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- (74) Fuldmægtig i Danmark: **Chas. Hude A/S, H.C. Andersens Boulevard 33, 1780 København V, Danmark**
- (54) Benævnelse: **TERAPEUTISK VACCINE TIL BEHANDLING AF DIABETES TYPE-1 HOS BØRN, ANVENDELSE AF CELLESORTER OG FREMGANGSMÅDE TIL AT MULTIPLICERE TREG-CELLER TIL FREMSTILLING AF TERAPEUTISK VACCINE TIL BEHANDLING AF DIABETES TYPE-1**
- (56) Fremdragne publikationer:
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DESCRIPTION

Field of the invention

[0001] The present invention concerns the new therapeutic vaccine prepared by an improved method for use in treatment of autoimmune diabetes type 1 in children prevents the disease effectively.

Prior Art

[0002] Diabetes type 1 (DM1A) is a genetically linked disease, however all researchers agree that direct damage to β cells in the pancreas is due to autoimmune reaction. Speaking in favour thereof are both the presence of antibodies towards β cell antigens, and lymphocytic infiltration to the islets of Langerhans, or the so-called *insulitis*, accompanied by increased β cell apoptosis.

[0003] The regulatory T-lymphocytes (Treg) form a specific population in the immune system. Although accounting for less than 1% of the leucocytes in the peripheral blood, they regulate the immune response so that swift elimination of harmful pathogens is possible while our own tissues remain protected. This is because Treg cells do not block other cells of the immune system when foreign pathogens are attacked, but prove strongly inhibiting when the immune system begins to destroy our own tissues and organs. Therefore by analogy, the immunosuppressive action of Treg cells sometimes earns them the name of the "intelligent steroids".

[0004] Reduced numbers of Treg cells in the organism are associated with transplant failures and incidence of allergic and autoimmune diseases. One of the diseases characterised by numerical deficiency of Treg cells, is diabetes type 1 where autoimmune attack destroys the patient's pancreas.

[0005] Known from the international publication WO 2004/110373 is a vaccine composition that comprises modified insulin B chain components suitable for use as immunogenic agents for treatment and prevention of type 1 diabetes.

[0006] Known from publication WO 2012/001099, on the other hand, is vaccine containing at least one enterovirus selected from the group including: Coxsackie viruses CAV4, CAV5, CAV6, and echovirus E18, or its component. The description states that the listed enteroviruses are linked to diabetes type 1, which opens new therapeutic and diagnostic possibilities.

[0007] Similarly, the description of WO 2012/001100 discloses vaccine comprising e.g. Coxsackie B virus CBV1 to prevent or treat diabetes type 1. It has been found that the virus is

strongly associated with the risk of contracting diabetes type 1.

[0008] Publications E.Allison Green et al: "Pancreatic Lymph Node-Derived CD4+CD25+ Treg Cells"; Salomon B et al: "B7/CD28 costimulations is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes"; E.A..Green: "CD4+CD25+ T regulatory cells control anti-islet CD8+ T cells through TGF-TGF- receptor interactions in type 1 diabetes", describe CD4+CD25+ phenotype transfer in mouse model of diabetes (different phenotype, cells are not expanded but directly transferred).

[0009] Publication Liu Weihong et al: "CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4(+) T reg cells" describes broader CD127^{low} to negative (low/-) phenotype and not ex vivo expanded cells and the results did not support the use of the cells in type 1 diabetes (no differences in the level of cells between type 1 diabetes subjects and healthy controls were found).

[0010] Publication Trzonkowski P. et al: "First-in-man clinical results of the treatment of patients with graf versus host disease with human ex vivo expanded CD4+CD25+CD127- T regulatory cells" describes the administration of the cells in graft versus host disease which is not an autoimmune phenomenon and the cells were not expanded in autologous inactivated human serum and not in the media described in the application.

[0011] Publication Natalia Marek et al: "The Time is Crucial for Ex Vivo Expansion of T Regulatory cells for Theraphy" describes the cultures with RPMI-1640 medium and human plasma.

