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METHOD FOR ASSESSING EFFICACY OF IMMUNOTHERAPY

Field of the invention

Background

Type 1 diabetes (T1D) treatment consists of lifelong administration of insulin, a replacement therapy which does not satisfactorily prevent serious complications. Efforts to delay or halt disease progression have been ongoing for several decades, clinical intervention trials with recent-onset T1D patients have shown no or limited efficacy [1-7], which highlights the complexity of translation from animal models to human T1D. Immunomodulation with autoantigens could potentially constitute the most specific and safe treatment for T1D. Subcutaneous administration of glutamic acid decarboxylase $(GAD)_{65}$ formulated with aluminum hydroxide (GAD-alum) showed efficacy in preserving residual insulin secretion in children and adolescents with recent-onset T1D [8] but subsequent phase II [9] and phase III [10] trials failed to reach their primary outcomes. However, significant efficacy was shown in pre-specified subgroups in the phase III study [10], and it has been shown that close administration of influenza vaccine might have influenced the study outcome [11]. Thus, it is most probable that treatment with GAD-alum might be beneficial but has not been sufficiently effective [12].

In order to render the presentation of GAD_{65} antigen to T cells in the lymph nodes more efficient than previously described [9, 10], GAD-alum was administrated into lymph nodes to six patients participating in an open-label clinical trial, DIAGNODE. Results from these patients showed that preservation of C-peptide in the patients appeared to be similar to promising results observed in patients from other immune intervention trials [24].

Summary of the invention

The present inventors have surprisingly found new biomarkers and combination of biomarkers that can be measured and analysed in patients receiving immunotherapy by administration of GAD to assess the efficacy of the immunotherapy.

Thus, in a first aspect, the invention relates to a method for assessing the efficacy of an immunotherapy administered to a patient, said immunotherapy comprising administration of GAD, comprising the following steps:

- measuring at least one of
  - GADA IgG subclass distribution;
  - GADA levels;
  - Distribution of cytokines secreted from lymphocytes; and
  - Lymphocyte proliferation in presence of GAD or CD3/CD28 beads;

  in a first blood, plasma or serum sample obtained from said patient at a first point in time and in a second blood, plasma or serum sample obtained from said patient at a second, later, point in time;
- Comparing the so obtained measurements;

wherein an increased relative amount of IgG_2, IgG_3, and/or IgG_4, or decreased relative amount of IgG_1 in the GADA IgG subclass distribution; increased GADA levels; an increased relative amount of IL-13 and/or IL-5 or a decreased relative amount of IFNγ and/or TNFα in the distribution of cytokines secreted from lymphocytes; and/or reduced lymphocyte proliferation in the presence of GAD or CD3/CD28 beads; as measured in the second sample as compared to as measured in the first sample, is indicative of an effective immunotherapy.

In one embodiment, at least two, at least three, or four of

- GADA IgG subclass distribution;
- GADA levels;
- Distribution of cytokines secreted from lymphocytes; and
- Lymphocyte proliferation;

are measured in said first and second blood, plasma or serum sample.

In one embodiment, the first sample is obtained before or at commencement of the immunotherapy, or 80-100, such as 90, days after commencement of the immunotherapy.

In one embodiment, the second sample is obtained at 160-200, such as 180, days after commencement of the immunotherapy.

In one embodiment, the immunotherapy comprises daily administration of vitamin D commencing at day 1, and intralymphatic injection of GAD at days 30, 60 and 90.

In one embodiment, GADA IgG subclass distribution is measured in said first and second samples and the so obtained measurements are compared, and wherein an increased relative amount of IgG_2 in the GADA IgG subclass distribution, as measured in the second sample as compared to as measured in the first sample, is indicative of an effective immunotherapy.

In one embodiment, GADA IgG subclass distribution is measured in said first and second samples and the so obtained measurements are compared, and wherein an increased relative amount of IgG_3 in

the GADA IgG subclass distribution, as measured in the second sample as compared to as measured in the first sample, is indicative of an effective immunotherapy.

In one embodiment, GADA IgG subclass distribution is measured in said first and second samples and the so obtained measurements are compared, and wherein an increased relative amount of IgG_4 in the GADA IgG subclass distribution, as measured in the second sample as compared to as measured in the first sample, is indicative of an effective immunotherapy.

In one embodiment, GADA IgG subclass distribution is measured in said first and second samples and the so obtained measurements are compared, and wherein a decreased relative amount of IgG_1 in the GADA IgG subclass distribution, as measured in the second sample as compared to as measured in the first sample, is indicative of an effective immunotherapy.

In one embodiment, GADA levels are measured in said first and second samples and the so obtained measurements are compared, wherein increased GADA levels, as measured in the second sample as compared to as measured in the first sample, are indicative of an effective immunotherapy.

In one embodiment, distribution of cytokines secreted from lymphocytes is measured in said first and second samples and the so obtained measurements are compared, wherein increased relative amount of IL-13 and/or IL-5, as measured in the second sample as compared to as measured in the first sample, is indicative of an effective immunotherapy.

In one embodiment,distribution of cytokines secreted from lymphocytes is measured in said first and second samples and the so obtained measurements are compared, wherein decreased relative amount of IFNγ and/or TNFα, as measured in the second sample as compared to as measured in the first sample, is indicative of an effective immunotherapy.

In one embodiment, lymphocyte proliferation in presence of GAD or CD3/CD28 beads is measured in said first and second samples and the so obtained measurements are compared, wherein reduced lymphocyte proliferation in the presence of GAD or CD3/CD28 beads, as measured in the second sample as compared to as measured in the first sample, is indicative of an effective immunotherapy.

In one embodiment, said immunotherapy comprises administration of GAD by means of intralymphatic injection, intradermal injection, subcutaneous injection, or oral administration.

In one aspect, the invention relates to a method for treatment or prevention of type 1 diabetes by means of immunotherapy, comprising the steps of

- administration of GAD to a subject;
- obtaining an assessment of the efficacy of the immunotherapy by a method according to any one of claims 1-14; and
- adjusting the dosage and/or administration route of GAD based on said assessment.

The invention also relates to GAD for use in such a method and to the use of GAD in the manufacture of a pharmaceutical composition for use in such a method.

Description of the drawings

Fig. 1: Overview of the GAD-alum treatment. (a) Type 1 diabetic patients (n=6) received a first injection of GAD-alum (4μg) into the lymph-nodes (LN), followed by two booster injections one

month apart. A control group of patients who participated in a another study (n=6) have received a first subcutaneous (SC) GAD-alum dose (20 μg) followed by a second injection after one month. All patients received in parallel Vitamin D (Calciferol), during the first 120 days the LN patients and for 450 days the SC group.

Fig 2: a) Mean values of GADA titres for patients who received GAD-alum injections ínto the lymph-nodes (LN, n=6) or subcutaneously (SC, n=6). (b) Change of the frequency (%) of IgG1, IgG2, IgG3, IgG4 GADA subclasses. Frequencies were calculated with respect to the combined sum of the 4 subclasses in each sample (i.e. total IgG). The median percentage respect to the total IgG is shown for each respective subclass. (c) GADA subclass relative contribution at baseline, 90 and 180 days for LN and SC groups.

Fig 3: GAD65-sinduced cytokine secretion upon in vitro PMBC stimulation. Patients received GAD-alum injections ínto the lymph-nodes (LN, n=6) or subcutaneously (SC, n=6). (a) Median levels of IL-13, IL-5, IL-10, IL-2, IL-17, TNF-α and IFN-γ (pg/ml) at baseline, 90 and 180 days for LN (black circles) and SC (white circles) patients were detected by Luminex in supernatants collected after 7 days culture in presence of medium or GAD65 (5μg/ml). GAD65-induced cytokine secretion is given after subtraction of spontaneous secretion from each individual. (b) Relative contribution (%) of the cytokines in the lymph-node patients (LN) and subcutaneous group (SC) at 90 and 180 days.

