Cellular and Humoral Immune Responses in Type 1 Diabetic Patients Participating in a Phase III GAD-alum Intervention Trial

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OBJECTIVE—GAD formulated in aluminum hydroxide (GAD-alum) has previously been shown to induce preservation of residual insulin secretion in recent-onset type 1 diabetes, but recent phase II and III GAD-alum trials failed to reach primary outcomes. The European phase III study was therefore closed after 15 months, and only a minority of patients completed the 30 months of follow-up.

RESEARCH DESIGN AND METHODS—This study was aimed to characterize cellular and humoral responses in the Swedish patients (n = 148) participating in the phase III trial, receiving four (4D) or two (2D) GAD-alum doses or placebo. Serum GAD_{65} antibody (GADA) levels, GADA IgG1–4 subclass distribution, cytokine secretion, and proliferative responses in peripheral blood mononuclear cells (PBMCs) were analyzed.

RESULTS—The GAD_{65}-induced cytokine profile tended to switch toward a predominant Th2-associated profile over time both in the 2D and 4D group. The groups also displayed increased GADA levels and PBMC proliferation compared with placebo, whereas GADA IgG subclass distribution changed in 4D patients.

CONCLUSIONS—Both 2D and 4D patients displayed GAD_{65}-specifc cellular and humoral effects after GAD-alum treatment, but at different time points and magnitudes. No specific immune markers could be associated with treatment efficacy.

Type 1 diabetes is regarded as an autoimmune-mediated disease in which pancreatic insulin-producing β-cells are destroyed, resulting in a lifelong dependence on exogenous insulin. Clinical intervention trials using different agents in recent-onset type 1 diabetic patients have shown various efficacies (1–7), which indeed highlights the complexity of translation from animal models to human type 1 diabetes. A phase II trial with GAD_{65} formulated with aluminum hydroxide (GAD-alum) showed efficacy in preserving residual insulin secretion in children and adolescents with recent-onset type 1 diabetes (8,9). However, subsequent GAD-alum phase II (10) and III trials (11) with different design failed to reach their primary outcomes. Significant efficacy was shown in some prespecified subgroups (11), so it cannot be excluded that treatment with GAD-alum might be beneficial in certain patient subgroups, alone or in combination with other therapies.

A large number of different potential therapies have been evaluated in humans but the effects on disease mechanisms remain unknown, and few immune correlates to clinical efficacy have been identified. Thus, to improve autoantigen treatment, it is of utmost importance to increase the understanding of the immunomodulatory effect of antigen-specific immunotherapy. In the previous phase II trial, GAD_{65} antibody (GADA) levels increased and remained elevated in patients who received two injections of GAD-alum compared with placebo (8,12), and a transient increase of GADA IgG4 and IgG3 subclasses was observed (13). In addition, the treatment induced an early Th2-associated response to GAD_{65}, followed by a wide range of Th1- and Th2-associated cytokines (14). These results suggest that an effect of GAD-alum could be mediated by induction of an early Th2-skewed immune response and generation of persistent GAD_{65}-specific cellular and humoral immune responses (15). In the phase III trial, two additional doses of GAD-alum were administered to one of the treatment groups to evaluate whether this could improve the clinical effect.

Since the phase III study failed to reach primary outcome, a main question is why the efficacy differed from the previous phase II study. It is also important to assess if differences of the immunomodulation induced by GAD-alum could explain the variable outcomes in the two trials. Although the phase III trial was closed after 15 months, a majority of the Swedish patients completed their 21-month visit, and a subgroup of patients completed the 30 months of follow-up, and those patients were included in the current study. Here we aimed to characterize GADA and insulinoma antigen 2 autoantibody (IA-2A) levels, GADA IgG1–4 subclass distribution, peripheral blood mononuclear cell (PBMC) cytokine secretion, and proliferative responses.

