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RESEARCH ARTICLE

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Immune response differs between intralymphatic or subcutaneous administration of GAD-alum in individuals with recent onset type 1 diabetes

Fabrícia Dietrich[1] | Hugo Barcenilla[1] | Beatriz Tavira[1] | Jeanette Wahlberg[2,3,4] | Peter Achenbach[5,6] | Johnny Ludvigsson[1,7] | Rosaura Casas[1]

[1]Division of Pediatrics, Department of Biomedical and Clinical Sciences, Faculty of Medicine and Health Sciences, Linköping University, Linköping, Sweden

[2]Department of Endocrinology in Linköping, Linköping University, Linköping, Sweden

[3]Department of Health, Medicine and Caring Sciences, Linköping University, Linköping, Sweden

[4]Faculty of Medical Sciences, Örebro University, Örebro, Sweden

[5]Institute of Diabetes Research, Helmholtz Zentrum München, Munich, Germany

[6]Forschergruppe Diabetes, School of Medicine, Technical University of Munich, Munich, Germany

[7]Crown Princess Victoria Children's Hospital, Linköping, Sweden

Correspondence
Rosaura Casas, Division of Pediatrics, Department of Biomedical and Clinical Sciences, Faculty of Medicine and Health Sciences, Linköping University, Linköping 581 85, Sweden.
Email: rosaura.casas@liu.se

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Abstract

Aims: Immunomodulation with autoantigens potentially constitutes a specific and safe treatment for type 1 diabetes (T1D). Studies with GAD-alum administrated subcutaneously have shown to be safe, but its efficacy has been inconclusive. Administration of GAD-alum into the lymph nodes, aimed to optimise antigen presentation, has shown promising results in an open-label clinical trial. Herein, we compared the immune response of the individuals included in the trial with a group who received GAD-alum subcutaneously in a previous study.

Materials and methods: Samples from T1D individuals collected 15 months after administration of either three doses 1 month apart of 4 μg GAD-alum into lymph nodes (LN, $n = 12$) or two doses 1 month apart of 20 μg subcutaneously (SC, $n = 12$) were studied. GADA, GADA subclasses, GAD_{65}-induced cytokines, peripheral blood mononuclear cell proliferation, and T cells markers were analysed.

Results: Low doses of GAD-alum into the lymph nodes induced higher GADA levels than higher doses administrated subcutaneously. Immune response in the LN group was characterised by changes in GADA subclasses, with a relative reduction of IgG1 and enhanced IgG2, IgG3, and IgG4 proportion, higher GAD_{65}-induced secretion of IL-5, IL-10, and TNF-α, and reduction of cell proliferation and $CD8^+$ T cells. These changes were not observed after subcutaneous (SC) injections of GAD-alum.

Conclusions: GAD-specific immune responses 15 months after lymph node injections of GAD-alum differed from the ones induced by SC administration of the same autoantigen.

KEYWORDS
autoantigen, GAD-alum, intralymphatic, lymph nodes, subcutaneous, type 1 diabetes

Abbreviations: FOXP3, forkhead box P3; GAD_{65}, glutamic acid decarboxylase; GADA, glutamic acid decarboxylase autoantibodies; GAD-alum, glutamic acid decarboxylase formulated in aluminium hydroxide; LN, lymph node; MMTT, Mixed Meal Tolerance Test; PBMCs, peripheral blood mononuclear cells; SC, subcutaneous; T1D, type 1 diabetes.

1 | INTRODUCTION

Although type 1 diabetes (T1D) incidence continues to increase worldwide,[1] no treatment able to stop or reverse the course of the disease has been found so far.[2-5] The search for effective interventions able to delay or stop the autoimmune destructive process is a main objective in the treatment of individuals with T1D. As immunomodulation with autoantigens may potentially constitute a specific and safe treatment, SC administration of glutamic acid decarboxylase $(GAD)_{65}$ formulated with aluminium hydroxide (GAD-alum) has been used with varied results in several clinical trials.[6-9] A meta-analysis performed with data from GAD-alum studies suggested a therapeutic benefit.[10,11]

In an attempt to optimise GAD_{65} presentation, GAD-alum was administrated directly into lymph nodes to six adults with T1D in an open-label pilot study.[12,13] Results from the small group of subjects receiving the treatment showed that intralymphatic injection of GAD-alum was safe and seemed to preserve C-peptide.[12] Assessment of the immune response in those individuals after 6 months showed that it differed from that observed in six patients who received GAD-alum subcutaneously in a previous trial.[13] As the treatment was tolerable with no adverse event, the study was then extended to 12 individuals, including also children. Data from this larger group has further supported that the treatment seems to preserve C-peptide secretion.[14] In the current study, we address the immune response in these 12 individuals after 15 months compared to a group of T1D patients who received GAD-alum subcutaneously.