Fig: 4 Proliferative responses to GAD65 and CD3/CD28 beads. Patients received GAD-alum injections into the lymph-nodes (LN, n=6) or subcutaneously (SC, n=6). PBMC were culture for 3 days with GAD_{65} (5μg/ml), CD3/CD28 beads or medium, and thereafter cells were pulsed with [^{3}H] thymidine and harvested. Proliferation is expressed as stimulation index (SI), calculated from the mean of triplicates divided by the mean of triplicates with medium alone.

Fig 5: T cell activation induced by GAD_{65}. PBMCs from baseline (day 1), 90 and 180 days from patients who received GAD-alum into the lymph node (n=6, LN, black circles) and subcutaneous (n=6, SC, white circles) were stimulated for 7 days with GAD_{65} (5μg/ml) or medium. (a) Percentage of GAD_{65}-activated $CD4^+CD25^+CD127^+$ T cells and (b) $CD8^+$ $CD25^+CD127^+$ T cells. (c) Mean percentage of $CD4^+$ $CD25^+CD127^{lo/-}$ $FOXP3^+$ (Treg) in resting samples (medium alone) and (d) induced by GAD_{65} stimulation. Mean percentage of $FOXP3^{lo}CD45RA$ non-suppressor regulatory T cells in (e) resting samples (medium alone) and (f) GAD_{65}-stimulated samples.

Fig 6: Change of differentiation state (%) of $CD4^+$ and $CD8^+$ T cells. PBMCs from baseline (day 1), 90 and 180 days from patients who received GAD-alum into the lymph node (n=6, LN, black circles) and subcutaneous (n=6, SC, white circles) were cultured for 7 days with GAD_{65} (5μg/ml) or medium. GAD_{65} induced changes of (a) Näive (T_N, $CD45RA^+CCR7^+$), (b) Central memory (T_{CM}, $CD45RA^-CCR7^+$), (c) effector memory (T_{EM}, $CD45RA^-CCR7^-$) and (d) terminally differentiated effector memory (T_{EMRA}, $CD45RA^+CCR7^-$) CD4 T cells. GAD-alum induced change of (e) näive (T_N, $CD45RA^+CCR7^+$), (f) central memory (T_{CM}, $CD45RA^-CCR7^+$) (g) Percentage effector memory (T_{EM}, $CD45RA^-CCR7^-$) and (h) terminally differentiated effector memory (T_{EMRA}, $CD45RA^+CCR7^-$) CD8 T cells. Lines represent mean trend.

Fig 7: Representative flow cytometry analysis from one patient who received lymph node injection of GAD-alum. Sample was collected at 180 days, and PBMC were cultured for 7 days in the presence of GAD_{65} (5μg/ml) or medium. The percentages of $CD4^+$, $CD8^+$ T cells and Tregs were assessed in resting (medium alone) and GAD_{65}-stimulated samples.

Fig 8: Heatmap was created to represent the immunological changes induced by GAD-alum treatment. Changes were calculated as the ratio of the values at 90 and 180 days respect to the baseline. Patients received GAD-alum injections ínto the lymph-nodes (LN, n=6) or subcutaneously (SC, n=6), and they were stratified from left to right according their clinical outcome at 180 days. Clinical variables are expressed as percentage of change from baseline (%). At 90 days, Max.stimulated and AUC c-peptide were not calculated as meal tolerance tests no performed, and are represented by "x". The greyscales illustrate the post-treatment increase of immunological variables in relation to baseline values.

Abbreviations and definitions

GAD: glutamic acid decarboxylase

GAD_{65}: 65 kDa form of glutamic acid decarboxylase

GADA: Glutamic acid decarboxylase autoantibodies

GAD-alum: glutamic acid decarboxylase formulated aluminum hydroxide

LN: lymph-node

PBMC: Peripheral blood mononuclear cell

SC: subcutaneous

Th: T helper

T1D: type 1 diabetes

Detailed description of the invention

The present inventors have studied how a number of biomarkers correlate with clinical outcome of immunotherapy using GAD. Our results described in the experimental section identify a plurality of biomarkers that can be used to assess the efficacy of a therapy using GAD. No specific immune signatures before treatment were identified among the biomarkers included in this study, but patients with improved metabolic outcome, i.e. lower HbA1c and insulin needs, and better preservation of C-peptide secretion seemed to have some common immune correlates.

A major pending question in T1D intervention trials is the identification of the mechanism behind treatment as well as the definition of pre- and post-treatment immune signatures. Based on the general consensus that T1D is due to the lack of tolerance and the involvement of autoreactive T cells, it has been expected that efficacy of immunotherapy with autoantigens should be accompanied by the induction of tolerance and loss of the immune response to the autoantigen in question. Here we observe that individuals with better clinical outcome after LN injections had an immune response characterized by rise of GADA, reduction of proliferation, and induction of predominant Th2-like responses, especially increase of IL-13, supported by the shift of IgG subclasses and GAD-induced cytokine secretion.

Although the direction of the immune responses to GAD_{65} were Th2-skewed in the LN group, there were however large inter-individual differences. Strikingly, GAD_{65}-induced cytokines were detected in the LN group after 180 days solely in patients with a best clinical outcome.

It was particularly interesting that patients displaying a better clinical outcome were characterized by GAD-induced T-cell responses deviated towards a Th2 cytokine profile, both after SC and LN treatment. T-cell responses in the LN patients did not show a tolerogenic deviation, but rather a Th2-associated profile, and some Th1 cytokines were also produced. However, Th2 cytokines were more dominant in the responders in the LN group than in the SC patients. We have previously shown that the cytokine secretion was characterized by a broad cytokine profile short after SC injection of 2 doses of GAD-alum, and cytokine secretion tended to switch towards a more predominant Th2-associated profile over time [37]. In that study, administration of further doses increased the secretion levels, but did not affect the quality of the cytokine response, and cytokine profile was similar in patients receiving 2 or 4 doses of GAD-alum [37]. Thus, predominant secretion of IL-13 after the third LN injection cannot be explained by an extra dose but rather by the administration route. Intra-lymphatic administration delivers more antigen to the site of immune response induction [38] and the difference in antigen dose available for stimulation of antigen specific T cells may also lead to increased Th2 response. The adjuvant aluminum effect is associated with the induction of Th2 responses [39], and preferentially induce humoral rather than cellular immune responses, thus alum has been the adjuvant of choice to minimize the possibility of promoting-mediated b-cell destruction with GAD_{65}.

A number of biomarkers of clinical outcome have thus been identified.

Individual immunological changes induced by GAD-alum treatment were calculated as the ratio of GAD_{65}-induced immune responses at 90 and 180 days respect to specific immune responses pre-treatment, and patients were stratified for each treatment according to their metabolic and c-peptide preservation (Figure 8).

Baseline immunological parameters did not show any pre-treatment feature that seemed to be related to the clinical outcome.

Representation of induced changes of antigen-induced cytokine secretion post treatment showed that Th1 and Th2 associated cytokines were induced by GAD_{65} stimulation after 90 days in most of the patients, independently of the administration route. However, changes on GAD_{65}-induced cytokines at 180 days were detectable in the LN patients with the best clinical response (n=3), i.e., lower HbAc1, decreased insulin intake and best preservation of c-peptide secretion. Intriguingly, activated CD4 cells but no CD8 were observed in these three patients. Reduction of IgG_1 and enhancement of IgG_2 and IgG_4 was most pronounced in the patient with the best clinical response (patient 1), and this patient accounted for the observed increase of IgG_4 in the LN group (Figure 8).

GAD_{65} induced cytokine secretion in the patient with best clinical response in the SC group (patient 1) resembled that observed in the LN patients with best response, but in the SC treated patient IgG1 was the predominant GADA subclass, and both activated CD4 and CD8 cells were detected.

Calculation of the ratio of Th2 (IL-13 and IL-5)/Th1 (IFN-γ and TNF-α) cytokines at 180 days for the better responders in both groups (n=2 LN and n=1 in SC) revealed that Th2 response was three times stronger in LN patients than in the SC group (ratio: 6.44 LN vs 2.24 SC).

Experimental

The experiments provided below are intended to illustrate some aspects and embodiments of the invention. They should not be construed as limiting the scope of the invention, which is that of the appended claims.

