infusion) and antihistamine (eg diphenhydramine intravenously 50mg immediately prior to infusion, klmastine intravenously 2mg immediately prior to infusion) (Roche 2012). Due to the possibility of hyperglycaemia, premedication with glucocorticosterone will not be used.

Patients treated with rituximab should be provided with a patient warning card when each infusion is given.

5.3.2b. Storage of the rituximab preparation

The expiration is 30 months. Store in a fridge (2°C - 8°C). Keep the container in the outer carton in order to protect it from sunlight.
The rituximab solution prepared for infusion is physically and chemically stable for 24 hours at 2°C - 8°C and then 12 hours at room temperature. From a microbiological point of view, the prepared solution for infusion should be used immediately. If not used immediately, it is the responsibility of the person administering the medication to determine the time and

storage conditions of the reconstituted solution prior to use and have to not be more than 24 hours at 2°C - 8°C unless dilution has occurred under controlled and attested conditions. aseptic. Any unused medicinal product or waste material should be disposed of in accordance with local requirements.

5.3.3. The other medicines during the study

Insulin

Patients should be treated with insulin in a routine manner in accordance with the standards of the Pediatric Section of the Polish Diabetes Association or equivalent standards of diabetes care.

Vaccinations

All vaccinations should be postponed for no less than 12 months after the last administration of Tregs or rituximab.

Contraception

Due to the lack of sufficient data on the effects of preparations on pregnancy and lactation, patients over 15 years of age must engage in sexually acceptable contraception approved by the physician conducting the study.

Other medicines

Unless they are used chronically (see exclusion criteria), other medicinal preparations can be taken by patients. The fact of such therapy should always be recorded in the study documentation.

5.4. Follow up of the patients

Follow up visit schedule (±30days from +3months)

	R[1]	A[2]	0d[3]	+14d[4]	+22d	+29d	+36d	+3m[2]	+3m[prim,3]	+6m	+9m	+1y	+15m	+18m	+21m	+24m
Clinical examination	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Insulin requirement	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
HbA1c (%)	x	x		x			x	x		x	x	x	x	x	x	x
C-peptide	x	x		x			x	x		x	x	x	x	x	x	x
C-peptide MMTT and glucagon test	x	x[5]						x[5]		x		x		x		x
Blood test+CRP+urine test		x	x	x	x	x	x	x	x							
Quality of life	x						x	x	x	x		x				x
Immunophenotype: Tregs + B lymphocytes		x	x	x			x	x	x	x	x	x	x	x	x	x
Autoantibodies	x	x		x				x		x		x		x		x
Cytokines		x		x			x	x		x	x	x	x	x	x	x
Molecular tests		x		x			x	x		x	x	x	x	x	x	x

1 - recruitment
2 - blood drawing
3 - Tregs infusion
4 – anti-CD20 antibody in TregsCD20 group +14, +22, +29, +36 day
5 – functional tests during blood drawing
□ – this protocol only in Medical University of Gdansk

5.4.1 Inclusion to study

Day R

Patients aged 8-16 years can be included in the study, in whom Type 1 diabetes was detected not earlier than 2 months before the application [Qualification FormTregVac2 (Annex 1)].

To include the patient in the study:
1. Inform the patient and parents/guardians about the assumptions, objectives and course of the examination and give the informed consent and consent forms for data processing (attachments 7 and 8); sufficient time should be provided to read the forms.
2. Obtain signed consent forms and consent for data processing signed by parents (and a child over 16 years old), one copy of each form remains with parents
3. Interview with special attention to compliance with the schedule of preventive vaccination (especially BCG vaccination against tuberculosis), time from the onset of DM1 symptoms, insulin doses, other medicines (especially chronic), other chronic diseases - especially those excluded from the study:

- Other than autoimmune type 1 diabetes
- IgA deficiency or other genetic defect present
- Hypersensitivity to rituximab or other components of the preparation
- Hypersensitivity to penicillin and/or streptomycin
- Presence or history of active infection including hepatitis B, hepatitis C, HIV, tuberculosis (TB) or syphilis (the laboratory evidence)
- Presence or history of active EBV virus infection (the laboratory evidence
- Presence or history of active systemic fungal infection
- Any history of malignancy
- Anemia, lymphopenia, neutropenia or thrombocytopenia below the lower limits of the reference range during the 6 weeks before study
- Known hypercoagulative state.
- Medical treatment requiring chronic use of drugs other than insulin longer then 3 months
- Treatment with any anti-diabetic medication other than insulin within 4 weeks of enrolment
- Diabetic retinopathy.
- Arterial hypertension.
- Presence or history of macroalbuminuria
- For female subjects older than 15 years positive pregnancy test, unwillingness to use effective contraceptive measures for the duration of the study and 4 months after discontinuation, when appropriate.

- For male subjects: intent to procreate during the duration of the study or within 4 months after discontinuation when appropriate.
- Excessive anxiety of the patient or parents related to the procedures.
- Any medical condition that, in the opinion of the investigator, will interfere with safe participation in the trial.
- For parents and children older than 15 years: known active alcohol or substance abuse.

4. Ensure the involvement of the patient and parents in the intensive diabetes management defined as self monitoring of glucose values no less than three times/ day and ability of the administration of insulin injections (check the blood glucose diary, when appropriate).

5. The clinical examination, mainly check appropriate venous access for blood drawing, the height and weight, body temperature, blood pressure and heart rate.