2 | MATERIALS AND METHODS

2.1 | Study design, individual recruitment, and procedures

The therapy with GAD antigen into lymph-nodes study (DIAGNODE-1) is a single centre open-labelled pilot clinical trial. A total of 12 children and young adults with recent-onset T1D (4 females, 8 males; 12.6-23.1 years old) were eligible if fasting C-peptide was ≥0.12 nmol/L (0.36 ng/mL) and elevated levels of GAD_{65} antibodies were present. Each patient received a primary injection of 4 μg each of GAD-alum (Diamyd Medical) into an inguinal lymph gland, followed by two booster injections with 1-month interval. The injections were administrated by help of ultrasound technique, so-called needle-guide. The patients received also Vitamin D (Calciferol) in oral solution (2000 U/day) for 4 months, starting 1 month prior to first GAD-alum injection. The patients have been evaluated at baseline, 6 and 15 months with clinical examination, blood samples for immune function, and a Mixed Meal Tolerance Test (MMTT).[15]

Another group of recent-onset T1D individuals were selected from a previous multicentre, randomised, four-arm, double-blind, placebo-controlled clinical trial, DIABGAD, described elsewhere (NCT01785108, https://clinicaltrials.gov/).[16] At time of screening, T1D patients ($n = 60$) with <4 months diabetes duration, aged 10.0 to 17.9 years old, fasting serum C-peptide ≥0.12 nmol/L and positive for GAD_{65}-autoantibodies (GADA), but <50,000 U/mL, were randomised in four arms. Twelve individuals were selected from the arm who received two SC injections of GAD-alum, 20 μg each, 1 month apart. They also in parallel received Vitamin D 2000 U/day per os.[16]

The safety of the treatments was evaluated in both studies, as well as preservation of residual beta cell function by the change in fasting C-peptide and C-peptide (90 minutes value and area under the curve (AUC)/120) during an MMTT from baseline, and effect on HbA1c and insulin dose.[12,14,16]

The trials were approved by the Research Ethics Committee, Linköping University, Sweden (DIAGNODE-1: Dnr 2014/153-31; DIABGAD-1: Dnr 2012/417-32), and by the Medical Product Agency, Uppsala, Sweden (DIAGNODE-1: Dnr 5.1-214-54385; DIABGAD-1: Dnr 2012-003251-11). All participants and their parents/caregivers gave their consent after oral and written information.

2.2 | Blood samples

Laboratory analyses were performed at Linköping University, Sweden. Blood samples were drawn during the morning hours, and peripheral blood mononuclear cells (PBMCs) were isolated within 24 hours using Leucosep (Greiner Bio One), according to the manufacturer's instructions.

2.3 | GAD autoantibodies

Serum GAD autoantibodies (GADA) were estimated in duplicate by radio-binding assay, using ^{35}S-labelled recombinant human GAD_{65} ($rhGAD_{65}$).[17]

2.4 | GADA-subclasses

GADA IgG 1, 2, 3, and 4 subclasses were measured by radio-binding assays[18] using IgG subclass-specific biotin-labelled mouse-anti-human monoclonal antibodies bound on Streptavidin Sepharose High Performance beads (GE Healthcare Life Sciences, Freiburg, Germany).[13] Results were expressed as delta cpm (IgG subclass-specific cpm – anti-rat IgM cpm), and converted to arbitrary units (AUs) proportional to the GADA IgG subclass-specific delta cpm of a local standard serum.

2.5 | Cell culture

PBMCs were cultured with 5 μg/mL $rhGAD_{65}$ (Diamyd Medical, Stockholm, Sweden) or in medium (AIM-V with β-mercaptoethanol) at 37°C in 5% CO_2, as previously described.[19] After 7 days incubation, the supernatants were collected for cytokine secretion analysis, and cells for flow cytometry.