Methods

Procedure

A group of T1D patients (n=6) participating in a pilot trial (NCT02352974), received a primary injection of 4 µg of GAD-alum (Diamyd Medical, Stockholm) into an inguinal lymph gland, followed by two booster injections of 4 µg each with one month interval [24]. In parallel they got Vitamin D 2000 U/d. Another control group of patients (n=6) who received two subcutaneous injections of GAD-alum, 20 µg each, one month apart, who also in parallel they got Vitamin D 2000 U/d, were selected under blind conditions, before code break, to match age and sex, and according to disponibility of samples among participants in a double blind placebo-controlled study described elsewhere (NCT01785108) (**Fig 1).**

Laboratory Tests

Laboratory analyses were performed at Linköping University, Sweden. Blood and serum samples were collected at baseline and after 30, 60, 90 and 180 days in LN group, and after 15, 45, 90 and 180 days in SC group. Samples were drawn during the morning hours and PBMCs were isolated within 24 h using Leucosep (Greiner Bio One) according to the manufacturer's instructions.

Serum antibodies and IgG subclasses

Serum GAD autoantibodies (GADA) and IA-2A were estimated in duplicate by means of a radio-binding assay, using ^{35}S-labeled recombinant human GAD_{65} as previously described [25]. Sepharose protein A was used to separate free from antibody-bound labelled GAD_{65}. A diabetes autoantibody standardization program (IASP) in which the laboratory participated has shown that GADA assay has a sensitivity of 70% and specificity of 100% and for IA-2A was 99% sensitivity and 100% specificity.

Total serum IgE was quantified using the ImmunoCap100Є system (Phadia AB, Uppsala, Sweden). The measuring range for the assay was 2–50 000 kU/L, and calibrators were run in duplicates to obtain a full calibration curve. Levels of total IgE ≥ 85 kU/L were regarded as positive.

Lymphocyte proliferation assay

For proliferation assays, PBMC were re-suspended at 10^6 cells/ml in AIM-V medium, and incubated in triplicates (2×10^5 cells/well) in round-bottom 96-well plates in presence of 5 µg/ml $rhGAD_{65}$ (Diamyd Medical, Stockholm, Sweden), CD3/C28 beads (Gibco, Life Technologies AS, Oslo, Norway) as positive control, and in medium alone. After 3 days, cells were pulsed for 18 hours with 0.2 µCi of [^{3}H] thymidine/well (Perkin Elmer), and thereafter harvested. Proliferation was recorded using a 1450

Wallac MicroBeta counter (Perkin Elmer, Shelton, CT, USA), and expressed as stimulation index (SI), calculated as the mean of triplicates for each culture condition divided by the mean of triplicates with medium alone.

Cytokine secretion assay

For cytokine quantification and flow cytometry, one million PBMC diluted in 1 ml AIM-V medium supplemented with 20 µM β-mercaptoethanol (Sigma-Aldrich, Saint Louis, MO, USA) were cultured for 7 days at 37°C in 5% CO_2 in the presence of 5 µg/ml rh-GAD_{65}. Additional, wells with medium alone and CD3/CD28 beads were used as negative and positive controls respectively for each sample. After 7 days, PBMC were separated from supernatants, and supernatants were preserved at -70 until used for multiplex fluorochrome analysis, and cells were directly used for flow cytometry.

The cytokines interleukin (IL)-2, IL-5, IL-10, IL-13, IL-17, tumour necrosis factor (TNF)-α and interferon (IFN)-γ were measured in cell culture supernatants using Bio-Plex Pro Cytokine Panel (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Data was collected using the Luminex 200 ™ (Luminex xMAP™ Corporation, Austin, TX USA). The lowest limit of detection was 1.38 pg/ml for IL-2, 1.27 pg/ml for IL-5, 0.14 pg/ml for IL-10, 0.43 pg/ml for IL-13, 1.54 pg/ml for IL-17, 2.1 pg/ml for IFN-γ and 3.71 pg/ml for TNF-α.. The specific antigen-induced cytokine secretion level was calculated by subtracting the spontaneous secretion (i.e. secretion from PBMC cultured in medium alone) from the one following stimulation with GAD_{65}.

Flow cytometry

For flow cytometry analysis, PBMC were incubated in AIM-V medium with β-mercaptoethanol at 37 °C, 5% CO2 for 7 days, with or without 5 µg/ml recombinant GAD_{65}. After incubation, cells were washed in PBS containing 0.1% BSA, and subsequently stained with Alexa-700-conjugated anti-CD3 (clone UCHT1, BD Biosciences), Pacific Blue-conjugated anti-CD4 (clone RPA-T4, BD Biosciences), allophycocyanin (APC)-H7-conjugated anti-CD8 (clone SK1, BD Biosciences), PerCP-Cy5.5-conjugated anti-CD45RA (clone HI100, BD Biosciences), phycoerythrin (PE)-conjugated anti-CCR7 (clone G043H7, Biolegend), FITC-conjugated anti-CD127 (clone eBioRDR5, eBioscience) and PE-Cy7-conjugated anti-CD25 (clone BC96, eBioscience). After surface staining, cells were fixed and permeabilized using FOXP3 staining buffer set (eBioscience), according to the manufacturer's instructions. Cells were then stained with APC-conjugated anti-FOXP3 (clone PCH101, eBiosciences) and acquired on a FACS Aria III (Becton Dickinson) running FACS Diva v8 software (Becton Dickinson). Data were analyzed using Kaluza v1.3 (Beckman Coulter).

Statistical analysis

Data distribution was tested using Komolgorov-Smirnov test. Variables that followed a normal distribution were presented as mean, and differences within groups were calculated by Paired samples test's. Differences between groups were calculated using T-student test. For non-normally distributed variables, non-parametric test were applied (Wilcoxon test related samples and Mann-Whitney test). Differences between categorical variables were calculated by Chi-square test (χ^2 – test). A probability level of <0.05 was considered statistically significant. Calculations were performed using IBM SPSS Statistics version 23 (IBM SPSS, Armonk, NY, USA) and graphical illustrations were made in GraphPad Prism 5 for Windows (GraphPad Software, La Jolla, CA, USA).

Results

Patients were stratified into those who received lymph-node (LN) or subcutaneous (SC) GAD-alum injections. Gender distribution was the same in both groups, while mean age was higher in LN patients (22 years) than in the SC group (14 years) ($p<0.001$). At baseline, both groups had similar baseline mean C-peptide (fasting, max. stimulated and AUC). Pre-treatment HbA1c values were higher in LN patients, who had lower insulin doses ($p>0.05$). GADA and IA-2A autoantibody levels did not differ between the groups (Table 1).

Follow up of the patients showed that fasting and stimulated c-peptide (AUC) remained stable at 180 days in the LN group, while glycated hemoglobin levels and insulin intake decreased [24]. Patients in SC group had a greater loss of stimulated c-peptide, as well as higher glycated hemoglobin levels and increased insulin intake (Table 1).

GADA titers and GADA subclasses analysis

GADA levels were enhanced after the second injection of GAD-alum both given SC and LN (Figure 2a). However, LN administration of low GAD-alum doses induced GADA levels 29 times higher than SC injection of higher doses.

We looked next to the GADA IgG 1-4 subclass distribution, calculating the frequencies of each subclass with respect to the combined sum of all the subclasses in each sample (i.e total IgG). Baseline GADA subclass distribution was similar in the two groups, being IgG_1 the most frequent, and followed by $IgG_3 > IgG_2 \approx IgG_4$ (Figure 2b). The proportion of IgG_1 decreased from baseline to 90 days both in the LN and SC groups, while the proportion of the other subclasses increased. Intriguingly, while GADA subclasses distribution in the SC group at 180 days was similar to that observed at baseline, the proportion of IgG1 in the LN group was further reduced, with a dramatically increase of IgG2, IgG3 and IgG4 (Figure 2c).