6. Ask to fill in / fill out with the patient the questionnaires of Quality of Life and perception of diabetes (Annexes 3 and 4)

7. Perform preliminary laboratory tests:

- Fasting plasma C-peptide
- After the fasting glucose below 126mg% to prepare a meal tolerance test (MMTT) or a glucagon test on two consecutive days
- HbA1C (%)
- fasting blood glucose level
- anti-GAD, ICA, IAA antibody level
- anti EBV IgM and IgG antibodies level

The laboratory tests performed during routine examinations at the diabetes department qualifying the center and the discharge from the hospital are sufficient for initial qualification. The results or discharge card should be sent to the Diabetology Department of the University Clinical Center in Gdańsk along with signed informed consent forms and consent to the processing of data to Professor Małgorzata Myśliwiec (see addresses and details of the coordinators). The final qualifications are carried out at the Department of Pediatrics of Diabetology and Endocrinology of UCK Gdańsk (designations in the Central Laboratory of UCK). Qualification results from the Central Laboratory of UCK can be used as laboratory tests for 'day A' (see later point 5.4.2 Day A)

8. The recruitment result is recorded on the qualification form of TregVac2 (Annex 1)

9. If the laboratory data are from the Central Laboratory of the UCK, fill out the patient's CRF document in the column 'Preliminary (blood donation -10 days) point A' (Annex No. 2 Form CRF_TregVac2).

5.4.2. Description of visits

The clinical evaluation of safety and efficacy based on clinical history, physical examination, laboratory tests and reporting by the patient and parents the side effects related to treatment.

Day A (-10 days)

Hospitalization for blood donation to Tregs expansion (2-3 days stay)

1. Conduct the medical interview. Once again confirm the willingness of the child and parents to participate in the study, confirm the informed consent form and the consent form for processing the data. The particular attention the inclusion and exclusion criteria. To verify the glycemic diary and insulin dose by parents (mean fasting blood glucose level from the last 7 days to CRF).

2. If not filled in on the „R" day, ask to fill in / fill out with the patient the questionnaires of Quality of life and perceptions of diabetes (Annexes 3 and 4)

3. The clinical examination, mainly check appropriate venous access for blood drawing, the height and weight, body temperature, blood pressure and heart rate.

4. To perform the Meal Tolerance Test (MMTT) and the glucagon stimulation test, store the blood from the tests.

5. Collect blood for tests (unless the results of the qualification from the Central Laboratory of the UCK from 'Day R' are available (see point 5.4.1. Day R of the task 6,7,8):

Central Laboratory of UCK:

• anti EBV IgM and IgG antibodies level
(the donation of blood for Tregs expansion in the Regional Blood Donation and Blood Treatment Center also antiHBV, Hbs, antiHCV, antiHIV, anti-syphilis)
• anti-GAD, ICA, IAA antibody levels
• HbA1c (%)
• Blood test
• CRP protein
• Urine test
• Blood from the Meal Tolerance Test (MMTT) and the glucagon stimulation test:
- 10x 2ml clotting tubes/coagulate tubes (MMTT test) + 10x 2ml sodium fluoride tubes (glucose)
- 2x 2 ml clotting tubes (glucagon stimulation test)
• The remaining tests from donations to the Tregs expansion

6. Complete the patient's CRF document (Appendix No. 2 CRF_TregVac2 Form)

Day 0

Hospitalization for administration of Tregs (1 day stay) - usually 14day after blood collection for expansion

1. Conduct the medical interview. Assessment of the incidence and severity of immediate Adverse Effects resulted from the administration of Tregs, reported diseases and symptoms and medications. To veryfy the involvement of the patient and parents in the intensive diabetes management defined as self monitoring of glucose values and ability of the

administration of insulin injections (mean fasting glucose from the last 7 days to CRF).

2. The clinical examination, mainly check appropriate venous access for Tregs administration, the height and weight, body temperature, blood pressure and heart rate.

3. Collect blood for tests:

Central Laboratory of UCK:

• Blood test
• CRP protein
• Urine test

Department of Clinical Immunology and Transplantology, GUMed, ul. Dębinki 7, building 27 2nd floor:

• 1x 4ml EDTA tube, immediately after Tregs lymphocyte administration

4. Complete the patient's CRF document (Appendix No. 2 CRF_TregVac2 Form)

Day +14

Hospitalization for **the first** dose of rituximab or placebo (1st day)

1. Contact the Department of Clinical Immunology and Transplantology GUMed, ul. Dębinki 7, building 27 2nd floor for the administration of rituximab or placebo.

2. Conduct an interview. Inform about the necessity of appearing for the next three times in the days of +22, +29 and +36 for administration of the antibody. Assessment **of the incidence and severity of possible Adverse Effects**, particularly infections, as well as, reported diseases, symptoms and medications. To verify the glycemic diary and insulin dose by parents (mean fasting blood glucose level from the last 7 days

3. The clinical examination, mainly check appropriate venous access for the puncture, the height and weight, body temperature, blood pressure and heart rate.

4. Collect blood for tests **before administration of rituksimab**:

Central Laboratory of UCK:

• anti-GAD, ICA, IAA antibody levels
• fasting plasma C-peptide and glucose level
• HbA1c (%)

• Blood test
• CRP protein
• Urine test

Department of Clinical Immunology and Transplantology, GUMed, ul. Dębinki 7, building 27 2nd floor:

• 2x 4ml heparin tubes for immunophenotyping
• 1 x 4ml EDTA tube for molecular tests
• 1x a 4ml trizol tube for molecular tests
• 2x 4ml clotting tubes for serum cytokine determination

5. Fill out the patient's CRF document (Annex No. 2 CRF_TregVac2 Form)

Day +22

Hospitalization for **the second** dose of rituximab or placebo (1st day)

1. Contact the Department of Clinical Immunology and Transplantology GUMed, ul. Dębinki 7, building 27 2nd floor for the administration of rituximab or placebo.
2. Conduct an interview. Inform about the necessity of appearing for the next two times in the days of +29 and +36 for administration of the antibody. Assessment of **the incidence and severity of possible Adverse Effects**, particularly infections, as well as, reported diseases, symptoms and medications. To verify the glycemic diary and insulin dose by parents.
3. The clinical examination, mainly check appropriate venous access for the puncture, the height and weight, body temperature, blood pressure and heart rate.
4. Collect blood for tests **before administration of rituksimab**:

Central Laboratory of UCK:

• Blood test
• CRP protein
• Urine test

5. Fill out the patient's CRF document (Annex No. 2 CRF_TregVac2 Form)

Day +29

Hospitalization for **the third** dose of rituximab or placebo (1st day)

1. Contact the Department of Clinical Immunology and Transplantology GUMed, ul. Dębinki 7, building 27 2nd floor for the administration of rituximab or placebo.
2. Conduct an interview. Inform about the necessity of appearing for the visit in the day+36 for administration of the antibody. Assessment of the incidence and severity of possible Adverse Effects, particularly infections, as well as, reported diseases, symptoms and medications. To verify the glycemic diary and insulin dose by parents.
3. The clinical examination, mainly check appropriate venous access for the puncture, the height and weight, body temperature, blood pressure and heart rate.
4. Collect blood for tests **before administration of rituksimab**:

Central Laboratory of UCK:

• Blood test
• CRP protein
• Urine test

5. Fill out the patient's CRF document (Annex No. 2 CRF_TregVac2 Form)

Day +36

Hospitalization for the quarter dose of rituximab or placebo (1st day)

1. Contact the Department of Clinical Immunology and Transplantology GUMed, ul. Dębinki 7, building 27 2nd floor for the administration of rituximab or placebo.

2. Conduct an interview. Assessment of the incidence and severity of possible Adverse Effects, particularly infections, as well as, reported diseases, symptoms and medications. To verify the glycemic diary and insulin dose by parents (mean fasting blood glucose level from the last 7 days.
3. To fill in / fill out with the patient the questionnaires of Quality of life and perceptions of diabetes (Annexes 3 and 4)

4. The clinical examination, mainly check appropriate venous access for the puncture, the height and weight, body temperature, blood pressure and heart rate.

5. Collect blood for tests:

Central Laboratory of UCK:

• fasting plasma C-peptide and glucose level
• HbA1c (%)

• Blood test
• CRP protein
• Urine test

Department of Clinical Immunology and Transplantology, GUMed, ul. Dębinki 7, building 27 2nd floor:

• 2x 4ml heparin tubes for immunophenotyping
• 1 x 4ml EDTA tube for molecular tests
• 1x a 4ml trizol tube for molecular tests
• 2x 4ml clotting tubes for serum cytokine determination

6. Fill out the patient's CRF document (Annex No. 2 CRF_TregVac2 Form)

Day +3 months

Hospitalization for the second blood donation to Tregs expansion (2-3 days stay)

1. Conduct the medical interview. Assessment of **the incidence and severity of possible Adverse Effects**, particularly infections, as well as, reported diseases, symptoms and medications. To verify the glycemic diary and insulin dose by parents (mean fasting blood glucose level from the last 7 days to CRF)

2. To fill in / fill out with the patient the questionnaires of Quality of life and perceptions of diabetes (Annexes 3 and 4)

3. The clinical examination, mainly check appropriate venous access for blood drawing, the height and weight, body temperature, blood pressure and heart rate.

4. To perform the Meal Tolerance Test (MMTT) and the glucagon stimulation test, store the blood from the tests.

5. Collect blood for tests:

Central Laboratory of UCK:

• anti-GAD, ICA, IAA antibody levels
(the donation of blood for Tregs expansion in the Regional Blood Donation and Blood Treatment Center also antiHBV, Hbs, antiHCV, antiHIV, anti-syphilis)

• fasting plasma C-peptide and glucose level (data from the 0 point of MMTT)
• HbA1c (%)
• Blood test
• CRP protein
• Urine test
• Blood from the Meal Tolerance Test (MMTT) and the glucagon stimulation test:
- 10x 2ml clotting tubes/coagulate tubes (MMTT test) + 10x 2ml sodium fluoride tubes (glucose)
- 2x 2 ml clotting tubes (glucagon stimulation test)
• The remaining tests from donation to the Tregs expansion

6. Complete the patient's CRF document (Appendix No. 2 CRF_TregVac2 Form)

Day +3 months prim

Hospitalization for administration of Tregs (1 day stay)

1. Conduct the medical interview. Assessment of **the incidence and severity of possible Adverse Effects**, particularly infections, as well as, reported diseases, symptoms and medications. To verify the glycemic diary and insulin dose by parents (mean fasting blood glucose level from the last 7 days to CRF)

2. To fill in / fill out with the patient the questionnaires of Quality of life and perceptions of diabetes (Annexes 3 and 4)

3. The clinical examination, mainly check appropriate venous access for Tregs administration, the height and weight, body temperature, blood pressure and heart rate.

4. Collect blood for tests:

Central Laboratory of UCK:

• Blood test
• CRP protein
• Urine test

Department of Clinical Immunology and Transplantology, GUMed, ul. Dębinki 7, building 27 2nd floor:

• 1 x 4ml EDTA tube for molecular tests

6. Fill out the patient's CRF document (Annex No. 2 CRF_TregVac2 Form)

Day +6 months

The control visit (hospitalization in Gdańsk for 2days)

1. Conduct the medical interview. Assessment of **the incidence and severity of Adverse Effects,** reported diseases and symptoms and medications. To verify the glycemic diary and insulin dose by parents (mean fasting blood glucose level from the last 7 days to CRF)

2. To fill in / fill out with the patient the questionnaires of Quality of life and perceptions of diabetes (Annexes 3 and 4)

3. The clinical examination, mainly check the height and weight, body temperature, blood pressure and heart rate.

4. To perform the Meal Tolerance Test (MMTT) and the glucagon stimulation test,

5. Collect blood for tests:

Central Laboratory of UCK:

• anti-GAD, ICA, IAA antibody levels

• fasting plasma C-peptide and glucose level (data from the 0 point of MMTT)
• HbA1c (%)
• Blood from the Meal Tolerance Test (MMTT) and the glucagon stimulation test:
- 10x 2ml clotting tubes (MMTT test) + 10x 2ml sodium fluoride tubes (glucose)
- 2x 2 ml clotting tubes (glucagon stimulation test)

Department of Clinical Immunology and Transplantology, GUMed, ul. Dębinki 7, building 27 2nd floor:

• 2x 4ml heparin tubes for immunophenotyping
• 1 x 4ml EDTA tube for molecular tests
• 1x a 4ml trizol tube for molecular tests
• 2x 4ml clotting tubes for serum cytokine determination

6. Fill out the patient's CRF document (Annex No. 2 CRF_TregVac2 Form)

Day +9 months

The control visit (outpatient)

1. Conduct the medical interview. Assessment of the incidence and severity of Adverse Effects, reported diseases and symptoms and medications. To verify the glycemic diary and insulin dose by parents (mean fasting blood glucose level from the last 7 days to CRF)

2. The clinical examination, mainly check the height and weight, body temperature, blood pressure and heart rate.

3. Collect blood for tests:

Central Laboratory of UCK:

• 1x clotting tubes - fasting plasma C-peptide
• 1x sodium fluoride tubes – fasting glucose
• 1x 4ml EDTA tube - HbA1c (%)

Department of Clinical Immunology and Transplantology, GUMed, ul. Dębinki 7, building 27 2nd floor:

• 2x 4ml heparin tubes for immunophenotyping
• 1 x 4ml EDTA tube for molecular tests
• 1x a 4ml trizol tube for molecular tests
• 2x 4ml clotting tubes for serum cytokine determination

Clinical centers outside of Gdansk - **ALL blood samples collected** (Central Laboratory of UCK and to the Department of Clinical Immunology and Transplantology) send by courier to: Department of Clinical Immunology and Transplantology, Medical University of Gdańsk, ul. Dębinki 7 80-210 Gdańsk, building 27 2nd floor, room 3.46, **with note: Piotr Trzonkowski TregVac2**

4. Fill out the patient's CRF document (Annex No. 2 CRF_TregVac2 Form)

Day +12 months

The control visit (hospitalization in Gdańsk for 2days)

1. Conduct the medical interview. Assessment of the incidence and severity of Adverse Effects, reported diseases and symptoms and medications. To verify the glycemic diary and insulin dose by parents (mean fasting blood glucose level from the last 7 days to CRF)

2. To fill in / fill out with the patient the questionnaires of Quality of life and perceptions of diabetes (Annexes 3 and 4)

3. The clinical examination, mainly check the height and weight, body temperature, blood pressure and heart rate.

4. To perform the Meal Tolerance Test (MMTT) and the glucagon stimulation test,

5. Collect blood for tests:

Central Laboratory of UCK:

• 1x clotting tubes - anti-GAD, ICA, IAA antibody levels

• fasting plasma C-peptide and glucose level (data from the 0 point of MMTT)
• 1x 4ml EDTA tube - HbA1c (%)

- Blood from the Meal Tolerance Test (MMTT) and the glucagon stimulation test:
  - 10x 2ml clotting tubes (MMTT test) + 10x 2ml sodium fluoride tubes (glucose)
  - 2x 2 ml clotting tubes (glucagon stimulation test)

Department of Clinical Immunology and Transplantology, GUMed, ul. Dębinki 7, building 27 2nd floor:

• 2x 4ml heparin tubes for immunophenotyping
• 1 x 4ml EDTA tube for molecular tests
• 1x a 4ml trizol tube for molecular tests
• 2x 4ml clotting tubes for serum cytokine determination

6. Fill out the patient's CRF document (Annex No. 2 CRF_TregVac2 Form)

Day +15 months

The control visit (outpatient)

1. Conduct the medical interview. Assessment of the incidence and severity of Adverse Effects, reported diseases and symptoms and medications. To verify the glycemic diary and insulin dose by parents (mean fasting blood glucose level from the last 7 days to CRF)

2. The clinical examination, mainly check the height and weight, body temperature, blood pressure and heart rate.

3. Collect blood for tests:

Central Laboratory of UCK:

• 1x clotting tubes - fasting plasma C-peptide
• 1x sodium fluoride tubes – fasting glucose
• 1x 4ml EDTA tube - HbA1c (%)

Department of Clinical Immunology and Transplantology, GUMed, ul. Dębinki 7, building 27 2nd floor:

• 2x 4ml heparin tubes for immunophenotyping
• 1 x 4ml EDTA tube for molecular tests
• 1x a 4ml trizol tube for molecular tests
• 2x 4ml clotting tubes for serum cytokine determination

Clinical centers outside of Gdansk - ALL blood samples collected (Central Laboratory of UCK and to the Department of Clinical Immunology and Transplantology) send by courier to: Department of Clinical Immunology and Transplantology, Medical University of Gdańsk, ul. Dębinki 7 80-210 Gdańsk, building 27 2nd floor, room 3.46, **with note: Piotr Trzonkowski TregVac2**

4. Fill out the patient's CRF document (Annex No. 2 CRF_TregVac2 Form)

Day +18 months

The control visit (hospitalization in Gdańsk for 2days)

1. Conduct the medical interview. Assessment of the incidence and severity of Adverse Effects, reported diseases and symptoms and medications. To verify the glycemic diary and insulin dose by parents (mean fasting blood glucose level from the last 7 days to CRF)

2. The clinical examination, mainly check the height and weight, body temperature, blood pressure and heart rate.

3. To perform the Meal Tolerance Test (MMTT) and the glucagon stimulation test,

4. Collect blood for tests:

Central Laboratory of UCK:

• 1x clotting tubes - anti-GAD, ICA, IAA antibody levels

• fasting plasma C-peptide and glucose level (data from the 0 point of MMTT)
• 1x 4ml EDTA tube - HbA1c (%)

- Blood from the Meal Tolerance Test (MMTT) and the glucagon stimulation test:
  - 10x 2ml clotting tubes (MMTT test) + 10x 2ml sodium fluoride tubes (glucose)
  - 2x 2 ml clotting tubes (glucagon stimulation test)

Department of Clinical Immunology and Transplantology, GUMed, ul. Dębinki 7, building 27 2nd floor:

• 2x 4ml heparin tubes for immunophenotyping
• 1 x 4ml EDTA tube for molecular tests
• 1x a 4ml trizol tube for molecular tests
• 2x 4ml clotting tubes for serum cytokine determination

5. Fill out the patient's CRF document (Annex No. 2 CRF_TregVac2 Form)

Day +21 months

The control visit (outpatient)

1. Conduct the medical interview. Assessment of the incidence and severity of Adverse Effects, reported diseases and symptoms and medications. To verify the glycemic diary and insulin dose by parents (mean fasting blood glucose level from the last 7 days to CRF)

2. The clinical examination, mainly check the height and weight, body temperature, blood pressure and heart rate.

3. Collect blood for tests:

Central Laboratory of UCK:

• 1x clotting tubes - fasting plasma C-peptide
• 1x sodium fluoride tubes – fasting glucose
• 1x 4ml EDTA tube - HbA1c (%)

Department of Clinical Immunology and Transplantology, GUMed, ul. Dębinki 7, building 27 2nd floor:

• 2x 4ml heparin tubes for immunophenotyping
• 1 x 4ml EDTA tube for molecular tests
• 1x a 4ml trizol tube for molecular tests
• 2x 4ml clotting tubes for serum cytokine determination

Clinical centers outside of Gdansk - ALL blood samples collected (Central Laboratory of UCK and to the Department of Clinical Immunology and Transplantology) send by courier to: Department of Clinical Immunology and Transplantology, Medical University of Gdańsk, ul. Dębinki 7 80-210 Gdańsk, building 27 2nd floor, room 3.46, with **note: Piotr Trzonkowski TregVac2**

4. Fill out the patient's CRF document (Annex No. 2 CRF_TregVac2 Form)

Day +24 months

The control visit (hospitalization in Gdańsk for 2days)

1. Conduct the medical interview. Assessment of the incidence and severity of Adverse Effects, reported diseases and symptoms and medications. To verify the glycemic diary and insulin dose by parents (mean fasting blood glucose level from the last 7 days to CRF)

2. To fill in / fill out with the patient the questionnaires of Quality of life and perceptions of diabetes (Annexes 3 and 4)

3. The clinical examination, mainly check the height and weight, body temperature, blood pressure and heart rate.
4. To perform the Meal Tolerance Test (MMTT) and the glucagon stimulation test,
5. Collect blood for tests:

Central Laboratory of UCK:
• 1x clotting tubes - anti-GAD, ICA, IAA antibody levels

• fasting plasma C-peptide and glucose level (data from the 0 point of MMTT)
• 1x 4ml EDTA tube - HbA1c (%)

- Blood from the Meal Tolerance Test (MMTT) and the glucagon stimulation test:
  - 10x 2ml clotting tubes (MMTT test) + 10x 2ml sodium fluoride tubes (glucose)
  - 2x 2 ml clotting tubes (glucagon stimulation test)

Department of Clinical Immunology and Transplantology, GUMed, ul. Dębinki 7, building 27 2nd floor:
• 2x 4ml heparin tubes for immunophenotyping
• 1 x 4ml EDTA tube for molecular tests
• 1x a 4ml trizol tube for molecular tests
• 2x 4ml clotting tubes for serum cytokine determination

6. Fill out the patient's CRF document (Annex No. 2 CRF_TregVac2 Form)

5.4.3. The control group

Patients who for various reasons have failed to provide any of the treatment provided for in the program (neither Tregs nor rituximab) despite meeting the inclusion criteria, should be proposed to continue to participate in the follow-up visits.
They should check-up on: 0, + 14 days, + 36 days, + 3 months, + 6 months, + 9 months, + 12 months, + 15 months, + 18 months, + 21 months, + 24 months. During these visits, the patient's assessment should be exactly as described in "5.4.2. Description of visits ", i.e. the same as for patients who received a full treatment protocol.

5.4.4. Patients without a full treatment protocol (the observation group)

Patients who for various reasons have failed to provide a full treatment protocol, i.e. two doses of Tregs and rituximab or placebo, should be proposed to continue to participate in the follow-up program.
They should check-up on: 0, + 14 days, + 36 days, + 3 months, + 6 months, + 9 months, + 12 months, + 15 months, + 18 months, + 21 months, + 24 months. During these visits, the patient's assessment should be exactly as described in "5.4.2. Description of visits ", i.e. the same as for patients who received a full treatment protocol.

5.5. Procedures

5.5.1. Documentation of the study

The document form for the patients of the trial is the Case Report Form CRF_TregVac2 (Appendix No. 2). In the document, enter the dates of all patients visits in the top row of the form (a tolerance of +/- 30 days from the planned date of the visit is acceptable starting from the day "+ 3month"). The dates of all appointments should also be noted on page 4 of Annex 7, "Information on the test and informed consent form", in a copy of the patient for the knowledge of the dates of the next examination. All data obtained in the study should be entered into this form within 7 days of obtaining the result. Information and patients results also be entered online on the study website. All results and information that can identify the patient will be entered in the confidential part of the password protected webpage.