Cytokines IL-2, IL-5, IL-10, IL-13, IL-17, interferon (IFN-γ), and tumour necrosis factor (TNF-α) were measured in cell supernatants using Bio-Plex Pro Cytokine Panel (Bio-Rad, Hercules, CA, USA). Data was collected using the Luminex 200™ (Luminex xMAP™ Corporation, Austin, TX, USA). The levels of antigen-induced cytokine secretion were calculated by subtracting the levels of spontaneous secretion (i.e. secretion from PBMCs cultured in medium alone) from the ones following stimulation with GAD_{65}.

For flow cytometry analysis, PBMCs were washed in phosphate-buffered saline solution containing 0.1% bovine serum albumin, and subsequently stained with a cocktail fluorochrome-conjugated monoclonal antibodies (Table S1). Cells were then fixed and permeabilised using FOXP3 staining buffer set (Thermo Fisher Scientific) according to the manufacturer's instructions, and incubated with anti-FOXP3 (Table S1). Data were acquired on a FACS Aria III (BD Biosciences) running FACS Diva v8 software (Becton Dickinson). Data were analysed using Kaluza v1.3 (Beckman Coulter). Induction of activated T cells was calculated as the difference between the percentage of $CD25^+$ T cells in GAD_{65}-stimulated cultures and the percentage of $CD25^+$ T cells in medium alone.

For the proliferation assays, PBMCs were incubated in triplicate, and culture conditions included also CD3/CD28 beads (~1 bead: 2 cells; Gibco, Life Technologies AS, Oslo, Norway). After 3 days, cells were pulsed with 0.2 μCi of ^3H thymidine/well (PerkinElmer, Waltham, MA, USA) for 18 hours. Then, cells were harvested and ^3H thymidine incorporation was recorded using a 2450 MicroBeta^2 Plate Counter (PerkinElmer, Waltham, MA, USA). Proliferation was expressed as stimulation index (SI) and calculated as the mean cells counts per minute (ccpm) of cells cultured in the presence of stimulus divided by the mean ccpm of cells cultured with medium alone. To calculate SI induced by antigen stimulation, proliferation from samples culture in medium alone was subtracted. For individuals that received SC injections of GAD-alum, proliferation was measured in available samples (10 out of 12).

2.6 | Statistical analysis

Clinical data were presented as mean ± SD, whilst immunological data were presented as median. Mann-Whitney *U* test was used to evaluate significant differences between lymph node (LN) and SC group. A probability level of <0.05 was considered statistically significant. Calculations were performed using GraphPad Prism 8.0.1 for Windows (GraphPad Software, La Jolla, CA, USA).

3 | RESULTS

3.1 | Clinical response

Patients were stratified into those who received LN or SC GAD-alum injections. Gender distribution was the same in both groups, whilst mean age was higher in LN patients than in the SC group ($p = 0.02$). Both groups had similar baseline mean C-peptide (fasting, max. stimulated, and AUC). There was no difference in pre-treatment HbA1c values, but insulin need (U/kg of body weight/24 hours) was higher for the SC group ($p = 0.01$). At 15 months, individuals who received LN treatment had a somewhat better clinical course, with both lower HbA1c ($p = 0.03$) and more often partial remission (IDAAC <9; $p = 0.03$; Table 1),[14] although the difference in C-peptide response was non-significant (Table 1).

3.2 | Immune response

Baseline levels of GADA did not differ between the two groups. However, at 15 months, the titres of GADA were 23 times higher in the LN group than those induced by SC administration of GAD-alum (Figure 1A; Table 1). Analysis of GADA subclasses revealed that pre-treatment levels were similar in the two groups, but the proportions of IgG2, IgG3, and IgG4 subclasses were significantly enhanced at 15 months in LN individuals as compared to the SC group (Figure 1B). Furthermore, whilst distribution of GADA subclasses remained unchanged in the SC group at 15 months, a reduction of IgG1 proportion and increase of IgG2, IgG3, and IgG4 proportions was observed in the LN group (Figure 1C).