To disregard a possible allergy-associated effect in response of GAD-alum, total IgE was measured at baseline and at 180 days. Results showed that baseline IgE levels were similar in patients receiving intralymphatic and subcutaneous injections, and levels were not affected by the treatment and remained unchanged after 180 days (data no shown).

Cytokine secretion and relative contribution

We next analyzed the cytokine secretion in PBMC supernatants collected after 7 days culture. Baseline cytokine secretion was similar in the two groups.

GAD_{65}-induced secretion of IL-5, IL-13 IFN-γ and IL-17 was increased at 90 days, both after the second GAD-alum SC and LN doses, together with IL-2 in the SC group. The third GAD-alum injection into the LN resulted in a predominant secretion of IL-13 and low levels of IFNγ at 180 days months. Meanwhile, IFNγ was the most secreted cytokine in the SC group at the same time point (Figure 3a).

We further assessed the relative contribution of each cytokine to the total GAD_{65}-induced cytokine secretion. In the LN group, a broad cytokine profile was observed at 90 days, following the second injection of GAD-alum, while cytokine secretion at 180 days, after the third injection, was dominated by the Th2-associated cytokine IL-13. In the SC group, cytokine profile was also characterized by a

broad cytokine secretion at 90 days with a predominant secretion of Th2 cytokines, but cytokine distribution shifted into a dominant Th-1-like response at 180 days (Figure 3b).

In vitro stimulation with GAD_{65}

GAD_{65}-induced proliferation was increased by the second injection of GAD-alum both into the LN and SC. The third injection to the LN group resulted in a reduction of proliferation at 180 days, while remained stable in the SC group. Proliferation induced by stimulation with CD3/CD28 beads showed the same distribution as the induced by GAD_{65} (Figure 4).

T cell immunophenotype

We monitored the differentiation state and GAD_{65}-induced activation of T cells. A representative illustration of the gate strategy followed for the analysis of CD8, CD4 and regulatory T cells is shown (figure 5).

GAD_{65} stimulation induced activated $CD25^{+}CD127^{+}$ T cells in both groups after the second injection of GAD-alum. In the LN, higher frequency of activated CD4 T cells was detected in 3 patients, while activation of CD8 T cells was moderate or not detectable. In the SC group in contrast, the proportion of activated CD8 T cells was more predominant, with weaker expression of GAD_{65}-activation of CD4 cells (Figure 6 a-b)

The analysis of $CD4^{+}FOXP3^{+}CD25^{hi}CD127^{low/-}$ Tregs showed that resting Treg did not vary through the study, but antigen recall induced an increase in cells with regulatory phenotype at 180 days in both groups (Figure 6 c-d). Further analysis with the addition of CD45RA revealed an increment in non-suppressive $FOXP3^{lo}$ $CD45RA^{-}$ T cells in both groups (Figure 6 e-f).

CD4 and CD8 T cells were further classified according to the expression of CD45RA and CCR7 as näive (T_N, $CD45RA^{+}CCR7^{+}$), central memory (T_{CM}, $CD45RA^{-}CCR7^{+}$), effector memory (T_{EM}, $CD45RA^{-}CCR7^{-}$) and terminally differentiated effector memory (T_{EMRA}, $CD45RA^{+}CCR7^{-}$) cells (Figure 7). Both groups, LN and SC, showed a progressive reduction in the proportion of näive CD4 and CD8 T cells after 90 days, while the frequency of memory and effector cells increased in GAD_{65} stimulated PBMC (Figure 7 a-h).

Table 1

				Baseline																				
	Id	Sex	Age	Fasting C-peptide	Max. Stim	AUC	Insulin intake	HbA1c	GADA (U/ml)	IgG_1 (%)	IgG2 (%)	IgG3 (%)	IgG4 (%)	IA-2 (U/ml)	IL-13 pg/ml	Il-5 pg/ml	IL-10 pg/ml	IL-2 pg/ml	IFN pg/ml	TNF pg/ml	IL-17 pg/ml	ΔFast C-peptide	ΔMax. Stim	ΔAUC	ΔInsulin intake	ΔHbAc
LN	1	M	23	0.12	0.61	0.43	0.29	66	929	70	29	0.4	0.9	704	n.d	8.6	0.95	n.d	n.d	n.d	n.d	108.3	13.1	30.2	-48.2	-31.8
	2	M	22	0.26	1.13	0.85	0.25	58	111	25	55	5.8	14.5	266	n.d	0.6	n.d	n.d	n.d	n.d	n.d	-23	7.9	-1.1	-4	-32.7
	3	M	21	0.26	0.62	0.42	0.29	103	968	64	9	11.5	14.7	497	n.d	n.d	n.d	n.d	n.d	n.d	n.d	-26.9	3.2	21.4	-41.3	-55.3
	4	F	21	0.25	1.26	0.74	0.45	68	2955	91	0.3	8.7	0.3	13.6	n.d	n.d	n.d	0.61	n.d	n.d	n.d	-4	-11.1	6.7	-64.4	-47
	5	M	23	0.16	0.73	0.38	0.55	78	14100	92	0.2	5.6	2.7	2.7	n.d	n.d	n.d	n.d	n.d	n.d	n.d	-25	-31.5	-13.1	-27.2	-44.8
	6	F	21	0.17	0.58	0.36	0.46	52	27450	43	17	17.9	21	2.7	n.d	0.6	n.d	n.d	n.d	n.d	n.d	-47	-31	-27.7	17.3	-15.3
SC	1	M	13	0.18	0.99	0.69	0.55	47	114	39	8	16	37	23 300	n.d	n.d.	0.25	n.d	n.d	n.d	n.d	77.7	-22.2	-5.7	-54.5	2.1
	2	F	17	0.59	0.9	0.8	1.36	68	786	64	6	19	10.2	n.d	n.d	0.3	n.d	n.d	1976	37	n.d	-18.6	7.7	6.2	9.5	-19.1
	3	M	17	0.24	0.77	0.6	0.6	65	667	87	1.4	10.4	1.4	2 880	969	80.5	3.74	n.d	n.d	n.d	10.2	-20.8	-15.5	-11.6	-46.6	-55.3
	4	F	11	0.15	0.56	0.46	0.38	50	70	10	59	28	3	619	n.d	n.d	1.35	n.d	n.d	0.78	1.3	153.3	-28.5	-19.5	65.7	48
	5	M	15	0.48	1.12	0.97	0.42	33	472	87	1.7	9	2.6	n.d	n.d	n.d	n.d	n.d	n.d	n.d.	n.d	-41.6	-32.1	-35.0	16.6	36.3
	6	M	11	0.18	0.61	0.44	0.48	41	1215	85	4.7	3.6	7	4.7	n.d	n.d	n.d	2.36	n.d	n.d	n.d	-94.4	-60.6	-65.9	41.6	39.0

Baseline C-peptide, insulin intake and HbA1c of the patients who received GAD-alum injections into the lymph-nodes (LN, n=6) or subcutaneously (SC, n=6). Pre-treatment GADA and IA-2 titers (U/ml), GADA IgG subclass relative distribution (%), and GAD_{65}-induced cytokine secretion levels (pg/ml) from each patient. M: male, F: female, n.d: non detectable levels.

Percentage of change (%) of C-peptide, insulin intake and HbA1c from baseline to 180 days. Data are represented as mean values, and differences between LN and SC groups were calculated by T-student test, and χ^2 for categorical variables (sex). Differences were considered significant for $p<0.05$.

CLAIMS

1. A method for assessing the efficacy of an immunotherapy administered to a patient, said immunotherapy comprising administration of GAD, comprising the following steps:
   - measuring at least one of
     - GADA IgG subclass distribution;
     - GADA levels;
     - Distribution of cytokines secreted from lymphocytes; and
     - Lymphocyte proliferation in presence of GAD or CD3/CD28 beads;

     in a first blood, plasma or serum sample obtained from said patient at a first point in time and in a second blood, plasma or serum sample obtained from said patient at a second, later, point in time;
   - Comparing the so obtained measurements;

   wherein an increased relative amount of IgG_2, IgG_3, and/or IgG_4, or decreased relative amount of IgG_1 in the GADA IgG subclass distribution; increased GADA levels; an increased relative amount of IL-13 and/or IL-5 or a decreased relative amount of IFNγ and/or TNFα in the distribution of cytokines secreted from lymphocytes; and/or reduced lymphocyte proliferation in the presence of GAD or CD3/CD28 beads; as measured in the second sample as compared to as measured in the first sample, is indicative of an effective immunotherapy.