[0012] Publication Federico Simonetta et al: "Increased CD127 expression on activated FOXP3+CD4+ regulatory T cells" describes increased expression of CD127 receptor on T regulatory cells while the application describes the cells that do not express CD127 receptor at all (CD127- cells).

[0013] Publication Ethan M.Shevach "Regulatory T Cells in Autoimmunity*" is a review that does not state anything about the phenotype and the way of expansion proposed in the application.

[0014] Publication Amy L.Putnam et al: "Expansion of human regulatory T-cells from patients with type 1 diabetes" describes the expansion of the product from broader phenotype of CD4+CD25+CD127^{low/-} cells expanded without autologous serum. As stated below, such a product is not as stable as the one from the application. In addition, its use in type 1 diabetes was not effective as described in Sci Transl Med. 2015;7(315):315ra189 (*see also discussion to EPO review*).

[0015] Publication WO 2007/127787 is a invention describing insulin-specific T regulatory cells which are derived through stimulation with insulin-like peptides. The cells differ from the phenotype of those from the application. In addition, these cells cannot be expanded to the

numbers from the application and their stability is not known.

[0016] Publication Marek Natalia et al: "Coating Human Pancreatic Islets with CD4+CD25highCD127- Regulatory T Cells as a Novel Approach for the local immunoprotection" is a description of the use of T regulatory cells in rejection of allogeneic tissue. The tissue comprise of pancreatic islets but this is not an autoimmune type 1 diabetes.

[0017] The above publications disclose approaches intended for treatment of insulin-dependent diabetes (not necessarily autoimmune type 1 diabetes), nevertheless they are different than the vaccine being the gist of this invention.

[0018] In order to increase the effectiveness of treating diabetes type 1 in children it is necessary to search for more effective and successful methods of treating the disease.

Basic of the invention and general disclosure

[0019] Unexpectedly, it has been found that the new therapeutic vaccine prepared by an improved method for use in treatment of autoimmune diabetes type 1 in children prevents the disease effectively. Administration of the vaccine according to this invention to patients results in an increase in the primary marker of the pancreas function, i.e. the C-peptide level. In addition, the sorter employed was originally dedicated to cell therapies, which enhances its safety.

[0020] The present invention relates to an improved method of preparing a vaccine comprising Treg cells CD3(+)CD4(+)CD25(high)CD127(-) comprising the following steps of:

- T cells (T lymphocyte) isolation from peripheral blood samples and T-cell monoclonal antibodies labeling;
- sorting T cells (T lymphocyte) according to the CD3(+)CD4(+)CD25[high]CD127(-)doublet(-)lineage(-)dead(-) phenotype using sorting device with a removable tube for experimental dosing, with the sorted cells being the Treg cell population of at least 97% purity;
- propagation of thus sorted *ex vivo* cells in the presence of artificial cells presenting antigen on culture medium in CellGro or X-VIVO supplemented with at least 1000 U / ml interleukin-2 and approximately 10% autologous inactivated serum, while artificial cells presenting antigen are magnetic sphere shape objects flattened by anti-CD3 and anti-CD28 antibodies in a 1: 1 ratio, while the *ex vivo* propagation time does not exceed 2 weeks;
- submitting the sample of thus obtained cells to tests for FoxP3 expression level, IFN-gamma inhibition assay and microbiological assays, as well as selecting for use as a vaccination cells expressing FoxP3 above 90%, positive result in IFN-gamma inhibition assay, and negative result in microbiological assays.

[0021] The vaccine prepared in accordance with the above method.

[0022] The vaccine prepared in accordance with the above method for use in treatment of autoimmune diabetes type 1 in children.

The figures:

[0023]

Fig.1 - presents the level of Treg cells CD3+CD4+CD25highCD127-FoxP3+ in children suffering from diabetes type 1, subject to the Treg cell therapy (n=10) over four months' observation. The value at point "-10 days" represents the day the blood was drawn for Treg cell isolation. The grey columns present the results obtained for children not administered the Treg lymphocyte infusion (control group; n=10). The values are given at their median, minimum, and the maximum levels. The statistically significant values ($p<0.05$) are marked with "**".