GAD_{65}-induced cytokine secretion and cell proliferation were similar in both groups pre-treatment. At 15 months, a difference between the groups included higher levels of GAD_{65}-induced IL-5, IL-10, and TNF-α in the LN individuals (Figure 2A), whilst the other analysed cytokines did not differ between the groups (Figure S1). In addition, GAD_{65}-induced PBMCs proliferation was almost undetectable in the LN group, and only observed in 4/12 individuals, whilst proliferation was 13 times higher in the SC group and detectable in 8/10 subjects (Figure 2B).

Analysis of GAD_{65}-induced T cell responses did not reveal change of activated $CD4^+CD25^+$ cells from baseline to 15 months. However, activated $CD8^+$ T cells were significantly reduced at 15 months in the LN group (Figure 2C). Differentiation of $CD4^+$ and $CD8^+$ T cells at 15 months had also distinct variations in the two groups, as the percentage of both $CD4^+$ and $CD8^+$ central memory cells (CM, $CD45RA^-CCR7^+$) was lower in LN individuals, whilst total effector cells (Eff, $CD45RA^{-/+}$ $CCR7^-$) were higher in the LN samples compared to individuals in the SC group (Figure 2D).

We did not detect modifications in the regulatory T cells ($CD4^+CD25^{hi}CD127^{low/-}$ $FOXP3^+$) in any of the groups after 15 months (Figure S2A). Furthermore, stratification of regulatory T cells according to the combined expression of FOXP3 and CD45RA showed a similar percentage of non-suppressive ($FOXP3^{low}CD45RA^-$), resting ($FOXP3^{low+}CD45RA^+$), and activated ($FOXP3^{high}CD45RA^-$) fractions in both groups (Figure S2B).

3.3 | Immunological profile

Radar chart was used for the combined visual representation of the immunological responses. Relative variations between the groups, given by mean scaled values of each parameter, highlights that higher

TABLE 1 Characteristics of individuals, according to the study group who received GAD-alum injections into the lymph node (LN, $n = 12$) or subcutaneously (SC, $n = 12$)

	Baseline			6 months			15 months		
Variable	LN	SC	*p*-Value	LN	SC	*p*-Value	LN	SC	*p*-Value
Gender distribution, *n* (%)									
Female	4 (33)	7 (58)	ns	–	–	–	–	–	–
Male	8 (67)	5 (42)	ns	–	–	–	–	–	–
Age (years)	19.08 ± 4.44	14.75 ± 2.67	0.02	–	–	–	–	–	–
Fasting C-peptide (nmol/L)	0.26 ± 0.12	0.26 ± 0.15	ns	0.23 ± 0.08	0.24 ± 0.15	ns	0.23 ± 0.05	0.19 ± 0.12	ns
C-peptide AUC (nmol/L)	0.55 ± 0.19	0.63 ± 0.22	ns	0.53 ± 0.16	0.51 ± 0.28	ns	0.44 ± 0.17	0.44 ± 0.32	ns
HbA1c (mmol/mol)	59.17 ± 18.89	49.42 ± 10.26	ns	42.58 ± 5.88	50.08 ± 11.01	0.03	44.92 ± 5.57	56.50 ± 13.91	0.03
Insulin dose (U/kg of body weight/24 hours)	0.36 ± 0.13	0.60 ± 0.32	0.01	0.29 ± 0.18	0.55 ± 0.36	0.02	0.34 ± 0.20	0.59 ± 0.26	ns
IDAAC	9.00 ± 1.89	9.06 ± 2.03	ns	7.22 ± 1.11	8.91 ± 2.09	0.03	7.64 ± 0.99	9.67 ± 2.15	0.02
IDAAC <9 (%)	7 (58)	7 (58)	ns	11 (92)	6 (55)	ns	11 (92)	5 (45)	0.03
Median GADA titres (U/mL)	978.50 ± 8250.08	726.50 ± 1669.01	ns	109,450.00 ± 289,050.52	4668.00 ± 19,635.53	<0.0001	54,970.00 ± 152,925.85	2351.50 ± 154,756.06	0.0005

Note: Plus–minus values are means ± SD, unless stated otherwise.

Abbreviations: AUC, area under the curve; GADA, 65-kD isoform of glutamic acid decarboxylase autoantibody; HbA1c, glycated haemoglobin; IDAAC, insulin dose adjusted HbA1c; ns, non-significant.