RESEARCH DESIGN AND METHODS

Subjects

The design and characteristics of the trial have been previously described (11). The

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study was a multicenter, randomized, double-blinded trial performed in nine European countries (Finland, France, Germany, Italy, the Netherlands, Slovenia, Spain, Sweden, and the U.K.). Patients (n = 334) aged 10–20 years with fasting C-peptide >0.1 nmol/L and detectable serum GADA were enrolled within 3 months of diagnosis. They received either four doses of 20 μg GAD-alum on days 1, 30, 90, and 270 (4D) or two doses of GAD-alum on days 1 and 30 followed by two doses of placebo on days 90 and 270 (2D), or four doses of placebo on days 1, 30, 90, and 270.

Only samples from the Swedish cohort (n = 148) were included in the current study and randomized to 4D (n = 49), 2D (n = 49), and placebo (n = 50). The group of patients who completed the 30-month visit (n = 45) was randomized as follows: 4D (n = 14), 2D (n = 15), and placebo (n = 16). Blood and serum samples were collected at day 0 and after 1, 3, 9, 15, 21, and 30 months. 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.

This study was performed according to the Declaration of Helsinki and was approved by the Research Ethics Committee at the Faculty of Health Sciences, Linköping University. Written informed consent was obtained from all patients, and for those <18 years of age, also from their parents.

Serum GADA titers and IgG1–4 subclass analysis

Serum GADA titers were centrally analyzed by BARC Laboratories (Ghent, Belgium), using an ELISA (GAD65 Antibody ELISA; RSR) (16). The accuracy of the assay has been assessed by the Diabetes Antibody Standardization Program (DASP) workshop 2010, with 90% sensitivity and 94% specificity.

The GADA IgG1, -2, -3, and -4 subclasses were measured using a modification of the conventional GADA assay, as previously described (13). The cutoff value for each subclass was determined using a GADA-negative control, which was run in duplicate in each assay. Results were expressed as counts per minute (cpm), and positivity of each sample was calculated by subtraction of the mean cpm value plus three times the SD obtained for the negative control.

IA-2A radiobinding immunoassay

Serum IA-2A titers were determined using a radiobinding assay using ^{35}S-labeled recombinant human GAD_{65}, as previously described (13). The assay was validated through the DASP workshop, and in 2010, the assay had 100% specificity and 66% sensitivity.

Cytokine secretion assay

One million PBMCs diluted in 1 mL AIM-V medium (Invitrogen) supplemented with 20 μmol/L β-mercaptoethanol (Sigma-Aldrich) were cultured for 7 days in the presence of 5 μg/mL GAD_{65}, 10 μg/mL IA-$2_{853–872}$ peptide (ProImmune), 100 ng/mL tetanus toxoid (TTX; Calbiochem) or in medium alone at 37°C in 5% CO_2. Interleukin-1β (IL-1β), IL-2, IL-5, IL-10, IL-13, IL-17, tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) were measured in cell supernatants using a Bio-Plex Pro Cytokine Panel (Bio-Rad) according to the manufacturer's instructions. Due to reagent incompatibilities, transforming growth factor-β1 (TGF-β1) was separately assayed using a Bio-Plex Pro TGF-β1 assay (Bio-Rad). Data were collected using the Luminex 200 (Luminex Corporation) and analyzed using MasterPlex QT software. The specific antigen-induced cytokine secretion was calculated by subtracting the spontaneous secretion (i.e., PBMCs cultured in medium alone). The lowest detection limit for each analyte was as follows: IL-1β (2.0 pg/mL), IL-2 (3.0 pg/mL), IL-5 (2.5 pg/mL), IL-10 (1.7 pg/mL), IL-13 (1.5 pg/mL), IL-17 (6.0 pg/mL), IFN-γ (30.0 pg/mL), TNF-α (4.0 pg/mL), and TGF-β1 (30.0 pg/mL).

Lymphocyte proliferation assay

Samples from baseline (n = 24), 1 month (n = 30), 3 months (n = 33), 9 months (n = 60), and 15 months (n = 44) were included in the assay based on availability. PBMC proliferative responses in the presence of GAD_{65} (5 μg/mL), IA-$2_{853–872}$ (5 μg/mL), or TTX (5 μg/mL; Statens Serum Institut) were analyzed as previously described (15) and expressed as stimulation index, calculated as the mean of triplicates in presence of stimulus divided by the mean of triplicates with medium alone.