5.5.2 Insulin requirements

Subjects will record their total daily insulin dose on self-monitoring diaries. Subject should be given exogenous insulin as needed to maintain:
- fasting capillary glucose levels <140 mg/dL (7.8mmol/L) at a minimum of 4 out of 7 days a week
- 2-hour post-prandial capillary glucose levels should not exceed 180 mg/dL (10.0 mmol/L) more than 3 times per week.
The results collected with the study schedule.

5.5.3. Glycemic control

Glycemic control will be assessed by HbAlc (%). The results collected with the study schedule. The self-control of glycemic levels will also be assessed.

5.5.4. The C-peptide

The function of the pancreas will be assessed by fasting blood glucose and C-peptide levels and after stimulation, the MMTT test; according to the study schedule.

Meal tolerance test (MMTT)

The fasting and stimulated blood glucose and C-peptide levels will be assessed in the meal tolerance test. Patients will be asked to refrain from a meal and not to take short-acting insulin from 8:00 P.M. before the test, is possible to administer long-acting insulin and drink water. Patients using the pump will remain at the basal insulin level. The next day, patients will report to the clinic, where they will check the level of fasting glucose. The level <70 mg / dl (3.89 mmol / L) or> 180 mg / dl (10mmol / L) will be disqualified and the test will be postponed. At the level of 70 -180 mg / dl (3.89 -10 mmol / L), the fasting glucose and C-peptide level will be assessed. Immediately after blood donation, the patient will receive 6mL per kg body weight (but no more than 300mL) of a standard high protein mix within 5 minutes (the start of the meal is the time "0"). At +15, +30, +60, +90, +120, +180, +210, and +240 minutes from the start of the meal, blood will be obtained to evaluate the stimulated glucose and C-peptide levels.

Glucagon stimulation test

Glucagon stimulation is the second test to assess the fasting and stimulated C-peptide level. Patients will be asked to prepare as above, i.e. refraining from a meal and not administering short-acting insulin from 8:00 P.M. before the test, is possible to administer long-acting insulin and drink water. Patients using the pump will remain at the basal insulin level. The next day, the fasting glucose level will be checked. The level <70 mg / dl (3.89 mmol / L) or> 180 mg / dl (10mmol / L) will be disqualified and the test will be. At the level of 70 -180 mg / dl (3.89 - 10 mmol / L) the fasting C-peptide level will be assessed. Immediately after blood donation, the patient will receive intravenously glucagon (0.5 mg if body weight ≤ 30 kg or 1.0 mg if weight> 30 kg) and 6 minutes later measurement of glucagon stimulated C-peptide.

5.5.5. Quality of life

To assess the quality of life two test will be used: : generic (attachment No. 4) and disease-specific measures (attachment No. 3)

Disease-targeted Measures

Psychometric (author) questionnaires measure various diabetes-related distress domains: emotional-burden, physician-related interpersonal distress, regimen-related distress and

diabetes-related interpersonal distress, attachment No. 3 „Psychological problems of children with DM1 ".

Generic Measures

Psychometric (author) questionnaires measure physical functioning, bodily pain, general health and vitality of child and parents, attachment No. 4 "Pediatric Questionnaire for the Quality of Life of Children and Youth"

5.5.6. Immunologic Testing

1. Cellular immunity – pharmacokinetics

The following phenotype, from the patient's whole blood, will be screened for the assessment of T regulatory cells: CD3/CD4/CD25/CD127/CD45RA/CD62L/FoxP3 by flow cytometry – pharmacokinetics, also.

The complete immunophenotype of the peripheral blood leukocyte populations will be performed according to the study schedule.

2. Autoantibodies

The level of autoantibodies, from patient's serum, as feature of immune response with confirmed link to the autoreactivity to islets: antiGAD, antiIA2, IAA, ICA will be followed on protocol for the duration of the study

3. Cytokines

To assess the balance of proinflammatory and anti-inflammatory and Th1/Th2/Th17 the expression of cytokines from patient's serum will be collected by multiset (over 15 cytokines in kit) by Luminex method or flow cytometry flexset, according to the study schedule.

5.5.7. Archived of materials

During the study patient's blood will be collected to obtain serum, blood cells, RNA, DNA processed and archived for study tests. At any request, the patient or paretns will be able to receive information about details for subjects regarding the archiving of samples.

5.5.8. Finish of the study

The '+ 24 month' visit is considered the completion of the study. Patients who have lost contact within two years of enrollment and failed to attend subsequent visits are considered to be discontinuing the study. Researchers should try to contact such patients and their parents / guardians after confirming the first abstention in the schedule and proposing to continue the study. If the patient decides to continue, should be kept in the study group until the end of the study, or '+ 24 month' visit.

6. Safety Monitoring

6.1. Clinical safety assessments

Clinical safety will be assessed by physical examination, measurement of vital signs, laboratory tests and occurrence of reportable adverse events.

6.2. Monitoring plan and data safety

Consenting process

All parents and patients ≥ 16 y.o. will have the cognitive ability to provide written consent. The information will be also given to the patients and the receive will be assessed and monitored. Assurance that the patient and parents understands the study will be obtained by routine evaluation of subjects' mental capacity as part of their routine physical examination. Informed consent is completed by physician and nurses on a routine basis by asking general questions to ensure the patient and parents are oriented to person, place and time. Additional questions will be asked and answers evaluated to discern the level of understanding of the patient and family with regard to what has and will transpire during the course of the treatment process. This will occur repeatedly during each follow-up visit during the course of the study. Consent will be discussed during the initial clinic visit and before any study procedure commences, only after the patient has fulfilled eligibility criteria and is considered by the principal investigator to be an appropriate subject for the study.