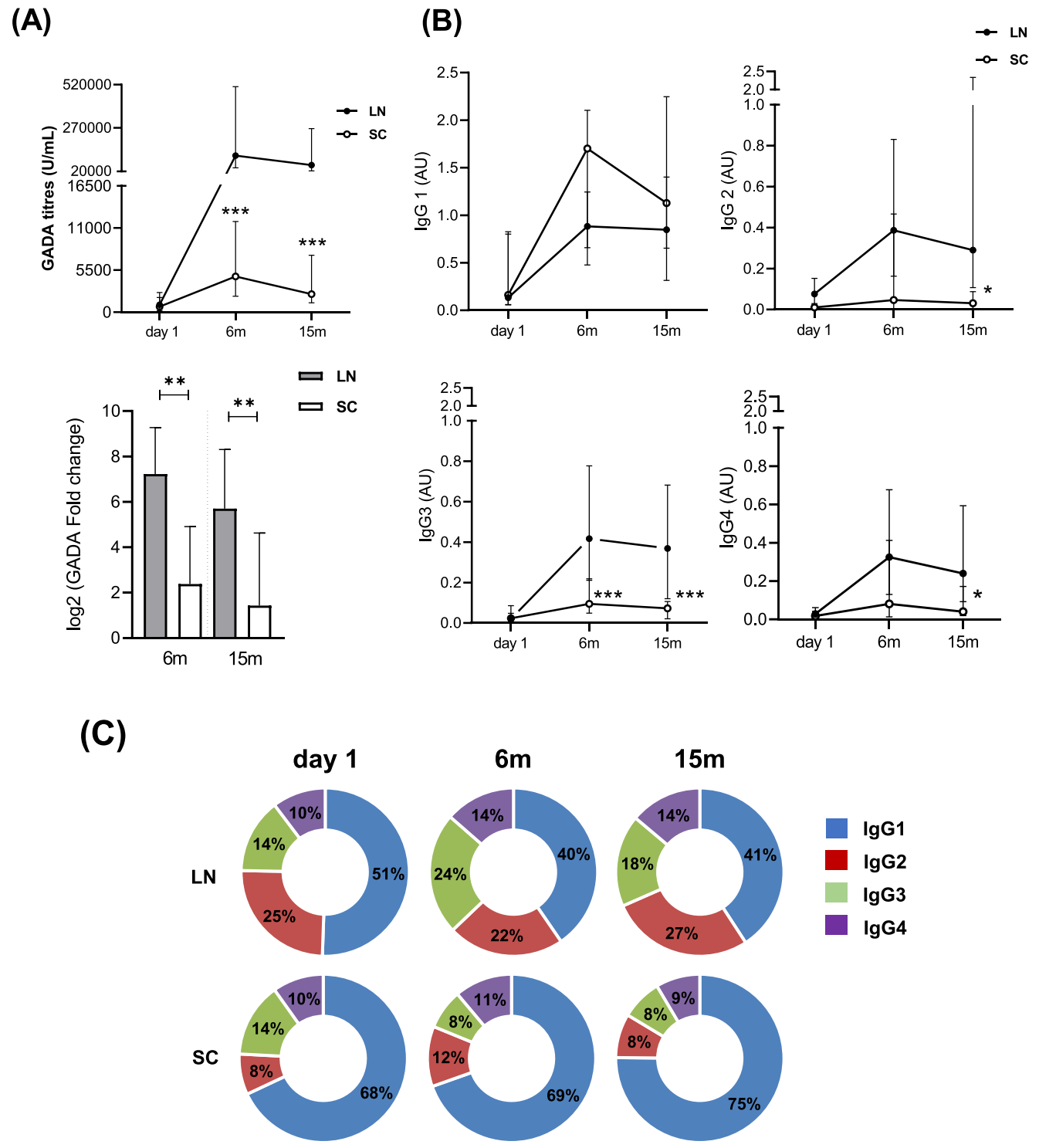

FIGURE 1 Immune responses induced by GAD-alum administrated into lymph nodes (LN, white circles or bars) or by subcutaneous injections (SC, black circles or grey bars) at baseline, 6 and 15 months. (A) Median values and fold change of GADA titres (U/mL). (B) Median levels of GADA IgG subclasses shown as arbitrary units (AUs). (C) Mean of GADA IgG subclasses relative distribution at baseline, 6 and 15 months. Frequencies of each subclass were calculated with respect to the combined sum of the four subclasses for each sample. Bars indicate interquartile range. Mann-Whitney *U* test. $^{*}p < 0.05$