Fig. 2 - presents the C-peptide, the daily insulin dose /kg BW(DDI/kg), and the HbA1C in the tested children with diabetes type 1, subject to the Treg cells therapy (n=10) over the four months' observation. The results for the patients not administered the cell preparation (control group; n=10) are presented in grey columns. The values are given at their median, minimum, and the maximum levels, and the statistically significant values ($p<0.05$) are marked with "**".

[0024] The invention is illustrated with the following embodiment, which is exemplary, i.e. not limiting in nature.

Exemplary embodiment:

[0025] 250ml of peripheral blood was sampled from each patient with the assistance from an anaesthesiologist. In the case of children whose body weight was less than 50 kg the sampled blood volume accounted for 0.5% of the body weight (BW). This concerns patients under the age of 18.

[0026] The collected blood was processed at the Regional Centre of Blood Donation and Treatment in Gdansk to extract the buffy coat and serum. Isolated from the buffy coat were peripheral blood mononuclear cells (PBMC) through centrifuging in the Ficoll/Uropolin concentration gradient. Lymphocytes T CD4+ were then separated by the immunomagnetic method (separation purity: 96-99%) using the CD4+ T enrichment kit and marked with the following monoclonal antibodies (mAb): anti-CD3, anti-CD4, anti-CD8, anti-CD19, anti-CD14,

anti-CD16, anti-CD25, and anti-CD127 (5ul mAb/10⁶ cells). Among the listed antibodies those which recognize antigens CD14, CD16, CD19, and CD8 were conjugated with the same dye. The purpose of that dying scheme was to exclude the cells positive with respect to the listed antigens (i.e. monocytes, NK cells, lymphocytes B and cytotoxic T lymphocytes) without the need to introduce additional fluorochromes, which reduces the undesirable phenomenon of fluorescent spectra overlapping. Then, the cells were sorted to separate Tregs using a sorting cytometer to the algorithm sorting the following phenotype: CD3(+)CD4(+)CD25(high)CD127(-)doublet(-)lineage(-)dead(-).

[0027] The adopted exemplary dying scheme (antibody; dye name acronym, full name of the dye)

- antiCD127 FITC (Fluorescein isothiocyanate)
- antiCD25 PE (phycoerythrin)
- antiCD16 PerCP (Peridinin Chlorophyll Protein Complex)
- antiCD19 PerCP (Peridinin Chlorophyll Protein Complex)
- antiCD8 PerCP (Peridinin Chlorophyll Protein Complex)
- antiCD14 PerCP (Peridinin Chlorophyll Protein Complex)
- antiCD4 APC (allophycocyanin)
- antiCD3 Pacific Blue / Pacific Blue

or equivalents evoked to emit fluorescent light in similar spectrum ranges.

[0028] The purity of the thus isolated Treg cells was ~100% [median(min-max): 98%(97-99)]. An important modification compared to our earlier procedure consisted in applying the Influx cell sorter designed in accordance with the good manufacturing practices (GMP). The sorter is fitted with a replaceable sample flow line, which eliminates the risk of sample cross-contamination among the patients. Moreover, applied was the CellGro medium meeting the GMP standards or X-VIVO. The medium was supplemented with autological inactivated serum (10%) and interleukin-2 (1000U/ml). Introduced into the culture were the so-called antigen-presenting artificial cells [magnetic beads coated with anti-CD3 and anti-CD28 antibodies in the 1:1 proportion. The cells were cultivated until the appropriate number was attained, though no longer than for 2 weeks [median(min-max): 10 days (7-12)].