2. The method according to claim 1, wherein at least two, at least three, or four of
   - GADA IgG subclass distribution;
   - GADA levels;
   - Distribution of cytokines secreted from lymphocytes; and
   - Lymphocyte proliferation;

   are measured in said first and second blood, plasma or serum sample.

3. The method according to claim 1 or 2, wherein the first sample is obtained before or at commencement of the immunotherapy, or 80-100, such as 90, days after commencement of the immunotherapy.

4. The method according to any one of claims 1-3, wherein the second sample is obtained at 160-200, such as 180, days after commencement of the immunotherapy.

5. The method according to any one of claims 1-4, wherein the immunotherapy comprises daily administration of vitamin D commencing at day 1, and intralymphatic injection of GAD at days 30, 60 and 90.

6. The method according to any one of claims 1-5, wherein GADA IgG subclass distribution is measured in said first and second samples and the so obtained measurements are compared, and wherein an increased relative amount of IgG_2 in the GADA IgG subclass

distribution, as measured in the second sample as compared to as measured in the first sample, is indicative of an effective immunotherapy.

7. The method according to any one of claims 1-6, wherein GADA IgG subclass distribution is measured in said first and second samples and the so obtained measurements are compared, and wherein an increased relative amount of IgG_3 in the GADA IgG subclass distribution, as measured in the second sample as compared to as measured in the first sample, is indicative of an effective immunotherapy.

8. The method according to any one of claims 1-7, wherein GADA IgG subclass distribution is measured in said first and second samples and the so obtained measurements are compared, and wherein an increased relative amount of IgG_4 in the GADA IgG subclass distribution, as measured in the second sample as compared to as measured in the first sample, is indicative of an effective immunotherapy.

9. The method according to any one of claims 1-8, wherein GADA IgG subclass distribution is measured in said first and second samples and the so obtained measurements are compared, and wherein a decreased relative amount of IgG_1 in the GADA IgG subclass distribution, as measured in the second sample as compared to as measured in the first sample, is indicative of an effective immunotherapy.

10. The method according to any one of claims 1-9, wherein GADA levels are measured in said first and second samples and the so obtained measurements are compared, wherein increased GADA levels, as measured in the second sample as compared to as measured in the first sample, are indicative of an effective immunotherapy.

11. The method according to any one of claims 1-10, wherein distribution of cytokines secreted from lymphocytes is measured in said first and second samples and the so obtained measurements are compared, wherein increased relative amount of IL-13 and/or IL-5, as measured in the second sample as compared to as measured in the first sample, is indicative of an effective immunotherapy.

12. The method according to any one of claims 1-11, wherein distribution of cytokines secreted from lymphocytes is measured in said first and second samples and the so obtained measurements are compared, wherein decreased relative amount of IFNγ and/or TNFα, as measured in the second sample as compared to as measured in the first sample, is indicative of an effective immunotherapy.

13. The method according to any one of claims 1-12, wherein lymphocyte proliferation in presence of GAD or CD3/CD28 beads is measured in said first and second samples and the so obtained measurements are compared, wherein reduced lymphocyte proliferation in the presence of GAD or CD3/CD28 beads, as measured in the second sample as compared to as measured in the first sample, is indicative of an effective immunotherapy.

14. The method according to any one of claims 1-13, wherein said immunotherapy comprises administration of GAD by means of intralymphatic injection, intradermal injection, subcutaneous injection, or oral administration.

15. A method for treatment or prevention of type 1 diabetes by means of immunotherapy, comprising the steps of
    - administration of GAD to a subject;
    - obtaining an assessment of the efficacy of the immunotherapy by a method according to any one of claims 1-14; and
    - adjusting the dosage and/or administration route of GAD based on said assessment.

16. GAD for use in a method according to claim 15.

ABSTRACT

The present invention relates to a method for assessing the efficacy of an immunotherapy administered to a patient, said immunotherapy comprising administration of GAD, comprising the following steps: measuring at least one of GADA IgG subclass distribution; GADA levels; Distribution of cytokines secreted from lymphocytes; and Lymphocyte proliferation in presence of GAD or CD3/CD28 beads; in a first blood, plasma or serum sample obtained from said patient at a first point in time and in a second blood, plasma or serum sample obtained from said patient at a second, later, point in time; and comparing the so obtained measurements.

(No fig)

Fig 1 1/8

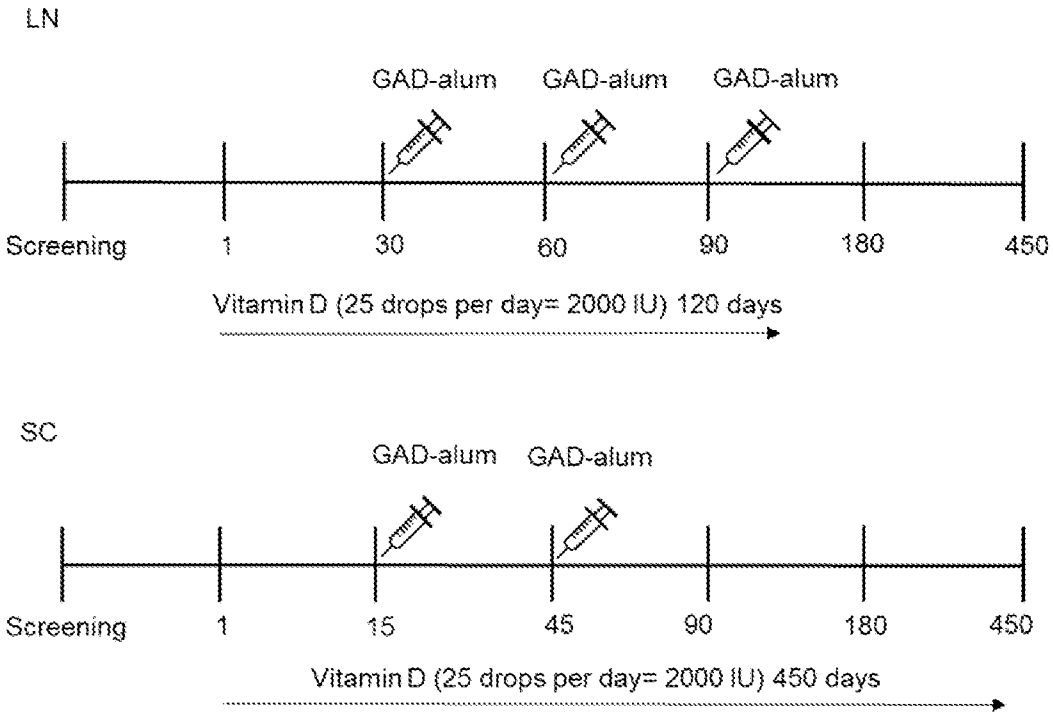
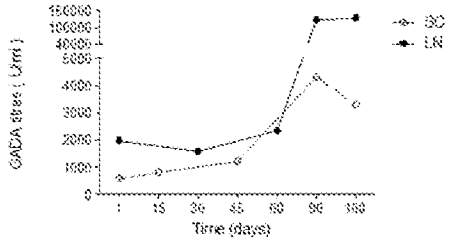
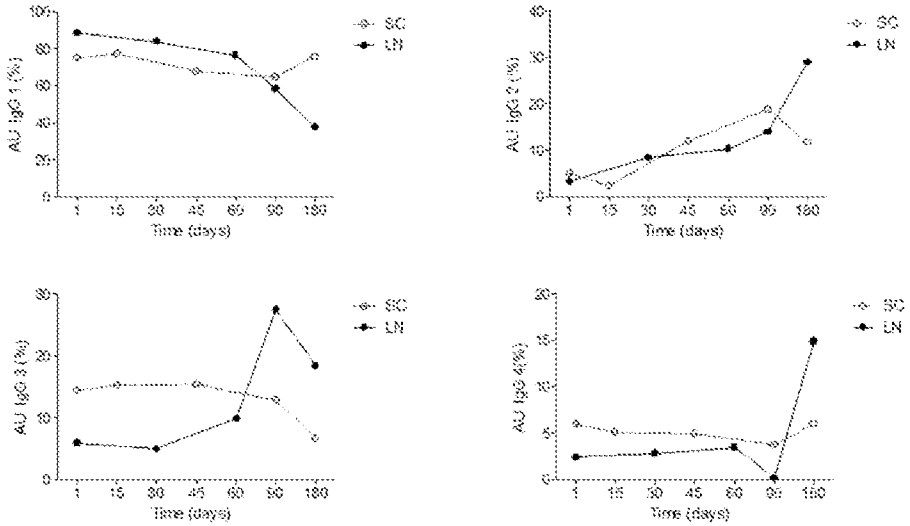