GADA levels at 15 months in the LN group were characterised by increase of IgG3 and IgG4 subclasses, and to a lesser extent of IgG2 and IgG1, whereas the small increment of GADA in SC individuals was mainly driven by IgG1 (Figure 3A). Another clear difference in the GAD_{65}-induced cytokine secretion between the groups was the predominant secretion of IL-10 in the LN group (Figure 3B,C), accompanied by the reduction of both GAD_{65}-induced proliferation and $CD8^{+}$ T cell activation in the same group (Figure 3C).

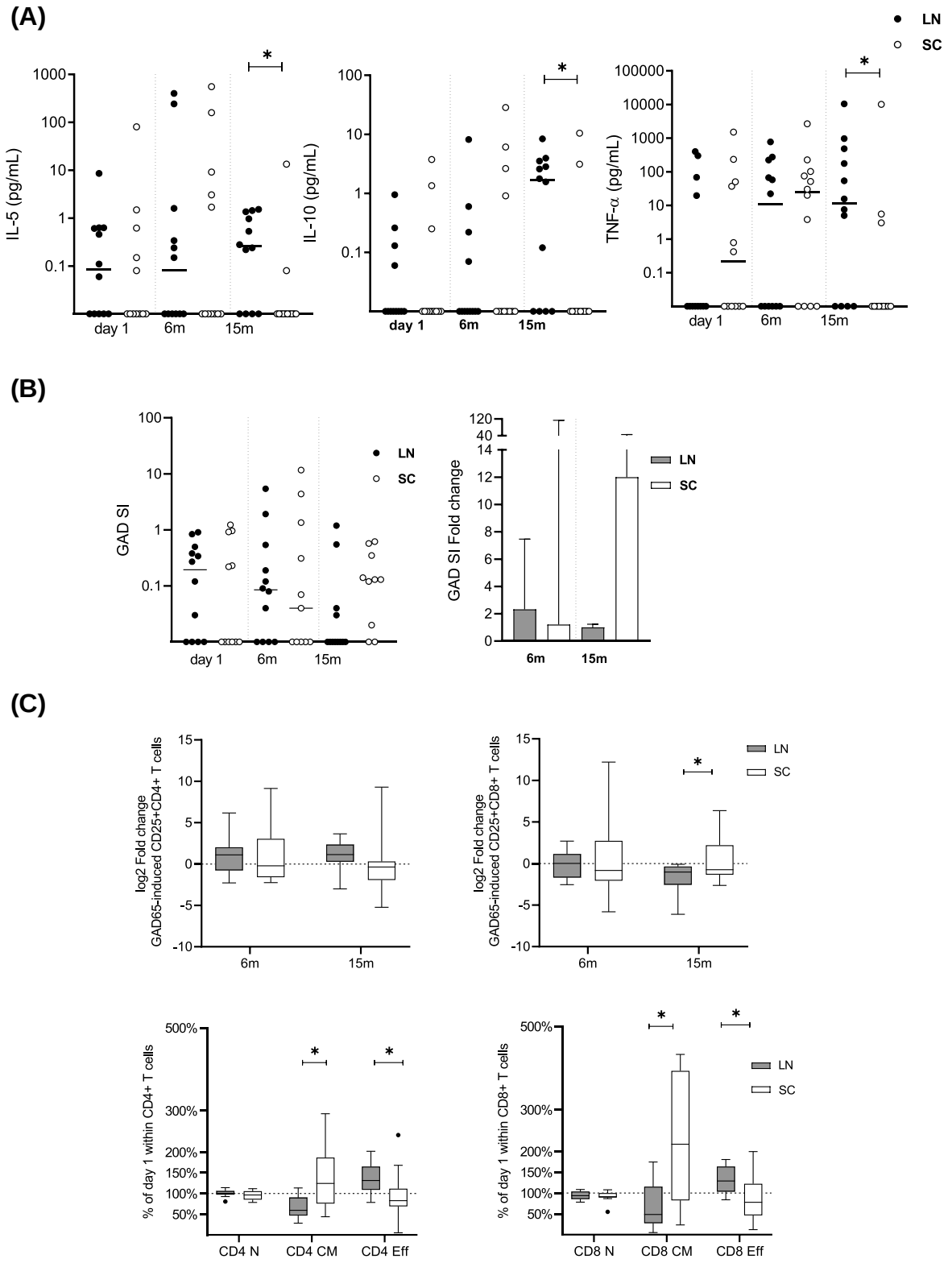

FIGURE 2 Cytokine secretion, cell proliferation, and T cell markers upon *in vitro* PMBCs stimulation induced by GAD-alum administrated into lymph nodes (LN, white circles or bars) or by subcutaneous injections (SC, black circles or grey bars). (A) Median levels of GAD_{65}-induced IL-5, IL-10, and TNF-α cytokines at baseline, 6 and 15 months detected by Luminex in PBMCs supernatants after 7 days culture in presence of medium or GAD_{65} (5 μg/mL). Levels of GAD_{65}-induced cytokine secretion were calculated by subtraction of spontaneous secretion from each individual, and expressed as pg/mL. (B) Median values and fold change of GAD_{65}-induced proliferation at baseline, 6 and 15 months were calculated from mean counts of triplicates in the presence of stimulus divided by the mean of triplicates from cells cultured with medium alone, and expressed as stimulation index (SI). Horizontal lines indicate the median. Bars indicate interquartile range. (C) Change in the induction of activated $CD25^+CD4^+$ and $CD25^+CD8^+$ T cells upon GAD_{65} stimulation expressed as log2 fold change from baseline to 6 and 15 months. Whiskers represent minimum and maximum. (D) Percentage of change from baseline to 15 months in the proportion of naïve (N, $CD45RA^+CCR7^+$), central memory (CM, $CD45RA^-CCR7^+$), and effector (Eff, $CD45RA^{-/+}CCR7^-$) within $CD4^+$ and $CD8^+$ T cells. Horizontal lines indicate the median. Whiskers according to Tukey. Mann-Whitney *U* test. $^*p < 0.05$

FIGURE 3 Radar chart representation of the immune response at baseline and 15 months in T1D individuals who received GAD-alum injections into the lymph node (LN, blue) or subcutaneously (SC, red). Relative variation of (A) GADA and IgG1, IgG2, IgG3, and IgG4 subclasses. (B) IL-2, IL-5, IL-10, IL-13, IL-17, IFN-γ, and TNF-α cytokines. (C) IL-10, GAD_{65}-induced proliferation and $CD8^+$ T cell activation. Relative variation was calculated using median scaled values

4 | DISCUSSION

The present study shows clear differences in the immunological response detected 15 months after treatment in T1D individuals receiving GAD-alum into the lymph nodes compared to a group of individuals who received higher doses of GAD-alum subcutaneously. Main differences included a switch in GADA subclass distribution, enhanced IL-10 secretion as well as the reduction of GAD_{65}-induced proliferation and $CD8^+$ T cell activation in the LN group. These changes were accompanied by a somewhat better