[0029] The above indicated modifications allowed the attainment of substantially improved stability and quality of the cultured Treg cells in the final product. The actual application of the preparation in therapy was conditional on satisfaction of the following criteria: factor FoxP3 expression above 90% [median(min-max)=93%(90-97)], positive result of the IFN γ production inhibition test, and negative results of microbiological tests - no genetic material of the HBV, HCV, or HIV viruses, and no bacterial contamination in the culture supernatants. Before infusion, the cells were washed with PBS, the magnetic beads removed, and administered in slow intravenous injection in 250ml 0.9% NaCl under supervision of the anaesthesiologist within 1h after the product release. The therapeutic dose was 20x10⁶/kg BW (n=6), or 10x10⁶/kg BW (n=4; whenever no higher number of cells had been achieved upon cultivation

for 2 weeks), or $30 \times 10^6/\text{kg}$ body weight. The control group was made up of patients who met all above-listed criteria of inclusion in the test, except for appropriate venous access, hence were not treated with the Treg vaccine. The test was not randomised, nor was there a blank sample introduced, and the children of the control group were not subject to any medical intervention related to the pending tests (blood sampling, simulated transfusion, or the like). Table 1 provides the characteristics of the tested groups. The test endpoints were as follows: the fasting C-peptide level, the HbA1c concentration, the insulin requirement, especially the daily dose (DDI)= 0.5 UI/kg BW adopted as the remission indicator. The test was conducted in accordance with the procedure approved by the Independent Research Bioethics Committee at the Medical University of Gdańsk (NKEBN/8/2010). A written consent to the above procedure was obtained from each patient and the parents.

[0030] None of the patients was observed to develop any serious infections, episodes of acute hyper-/hypoglycaemia, or any other undesirable side effects of the Tregs vaccine at any time over the test period. In case of one patient the Treg cell infusion date coincided with flu diagnosed a day after the Treg cells had been administered.

[0031] Beginning on the infusion date and continuously afterwards the recorded Treg lymphocyte per cent level in the peripheral blood was significantly increased (Wilcoxon test, $p=0.04$) (Fig. 1).

[0032] Two weeks after the Treg cell infusion all patients subject to the therapy were observed to demonstrate substantially reduced demand for exogenous insulin and a reduced HbA1c level (Fig. 2).

[0033] The first significant differences between the test group and the patients of the control group were observed six months after formulation of the diabetes diagnosis (5-6 months after the Treg cell infusion). The treated patients continued in the remission phase [DDI median(min-max)= 0.24 UI/kg BW ($0-0.55$)], whereas the control group experienced the end of remission [DDI median(min-max)= 0.55 UI/kg BW ($0.43-0.69$)] (Mann-Whitney U test, $p=0.03$). In addition, the children treated with Treg cells proved to have a significantly higher level of C-peptide [median(min-max): 0.65 ng/ml ($0.46-2.11$) vs. 0.40 ng/ml ($0.15-0.54$)] (Mann-Whitney U test, $p=0.04$) (Fig. 3). No differences with respect to therapy effectiveness were observed in the patients who had been administered Treg cells dosed at $20 \times 10^6/\text{kg BW}$ or $10 \times 10^6/\text{kg BW}$. Therefore, all results of the test group are presented en block.

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Patentkrav

1. Terapeutisk vaccine til brug i behandling af diabetes type 1 hos børn, kendetegnet ved, at den indeholder:

- Treg-celler CD3 (+) CD4 (+) CD25 (høj) CD127 (-)