Statistical analysis

As datasets were determined to be significantly different from a Gaussian distribution using Shapiro-Wilk test, nonparametric tests corrected for ties were used. Unpaired analyses were performed using the Kruskal-Wallis test followed by Mann-Whitney U test, and correlations were analyzed with Spearman rank correlation coefficient test. Differences within groups were calculated by Friedman test followed by Wilcoxon signed rank test. A probability level of <0.05 was considered statistically significant. Calculations were performed using IBM SPSS Statistics version 20 (SPSS Inc.).

RESULTS

Baseline characteristics

Patient baseline characteristics in the Swedish study population (n = 148) were well balanced among the three treatment groups, and the subgroup of patients followed for 30 months (n = 45) did not differ from the Swedish cohort or from the entire study cohort (n = 334) (Supplementary Table 1).

GAD-alum treatment enhances GADA levels

In line with our results from the previous phase II trial (8), GADA titers increased after GAD-alum treatment, both in the 4D and 2D groups compared with placebo, and titers remained elevated throughout the study period (Fig. 1*A*). GADA titers peaked at 3 months in the 2D ($P <$ 0.001) and 4D ($P < 0.001$) groups. The extra injections of GAD-alum administered to the 4D group further boosted GADA levels, which were enhanced in 4D patients at 15 months compared with the 2D group (P = 0.024). In addition, GADA fold change from baseline was also higher in the 4D patients at 15 and 21 months compared with the 2D (P = 0.007 and P = 0.012, respectively) and placebo ($P < 0.001$ and $P < 0.001$, respectively) groups (Fig. 1*B*). In contrast, both GADA levels and GADA fold change decreased from baseline to 21 months within the placebo group (data not shown). Moreover, IA-2A titers did not differ between the treatment arms at any time point (data not shown), whereas an overall decrease within each group during the study period was observed (Fig. 1*C*).

Altered GADA IgG1–4 subclass distribution after treatment

As GAD-alum treatment transiently reduced GADA IgG1 and enhanced IgG3/IgG4 subclasses during the phase II trial (13), we further assessed the subclass distribution in the current study. At baseline, GADA IgG1 was the most frequent subclass in all groups, followed by IgG3 > IgG4 >IgG2 (Fig. 1*D–F*). This hierarchy was evident for the 4D group until 9 months, when these patients displayed