clinical course in the individuals who received intralymphatic treatment.

Although GADA titres peaked in both groups, levels were higher after 15 months in the LN individuals, despite administration of lower doses of GAD-alum, in line with previous results showing higher GADA levels at 6 months[13] Switch of GADA subclasses distribution in the LN group was due to the reduction of IgG1 proportion and increased proportion of IgG2, IgG3, and IgG4 subclasses. This is an interesting finding, as subclass frequencies can be associated with Th1/Th2 responses, and higher IgG_2 and IgG_4 responses to tetanus vaccine in autoantibody negative children correlated with the secretion of IL-4 and IL-13 Th2-associated cytokines.[20] Results from previous studies have shown a transient increase of IgG3 and IgG4 together with a reduction of IgG1 after SC administration of GAD-alum, but the effect did not last.[17,19] Enhanced levels of IL-5 observed at 15 months might explain the switch of subclasses in GADA induced after treatment, as IL-5 is known to stimulate differentiation of B cells into antibody-secreting cells[21] and could also stimulate Ig-isotype switching.[22] It was interesting that the increment of GADA was accompanied by a reduction of both cell proliferation and $CD8^+$ T cell activation in LN individuals. This is in line with results from a prevention trial where intranasal insulin was administered to individuals at-risk for T1D, suggesting an immune tolerance effect.[23] A stepwise increase of IL-10 was also part of the immunological changes. Amongst its broad and potent anti-inflammatory effects, IL-10 is known to inhibit activation, proliferation, and production of pro-inflammatory cytokines on T cells, as well as to promote survival, proliferation, differentiation, and induce IgG4 production by B cells.[24,25] Levels of TNF-α were also increased at 15 months in the LN group. This cytokine is known for having both pro- and anti-inflammatory effects on the regulation of the immune response depending on the microenvironment.[25] The reduction of $CD8^+$ T cell activation in the LN group occurred together with a shift towards a predominant effector phenotype both in $CD4^+$ and $CD8^+$ T cells. In contrast, T cells from SC individuals maintained a predominant central memory phenotype. These changes in T cell phenotypes induced after different administration routes are important for the recall responses, as effector cells rapidly secrete cytokines upon antigen stimulation whilst central memory T cells have better proliferative capacity.[26] As we did not observe changes in regulatory T cells, our results suggest that part of the immunological effect observed in LN individuals may be driven by GAD-specific responses rather than by regulatory mechanisms. However, it cannot be excluded that the scarce number of GAD_{65}-specific regulatory T cells precluded their identification.