LN
GAD-alum
GAD-alum
GAD-alum
Screening
1
30
60
90
180
450
Vitamin D (25 drops per day= 2000 IU) 120 days
SC
GAD-alum
GAD-alum
Screening
1
15
45
90
180
450
Vitamin D (25 drops per day= 2000 IU) 450 days

Fig 2

a


GADA titres (U/ml)
Time (days)
SC
LN

b


AU IgG 1 (%)
AU IgG 2 (%)
AU IgG 3 (%)
AU IgG 4(%)
Time (days)
SC
LN

c

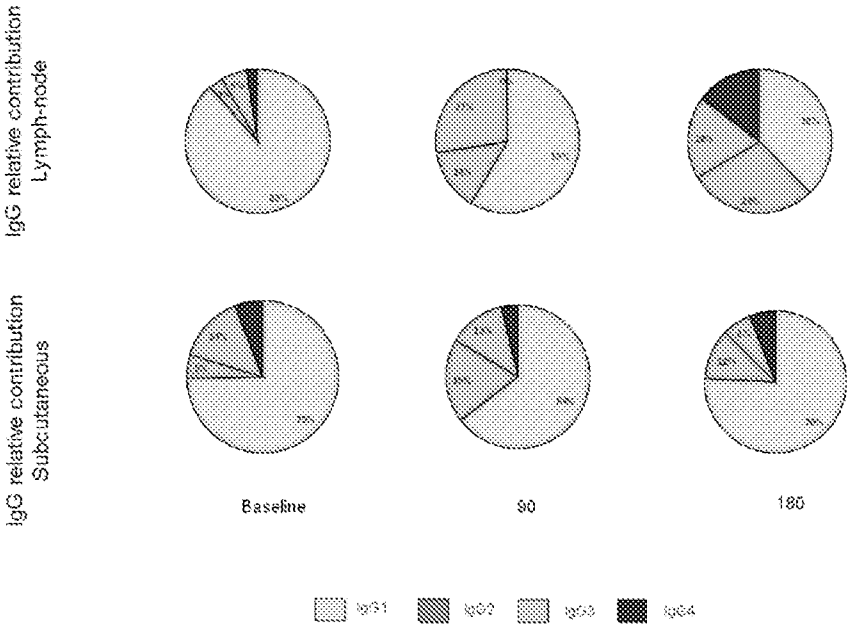
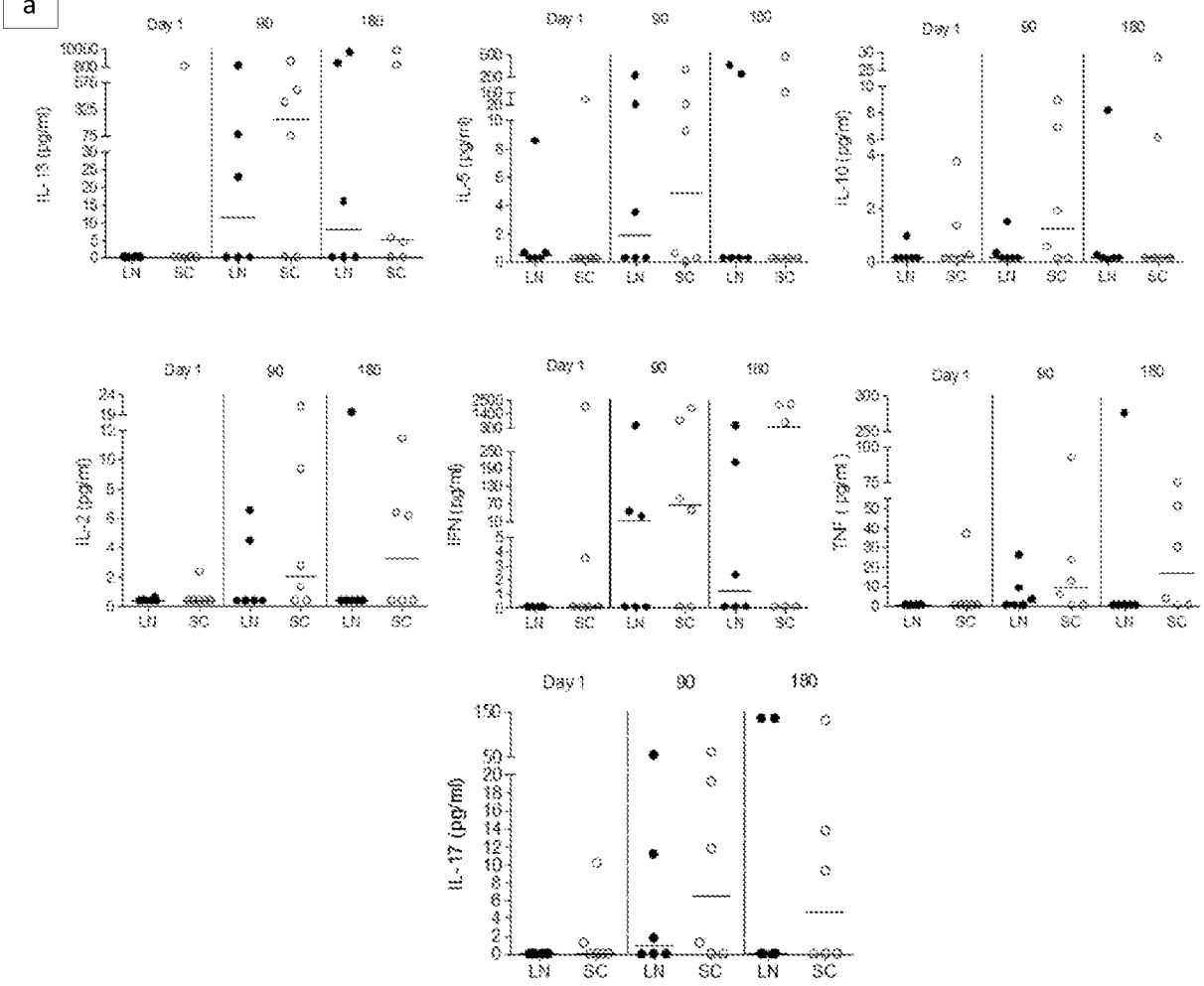

IgG relative contribution
Lymph-node
IgG relative contribution
Subcutaneus
Baseline
90
180
IgG1
IgG2
IgG3
IgG4