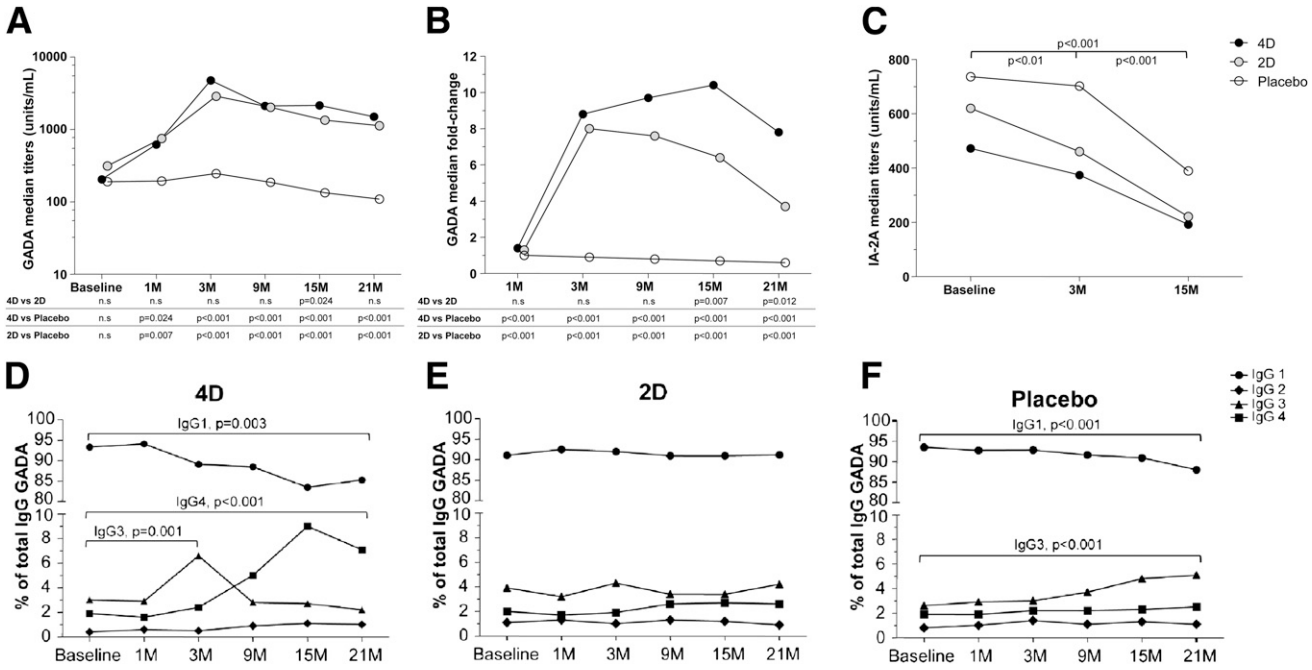


Figure 1—*Changes in autoantibody titers and GADA IgG1–4 subclass distribution. GADA median titers* (A)*, GADA median fold-change from baseline* (B)*, and IA-2A median titers* (C) *in 4D* (n = *49, black circles), 2D* (n = *49, gray circles), and placebo* (n = *50, empty circles). The median GADA IgG subclass distribution is presented as a percentage of total IgG for 4D* (D)*, 2D* (E)*, and placebo* (F)*. Results were expressed as cpm, and positivity of each sample was calculated by subtraction of the mean cpm value plus three times the SD obtained for the negative control. Significant differences are indicated as* P *values. M, months.*

significantly increased proportions of IgG4. Thus, 4D patients displayed significantly increased IgG4 frequencies at 9, 15, and 21 months, compared with 2D and placebo, which resulted in a changed subclass hierarchy (IgG1 >IgG4 >IgG3 >IgG2) for this group (data not shown). Further, the proportions of the IgG4 subclass at 9, 15, and 21 months were also significantly higher within the 4D group compared with baseline ($P < 0.001$), whereas in contrast, the proportions of IgG1 decreased from baseline to 3, 9, 15, and 21 months ($P = 0.003$) (Fig. 1*D*). In addition, a transient increase in IgG3 was observed at 3 months ($P = 0.001$) within the 4D group, when total GADA levels peaked and IgG1 started to decrease. No change in any of the subclasses was observed within the 2D group at any time point (Fig. 1*E*). In the placebo group, IgG3 increased at 3, 9, 15, and 21 months compared with baseline ($P < 0.001$), whereas the proportion of IgG1 decreased throughout the study period ($P < 0.001$) (Fig. 1*F*).

GAD_{65} stimulation of PBMCs induces cytokine secretion in GAD-alum–treated patients

Since an early Th2-associated response to GAD_{65} was observed in GAD-alum–treated patients in the phase II study (14), we next analyzed the cytokine profile in PBMC supernatants after antigen challenge. There was no difference in cytokine secretion between the three treatment arms at baseline. One month after the first GAD-alum injection, in vitro stimulation with GAD_{65} induced higher secretion of IL-2, IL-5, IL-10, IL13, IFN-γ, and TNF-α both in 2D and 4D patients compared with placebo (Fig. 2*A*), and IL-5, IL-10, IL13, IFN-γ, and TNF-α remained higher in the 2D and 4D groups at the following visits at 3, 9, 15, and 21 months. Higher secretion of IL-1β and IL-17 was detected at 3 months in the 2D and 4D groups and remained higher compared with placebo at 9, 15, and 21 months. Secretion of TGF-β followed the same pattern in all treatment groups without statistically significant differences at any time point. We further assessed the relative contribution of each cytokine to the total GAD_{65}-induced secretion. Both 2D and 4D patients displayed a cytokine profile that tended to switch from a wide cytokine profile toward a more predominant Th2-associated profile from baseline to 21 months (Fig. 2*B*).