It is likely that a stronger immune response after intralymphatic administration of low doses is explained by administration route, as the immunogenic environment in the lymph nodes increase antigen presentation.[26-28] Although differences in the immunological response in this study are in line with the observed in samples from 6 adults after 6 months treatment,[13] the differences became more evident in this study with larger number of subjects also including children. One could speculate that an extra dose of GAD-alum into the lymph nodes might explain the difference in the immune response between the groups. However, we have previously shown that further doses of SC injections of GAD-alum did not affect the quality of the immune response.[17,19] Slight age difference between the LN and SC groups might be another underlying explanation for the differences, but it is unlikely as the immunological response pre-treatment was similar in both groups, and differences at 15 months were GAD_{65}-specific induced by the treatment.

5 | CONCLUSION

The immunological response induced by intralymphatic injections of small doses of GAD-alum 15 months after treatment differed from the response induced by SC injections of significantly larger doses of GAD-alum. Follow-up of the patients participating in DIAGNODE-1 showed a good C-peptide preservation at 6 and 15 months[12,14] Whether the immunological changes associated with clinical efficacy in these few patients might be regarded as a favourable immune outcome have to be addressed in larger double-blinded studies. Although this kind of treatment represent a promising therapeutic approach to increase the efficacy of autoantigen immunotherapy, administration of antigens directly into the lymph nodes is in an early stage. It is difficult to draw conclusive statements based on this small pilot study, but our finding further supports the future use of intralymphatic administration of GAD-alum in studies aiming at preservation of residual beta cell function.

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CONFLICT OF INTEREST

No potential conflicts of interest relevant to this article were reported. None of the authors in this article report any conflict of interest.

ETHICS STATEMENT

The DIAGNODE-1 and DIABGAD trials were approved by the Research Ethics Committee, Linköping University, Sweden (DIAGNODE-1: Dnr 2014/153-31; DIABGAD-1: Dnr 2012/417-32), and by the Medical Product Agency, Uppsala, Sweden (DIAGNODE-1: Dnr 5.1-214-54385; DIABGAD-1: Dnr 2012-003251-11). All participants and their parents/caregivers gave their consent after oral and written information.

AUTHOR CONTRIBUTIONS

Fabrícia Dietrich performed experiments, analysed data, and wrote the manuscript. Hugo Barcenilla performed experiments, analysed data, and prepared and edited the manuscript. Rosaura Casas designed data set and data analysis and prepared and edited the manuscript. Johnny Ludvigsson designed DIAGNODE and DIABGAD and reviewed the manuscript. Beatriz Tavira performed experiments and analysed data. Jeanette Wahlberg recruited and followed the participants. Peter Achenbach performed the analysis of GADA subclasses. All the authors read and approved the final version of the manuscript. Johnny Ludvigsson and Rosaura Casas are guarantors of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

DATA AVAILABILITY STATEMENT

The data sets are available from the corresponding author on a reasonable request.

ORCID

Fabrícia Dietrich https://orcid.org/0000-0002-6458-2359
Johnny Ludvigsson https://orcid.org/0000-0003-1695-5234

TRANSPARENT PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/dmrr.3500.

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