Confidentiality of data

Patients' identity will remain confidential throughout the study period unless disclosure is required by law. It has been included in the Informed Consent Form that the data may be shared by study personnel other than the Primary Investigator, representatives of the approved government agencies, the Ethics Committee, and other regulatory agencies. Patients will be identified only by a unique identifier number. All data records and computers containing information about subjects specifically relevant to this study will be maintained in a locked, secured office. All study results for studies performed by hospital laboratories for which the results are posted on the Hospital computer system will be maintained by a secured password system with all the confidentiality safeguards afforded to patients receiving routine, non-experimental care. The results of this study may be published and presented in scientific meetings, however, the patient will not be identified in such publications or presentations.

6.3. Monitoring for toxicity and adverse outcomes

The schedules for regular physician encounters and laboratory testing are detailed previously. The adverse effects will be classified according to „Common Terminology Criteria for Adverse Effects CTCAE ver. 4.0" (Annex No. 5). In urgent cases, parents, or patients will have the option of a phone call (phone number on the informed consent form) or a personal contact with the attending physician / researcher. In addition to regular review of this data, the study team will meet at intervals to review individual patient data and summary descriptive statistics on adverse events and measurements of treatment efficacy. Minutes will document each of these conferences and can be presented to the Ethics Committee for external evaluation.

Adverse effects

Definition of adverse effect (AE): any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medical treatment or procedure regardless of whether the sign, symptom or disease is considered related to the medical treatment or procedure.

Definition of serious adverse effect (SAE): any event that is fatal or life-threatening, that is permanently disabling, requires or extends hospitalization of the subject, represents a significant overdose or breach of protocol, or suggests that a drug or procedure used in a research protocol has produced congenital anomaly or cancer, or, in the opinion of the investigator, represents other significant hazards or potentially serious harm to the research subject or others.

Severity grading scale:

- Mild: adverse event of little clinical significance
- Moderate: adverse event of mild to severe significance; causing some limitation of usual activities
- Severe: see definition of SAE above

Attribution scale:

- Not related: clearly not related to the study
- Possible: may be related to the study
- Probable: likely related to the study
- Definite: clearly related to the study
- Unable to assess

Adverse effects of special importance in this study:

a. AE related with blood collection and administration of the Tregs

Blood collection and administration of the Treg product may be associated with the occurrence of adverse effects typical of peripheral vascular puncture and blood transfusion preparation. These are mainly excessive bleeding, vasovagal reactions, injection site reactions, haemolytic reactions, allergic reactions, fever, chills, erythema, purple, and acute lung injury. Diagnosis and treatment of these reactions will belong to the anesthesiologist assisting with the procedures.

b. ***Treg product contamination***

Physician notification of Tregs product contamination occurs as dictated by Microbiology Laboratory SOP by direct phone notification of the principal investigator (PI) for positive culture results. The PI's mobile number is included in the SOP for this protocol, available to technicians in the Microbiology Laboratory.

Upon learning of contamination in a batch already administered, the patient will be contacted by telephone by PI, the nurse coordinator, or another co-investigator, informed of the contamination, and queried regarding symptoms including but not limited to fever, chills, malaise and dizziness. Negative responses will result in verbal reassurance, counseling on signs/symptoms which should prompt the subject to contact our center and/or seek medical assistance, and the addition of a complete blood count (including white blood count and differential) and blood cultures x 2 at the next protocol-scheduled blood draw. Affirmative responses to any of these questions will result in requesting the subject to come immediately to The Medical University of Gdańsk Medical Centre, or, if not possible, to another medical facility for evaluation. Evaluation will include at least history and physical, CBC and blood cultures. Fever, leukocytosis, and/or positive blood cultures will result in hospitalization and the initiation of empirically chosen antibiotics (based on the positive culture results from the product) until the signs/symptoms resolve and/or positively identified organisms with antibiotic sensitivities allow specifically tailored antibiotic therapy.

The fluidics line from infusion is stored for 48 hours and will be sent to the Microbiology Laboratory for assessment when contamination or other adverse effects are reported.

c. AE related with immunosuppressive activity of Tregs

Tregs have immunosuppressive activity and therefore can potentially increase susceptibility to infection and the cancer cells growth. The mild / moderate viral infections were described within 48 hours after Tregs treatment, as well as worsening the paranasal sinusitis during the one-year follow-up of the Tregs administration. Regarding the risk of cancer, it has not been proven in the animal model, nor has a human tumor been detected after Tregs administration but because of the immunosuppressive mechanism of activity, theoretically such a risk exists and can not be ruled out.

d. Adverse effects related with administration of rituximab antibody (antiCD20) and management

The manufacturer provides the following most common side effects associated with the use of rituximab. Observed more often than 1 patient in 10 are:

1. Infections: bacterial and viral, upper respiratory tract infections, bronchitis, urinary tract infections,
2. Hematologic reactions: Neutropenia, leukopenia, febrile neutropenia, thrombocytopenia,
3. Infusion reactions: mainly fever, chills and feeling cold, angioneurotic edema, nausea, pruritus, rash, alopecia, fever, chills, asthenia, headache and lowered IgG level.

The less frequent complications listed in the medicine manufacturer's information (Roche 2012):
4. In the first 2 hours during the first infusion may occur fever, chills, feeling cold. If the reaction starts just after the infusion, it usually has the character of a hypersensitivity reaction, while in the case of its later development, within 1-2 hours from the beginning of the infusion, particular attention should be paid to **the cytokine release syndrome** (dyspnoea, often with simultaneous contraction bronchi and hypoxia, accompanied by fever, chills, muscle stiffness, urticaria and angioedema). Less likely to occur: pain at the injection site, blisters on the skin, pruritus, nausea, tiredness, headache, difficulty in breathing, swelling of the tongue or throat, runny nose or itching, vomiting, hot flush or palpitations, heart attack or a small number of platelets. The subject who has had heart disease or angina before treatment may get worse. If any of these symptoms occurs, should be notified immediately because there may be a necessity to slow down or stop the infusion.
5. Progressive multifocal leukoencephalopathy, due to which the patient should be monitored neurologically during follow-up visits during the study
6. Malignant neoplasms. Due to the mechanism of action, immunomodulatory drugs may increase the risk of malignant tumors. Based on limited experience in the use of rituximab in rheumatoid arthritis, it seems that currently available data do not support the increased risk of malignancies, but theoretically such a risk exists and can not be excluded.