Correlation analysis revealed an association of several GAD_{65}-induced cytokines with GADA fold change in the 2D group at 15 months (IFN-γ: $r = 0.64$, $P < 0.001$; IL-10: $r = 0.66$, $P < 0.001$; IL-13: $r = 0.66$, $P < 0.001$; IL-1β: $r = 0.50$, $P = 0.001$; IL-5: $r = 0.73$, $P < 0.001$; TNF-α: $r = 0.46$, $P = 0.003$) and 21 months (IFN-γ: $r = 0.44$, $P = 0.008$; IL-13: $r = 0.44$, $P = 0.008$; IL-2: $r = -0.44$, $P = 0.008$; IL-5: $r = 0.49$, $P = 0.003$; TGF-β1: $r = 0.48$, $P = 0.005$); however, this association was not observed in the 4D or placebo patients. To confirm that the effect of GAD-alum was antigen specific, spontaneous cytokine secretion as well as secretion after in vitro stimulation with TTX and IA-$2_{853–872}$ was studied. Spontaneous, TTX-induced, and IA-$2_{853–872}$–induced secretion did not differ between the three treatment groups (data not shown).

In vitro stimulation with GAD_{65} induces PBMC proliferation in GAD-alum–treated patients

In addition to cytokine secretion, the proliferative response to antigenic recall challenge was quantified. Proliferation in response to GAD_{65} was increased at 3, 9, and 15 months both in 2D ($P = 0.007$, $P < 0.001$, and $P < 0.001$, respectively) and 4D groups ($P = 0.001$, $P < 0.001$, and $P < 0.001$, respectively) compared with placebo (Fig. 3*A*). Furthermore, proliferation to GAD_{65} was higher in 4D compared with 2D at