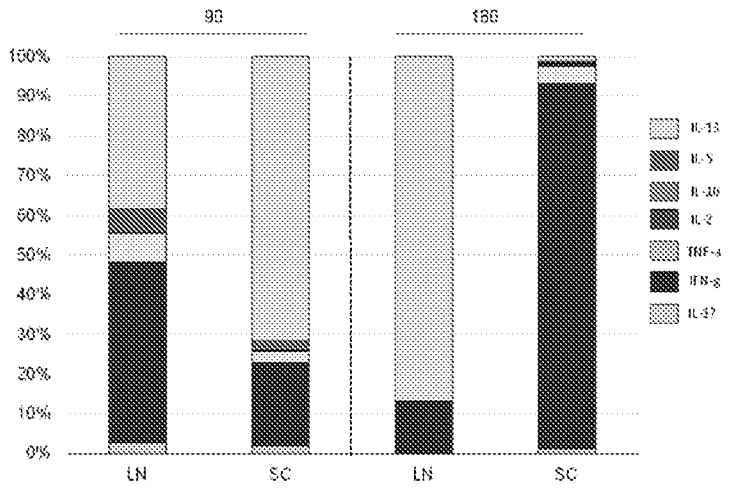
Fig 3

3/8

a


Day 1
90
180
LN
SC
IL-13 (pg/ml)
IL-5 (pg/ml)
IL-10 (pg/ml)
IL-2 (pg/ml)
IFN-g (pg/ml)
TNF-a (pg/ml)
IL-17 (pg/ml)

b


90
180
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
LN
SC
LN
SC
IL-13
IL-5
IL-10
IL-2
TNF-a
IFN-g
IL-17

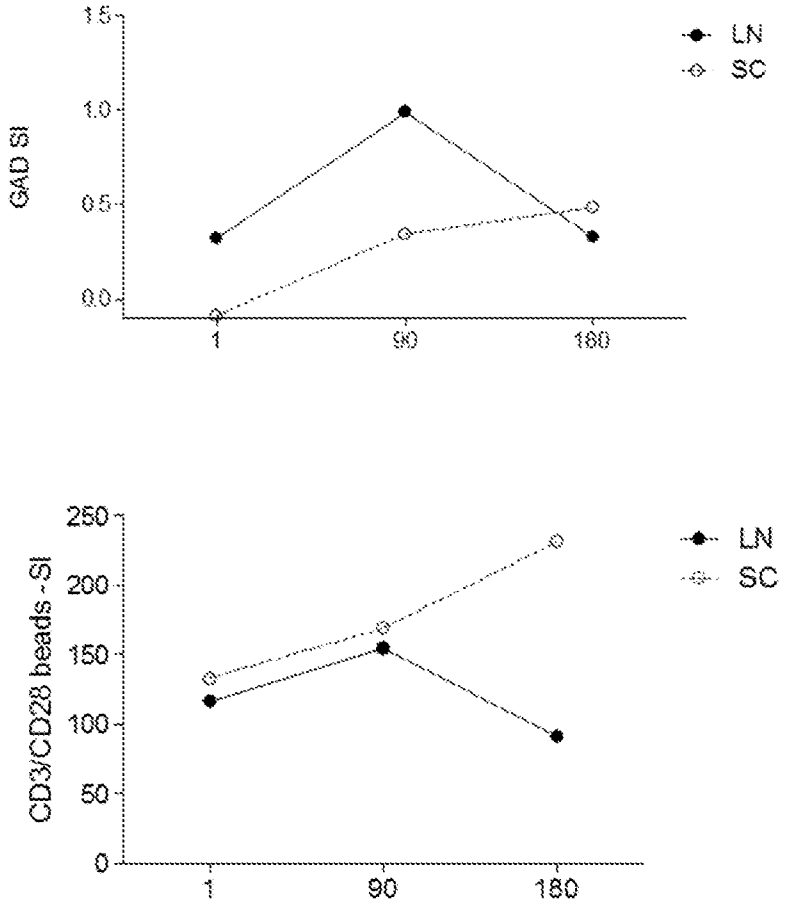

GAD SI
1.5
1.0
0.5
0.0
1
90
180
LN
SC
CD3/CD28 beads - SI
250
200
150
100
50
0
1
90
180
LN
SC