6.4. Adverse event reporting plan

The principal investigator, together with the researchers, will serve as the data safety monitor for this study according to "CTCAE ver. 4.0' (attachment no 5). The researcher reports to the persons responsible for the research on behalf of the sponsor: Professor Małgorzata Myśliwiec (mysliwiec@gumed.edu.pl), Professor Piotr Trzonkowski (ptrzon@gumed.edu.pl).

Serious adverse effect (SAE) occurring as part of this trial will be reported to the Ethics Committee, and to the Department of Registration of Medicinal Products, Medical Devices and Biocidal Products within 48 hours. The report will contain the current "Undesired Activity Reporting Form of the investigational medicinal product" available on the website http://www.urpl.gov.pl/pl-formularze-zgloszenia-dzialania-niepozadanego (attachment no 6). Follow-up written notification will be submitted within 10 days.

The other adverse effects will be classified and noted in the CRF_TregVac2 patient study form (Appendix 2) in the "comments" field (should contain the name and grade in accordance with the nomenclature from Annex 5 and description, e.g. duration, treatment) and reported together with the test results. The results and the study information will also be published in the public Internet domain in which the study will be registered.

7. Ethics and legal norms

7.1. Bioethical consent and legal norms

The study obtained the permission of the Independent Bioethics Committee for Scientific Research at the Medical University of Gdańsk (approval number NKBBN / 374/2012 of 25.10.2012 and NKBBN / 374-7 / 2014 dated 21.01.2014). Thus, it meets the requirements of legal acts regulating the principles of medical experiments of an international nature:

- Helsinki Declaration, "Ethical Principles of Conducting Medical Experiments with Participation of People" adopted in June 1964 last change in 2004),

- International Ethical Guidelines for Biomedical Research Involving Human Subjects, Council for International Organizations of Medical Sciences — CIOMS) in 2002,

-The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use; ICH Harmonised Tripartite Guideline for Good Clinical Practice, ICH-GCP) from 1996,

- Recommendation R (90) 3 of the Committee of Ministers of the Council of Europe on Medical Research on Human Beings, adopted on February 6, 1990.
- International Covenant on Civil and Political Rights, adopted by the UN General Assembly of December 16, 1966.

Convention for the Protection of Human Rights and the Dignity of the Human Being against Applications of Biology and Medicine: Convention on Human Rights and Biomedicine adopted by the Committee of Ministers of the Council of Europe in 1996.
- Additional Protocol to the Convention on Human Rights and Biomedicine relating to Biomedical Research open for signature in Strasbourg on 25 January 2005.
-Dyrektywa 2001/20 / EC of the European Parliament and of the Council of 4 April 2001 on the approximation of the laws, regulations and administrative provisions of the Member States relating to the implementation of the principle of good clinical practice in conducting clinical trials on medicinal products for human use . This directive has been implemented into Polish law by the Act of 20 April 2004.
- Commission Directive 2005/28 / EC of 8 April 2005 fixing the principles and detailed guidelines of good clinical practice for investigational medicinal products for human use

and national:
- The medical doctor and dentist professions Act of December 5, 1996 (unified text: Journal of Laws of 2005, No. 226, item 1943, as amended),
- Act - Pharmaceutical Law of September 6, 2001 (unified text: Journal of Laws of 2008, No. 45, item 271),
- Act on medical devices of April 20, 2004 (Journal of Laws of 2004, No. 93, item 896, as amended),
- Regulation of the Minister of Health on the detailed requirements of Good Clinical Practice of March 11, 2005 (Journal of Laws of 2005, No. 57, item 500).

7.2. Signing the informed consent form and other ethical responsibilities of the researcher

In order to start the examination, the patient and his / her legal guardians should be informed in detail about the assumptions, objectives, procedures and possible consequences of the examination, paying particular attention to the fact that it is NOT a routine medical procedure, but a medical experiment. Carers and patients should receive information about the study and a form of informed consent (Annex 7) and have time to reflect on the decision to join the study.

Examination procedures can start only after legal guardians (and a patient from the age of 16) have signed the forms:

1. Information on the examination and the form of informed consent (Annex No. 7)

2. Patient's consent for data processing (attachment No. 8)

Obtaining signatures and including the form of informed consent and consent to the processing of data are the responsibilities of the researcher. After obtaining the consent, the researcher informs the coordinator about the including in the study. In case of withdrawal of consent by the caregivers or the patient during the examination, the researcher informs the coordinator and makes an appropriate entry in the CRF_TregVac2 form (Appendix No. 2), and all patient data are withdrawn by the coordinator from the study databases.

Any problems not covered in this document should also be reported to the coordinators.

7.3. Finishing of the study

After the last visit of the last patient, a study report will be prepared, which will be forwarded to the Independent Bioethics Committee for Research at the Medical University of Gdańsk and the relevant control bodies.

7.4. Coordination of the study

The sponsor of the study is:

Medical University of Gdańsk, Skłodowskiej-Curie 3a,. 80-210 Gdańsk

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Attachments:

Annex 1: Qualification FormTregVac2
Annex 2: Case Report Form CRF_TregVac2
Annex 3: Diabetes-related scales "Psychological problems of children with type 1 diabetes"
Annex 4: QoL questionnaires "Pediatric Questionnaire for Quality of Life of Children and Youth"
Annex 5: Common Terminology Criteria for Adverse Effects CTCAE ver. 3.0
Annex 6: Form for reporting the adverse reaction of the investigational medicinal product
Annex 7: Information and informed consent form
Annex 8: Patient consent form for data processing