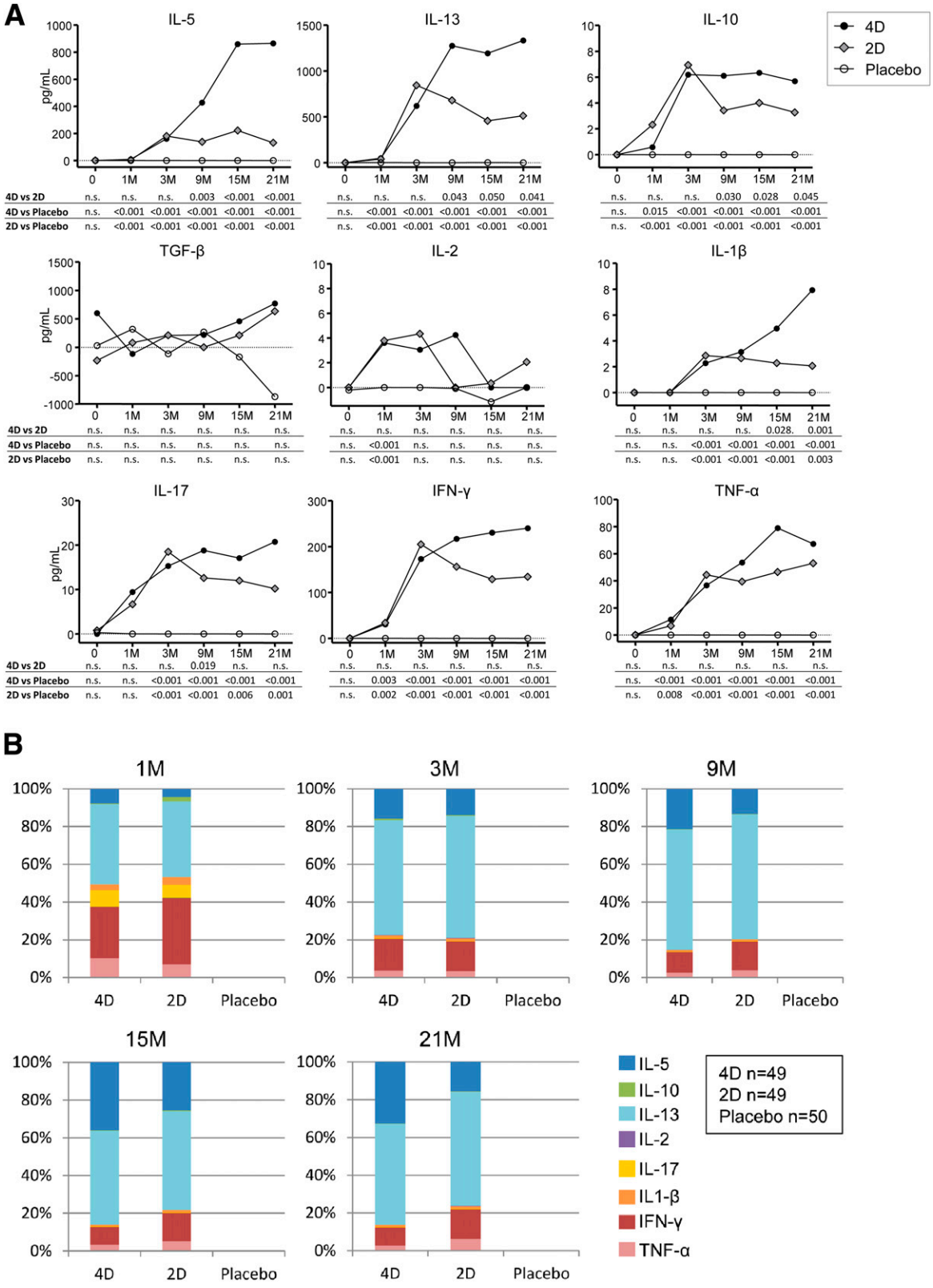
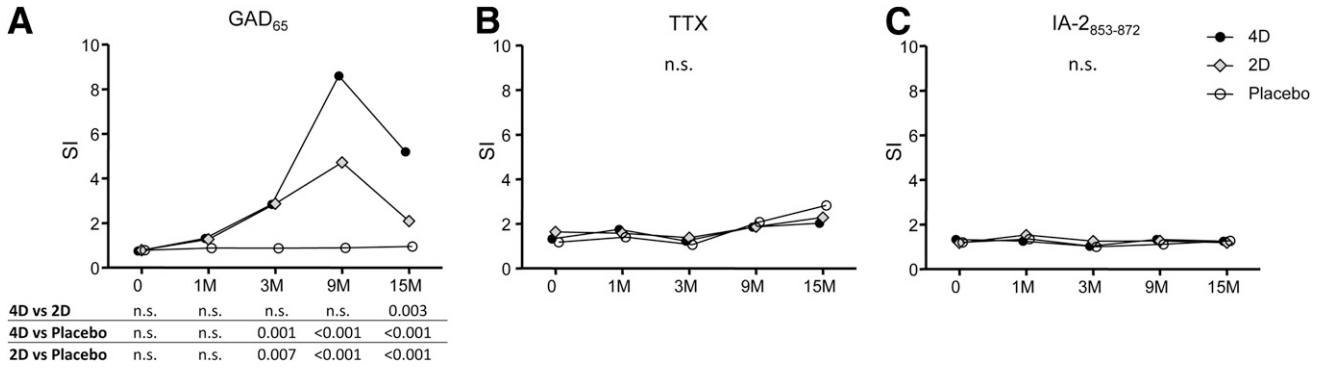


Figure 2—*Cytokine secretion upon in vitro PBMC stimulation.* A: *Median secretion of IL-1β, IL-2, IL-5, IL-10, IL-13, IL-17, IFN-γ, TNF-α, and TGF-β (pg/mL) from baseline to 21 months in the entire Swedish cohort, detected by Luminex in PBMC supernatants after 7-day culture with GAD_{65}, in patients receiving 4D* (n = *49, black circles), 2D* (n = *49, gray rhombuses), or placebo* (n = *50, empty circles). GAD_{65}-induced cytokine secretion is given after subtraction of spontaneous secretion.* B: *The relative contribution (%) of each cytokine to the GAD_{65}-induced secretion illustrated as bar chart from 1 to 21 months follow-up in 4D, 2D, and placebo groups. Significant differences are indicated as* P *values. M, months.*



	0	1M	3M	9M	15M
4D vs 2D	n.s.	n.s.	n.s.	n.s.	0.003
4D vs Placebo	n.s.	n.s.	0.001	<0.001	<0.001
2D vs Placebo	n.s.	n.s.	0.007	<0.001	<0.001