DRAWINGS

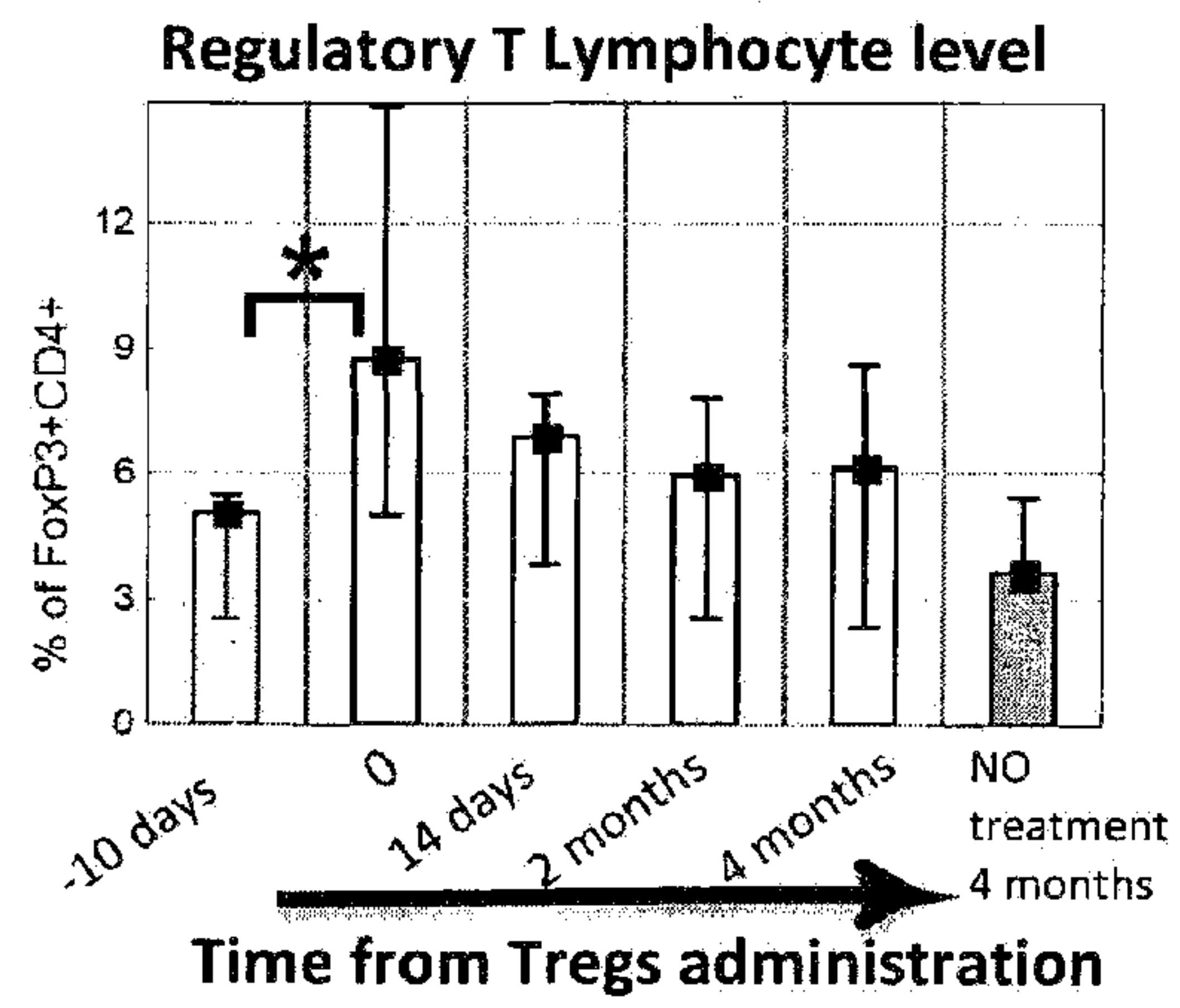


Fig. 1

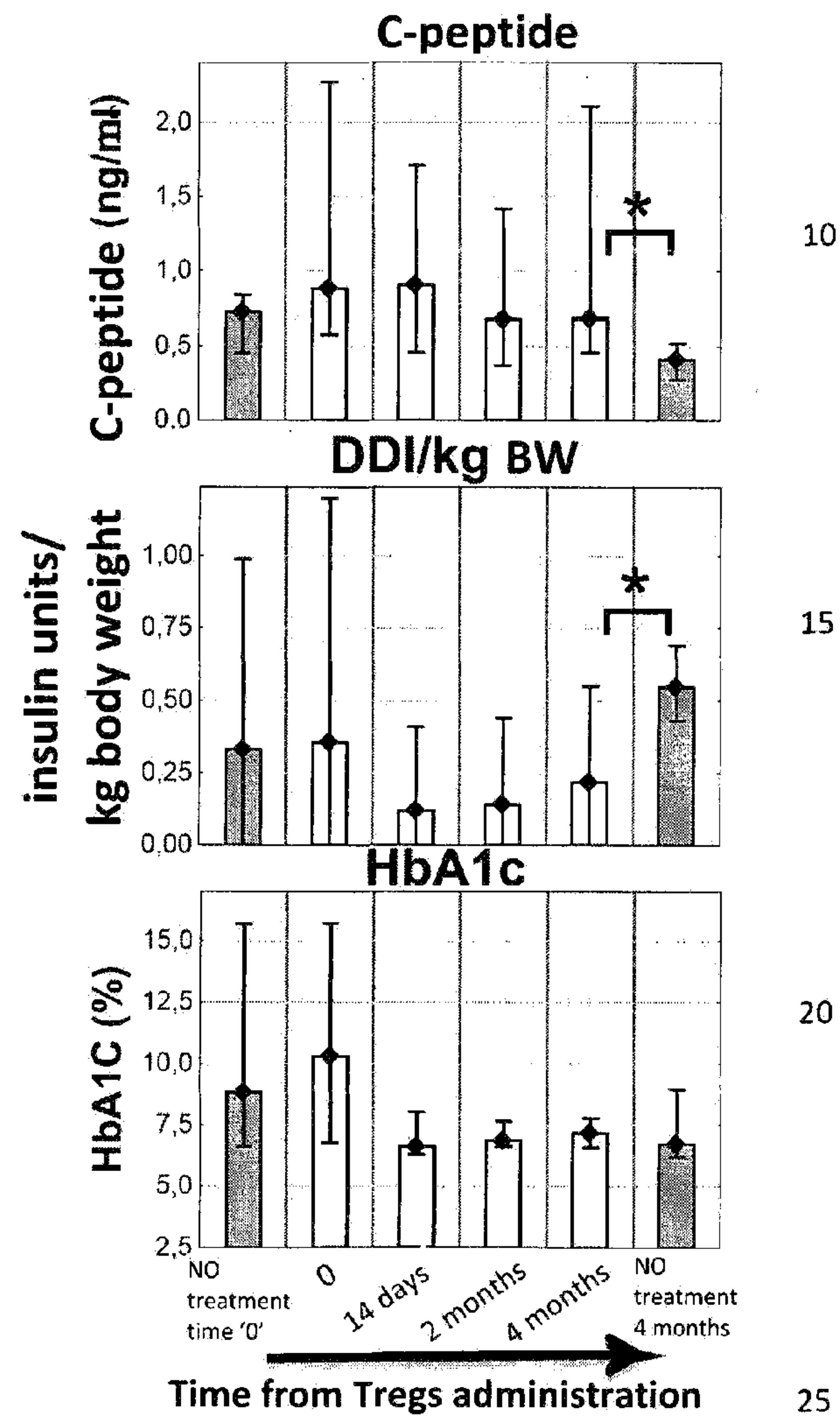


Fig. 2

TABLE 1: CLINICAL CHARACTERISTICS OF THE PATIENTS

	Treg treated (n=10)	Untreated control group (n=10)
Age (years) [median; min-max]	12.2; 8-16	11.8; 7-16
BMI [median; min-max]	16.9; 14.2-21.5	16.9; 14.2-20.7
Glycaemia on empty stomach at diagnosis (mg%) median; min-max]	354; 151-588	354; 151-598
Polydipsia at diagnosis (number of patients)	5	8
Polyuria at diagnosis (number of patients)	5	8
Body weight loss at diagnosis (number of patients)	4	3
Capillary blood pH at diagnosis [median; min-max]	7.39; 7.36-7.46	7.39; 7.34-7.53
Capillary blood pO ₂ at diagnosis (mmHg) [median; min-max]	69.3; 63.4-88.0	69.0; 56.9-86.2
Capillary blood pCO ₂ at diagnosis (mmHg) [median; min-max]	39.1; 28.0-41.8	38.0; 24.0-41.0
HCO ₃ at diagnosis (capillary blood - mmHg) [median; min-max]	23.85; 18.8-25.0	23.3; 21.3-25.2
Acid base balance (BE - mEq/l) [median; min-max]	-0.55; -7.8-1.0	-0.6; -7.0-0.9
Capillary blood SatO ₂ at diagnosis (%) [median; min-max]	94.1; 90.2-97.3	95.5; 92.4-98.0
Ig anti-GAD65 (number of patients)	10	10
Ig anti-ICA (number of patients)	5	5
Ig anti-IAA (number of patients)	9	8