Fig 5

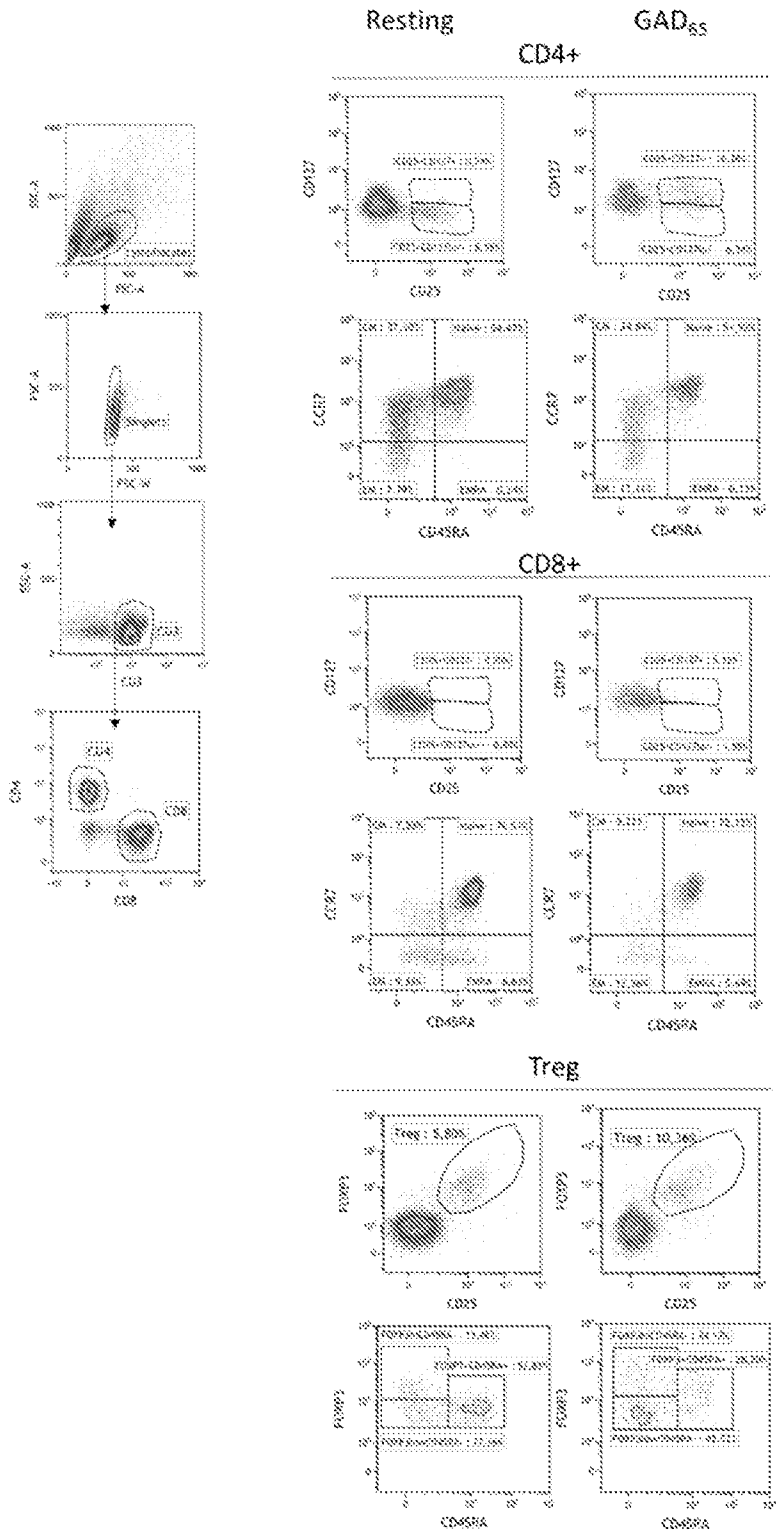


Fig 6

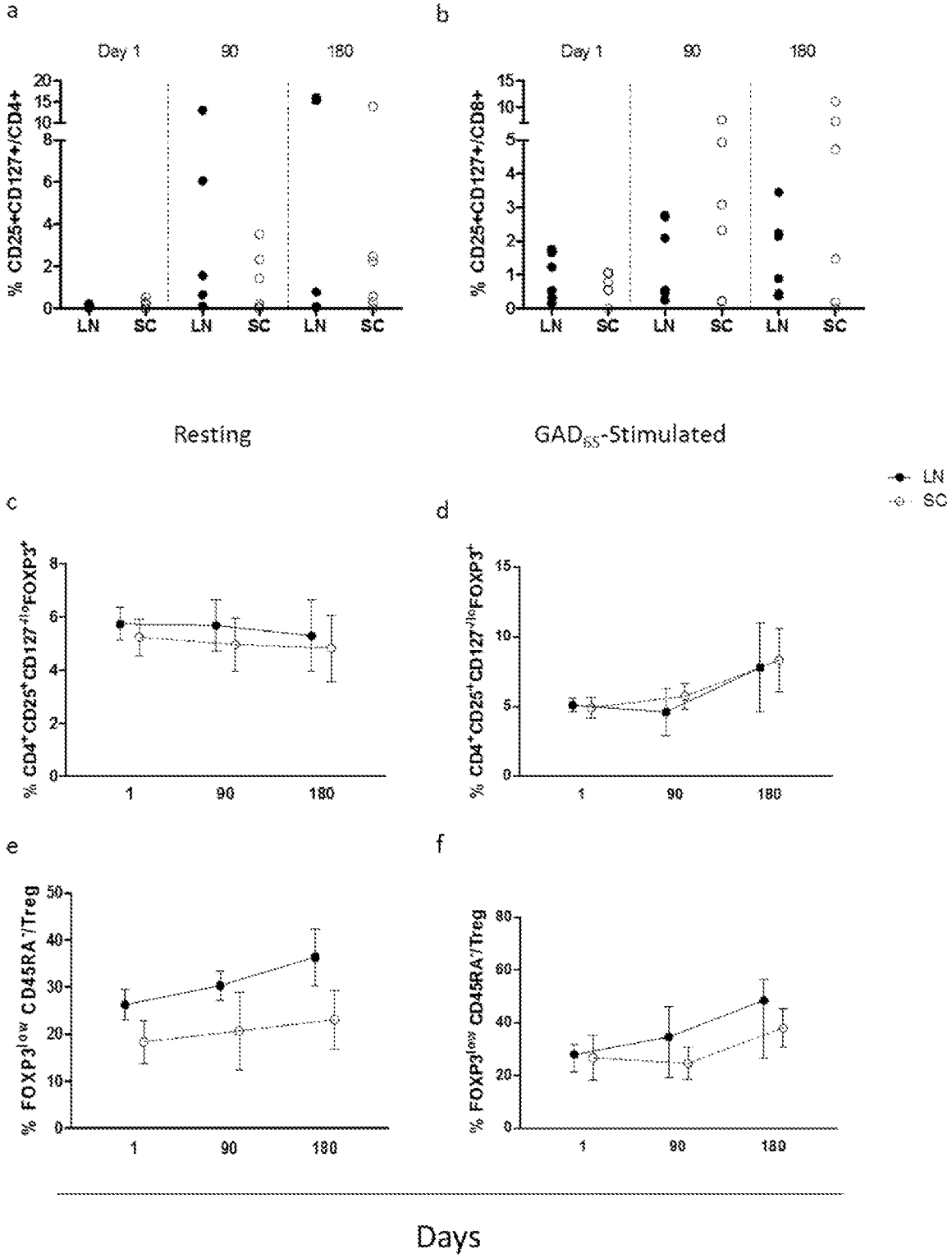

a
b
Day 1
90
180
% CD25+CD127+/CD4+
% CD25+CD127+/CD8+
LN
SC
Resting
GAD65-Stimulated
c
d
e
f
% CD4+CD25+CD127-/loFOXP3+
% FOXP3low CD45RA-/Treg
Days

Fig 7

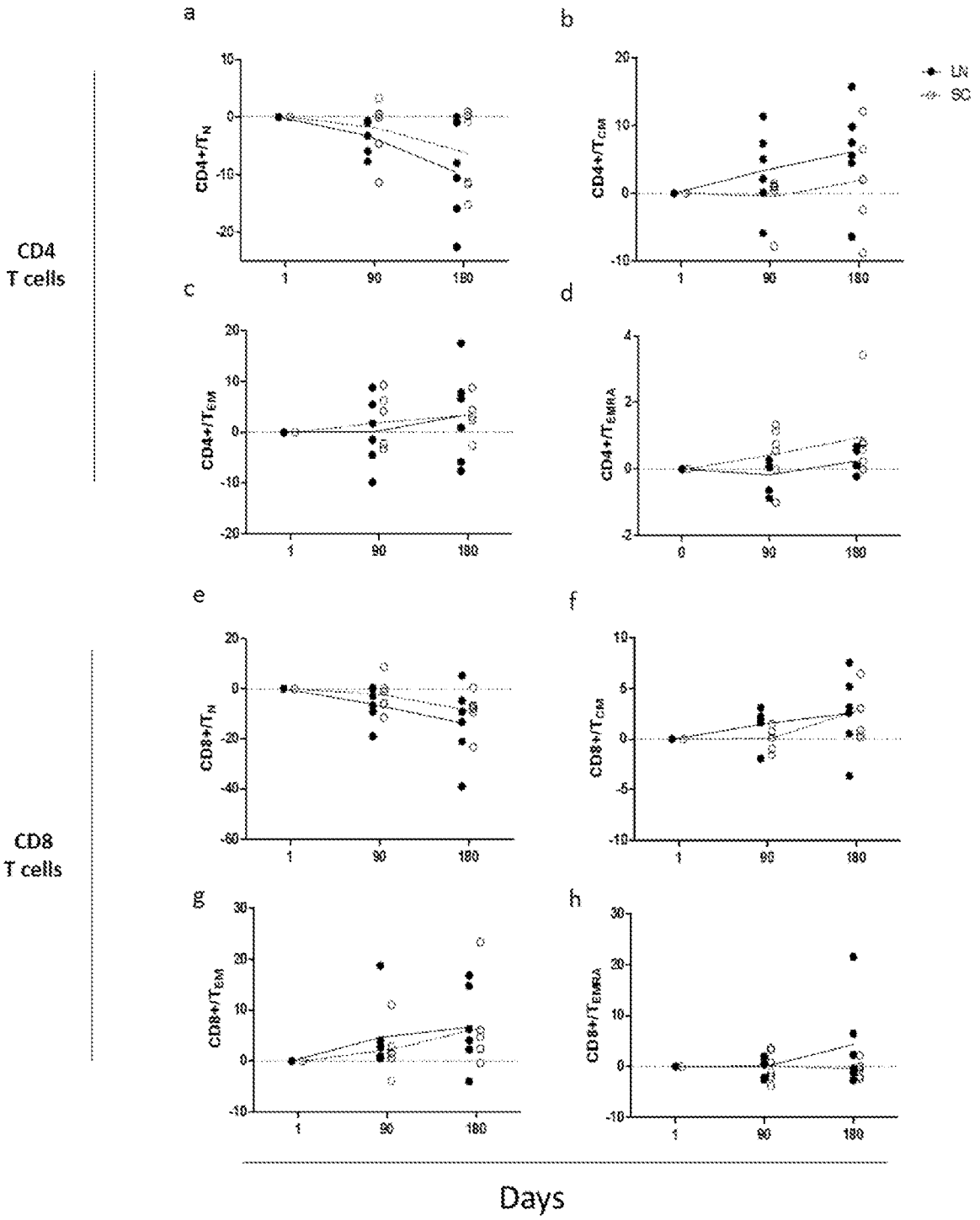

a
b
c
d
e
f
g
h
IV
SC
CD4+/TN
CD4+/TCM
CD4+/TEM
CD4+/TEMRA
CD8+/TN
CD8+/TCM
CD8+/TEM
CD8+/TEMRA
1
90
180
CD4 T cells
CD8 T cells
Days

Fig 8

Lymph-node
Subcutaneous
Patient
GADA
GADA
IgG1 %
IgG2 %
IgG3 %
IgG4 %
IL-13
IL-5
IL-10
IL-2
IFN
TNF
IL-17
CD4+CD25+CD127+
CD8+CD28+CD127+
Fasting C-peptide %
Max. Stimulated %
AUC %
Insulin intake %
HbA1c %
Legend heatmap