Figure 3—*PBMC proliferation. Median proliferative responses to GAD_{65}* (A)*, TTX* (B)*, and IA-$2_{853–872}$* (C) *from baseline to 15 months in patients receiving four doses of GAD-alum (4D, black circles), two doses of GAD-alum (2D, gray rhombuses), or placebo (empty circles). Proliferation is expressed as stimulation index (SI) calculated from the mean of triplicates in the presence of stimulus divided by the mean of triplicates with medium alone. Significant differences are indicated as* P *values. M, months.*

15 months ($P = 0.003$). There was no difference between the groups upon stimulation with the control antigens TTX and IA-$2_{853–872}$ (Fig. 3*B* and *C*), further confirming the specific effect of GAD-alum treatment.

Immune responses in the subgroup of patients followed for 30 months

Analysis of GADA titers, fold change, and IgG subclass distribution in the subgroup of patients followed for 30 months revealed a pattern similar to that observed for the whole Swedish cohort. Thus, GADA levels were higher in 4D and 2D groups compared with placebo (Supplementary Fig. 1*A*), and the GADA fold change was higher in 4D, with a trend in 2D, compared with placebo patients (Supplementary Fig. 1*B*). Further, there was a trend toward increased IgG4 and decreased IgG1 levels within the 4D group (Supplementary Fig. 2*A–C*).

GAD_{65}-induced secretion of IL-1β, IL-5, IL-10, IL-13, IL-17, IFN-γ, and TNF-α was higher in 2D and 4D patients compared with placebo (Supplementary Fig. 3). Both 2D and 4D patients displayed a cytokine profile that tended to switch from a wide cytokine profile toward a more predominant Th2-associated profile from baseline to 30 months (Supplementary Fig. 4).

Association of biomarkers with clinical parameters

No associations were observed between clinical parameters (i.e., fasting or stimulated C-peptide) and cytokines, GADA/IA-2A levels, or GADA IgG1–4 subclass distribution at any of the time points.

CONCLUSIONS—Here we aimed to study the immunomodulatory effect of two and four injections of GAD-alum and to identify immune markers that might explain or predict efficacy of treatment in patients participating in a phase III European study. The results of the trial showed that treatment with GAD-alum did not significantly reduce the loss of stimulated C-peptide or improve clinical outcomes over a 15-month period (11). The lack of clinical efficacy in the study was disappointing, but other recent trials have also failed to show efficacy, which highlights the difficulties to translate findings from animal models to humans. Indeed, the failure of recent GAD-alum III trials does not preclude the possibility that the treatment might be useful during prevention of type 1 diabetes in high-risk individuals (17,18) and/or in combination therapies with complementary agents at onset of type 1 diabetes. Better understanding of the immunological effect of the treatment is crucial for further development of successful intervention or prevention therapies.

In line with our previous results from the phase II trial (8,14,15,19), GAD-alum had a specific immunomodulatory effect, indicated by in vitro cytokine production and proliferation upon GAD_{65} stimulation. Cytokines play a key role in modulation of immune responses, and previous studies have suggested a shift from Th1 to Th2 as a mechanism of action of antigen-based immunotherapy in murine (20,21) and human type 1 diabetes (14,22,23). In contrast to our previous results showing an early Th2 immune deviation in response to GAD_{65} (14), the cytokine profile in the current study rather tended to switch from a wide spectrum of cytokines toward a more pronounced Th2-associated profile over time. Variations in the cytokine secretion between the studies could be explained by shorter time for in vitro antigen stimulation and usage of cryopreserved cells in the phase II study. However, when assessing the relative contribution of each cytokine, the cytokine profiles were similar in both trials. Thus, even if the early cytokine response differed between the two studies, the overall cytokine profiles resembled each other.

Treatment with GAD-alum raised GADA levels both in 2D and 4D patients, and after peaking at 3 months, the titers remained elevated throughout the study, which is in agreement with our previous findings (8). In addition, GADA IgG1–4 subclass distribution remained similar in all groups until 9 months, when the proportion of IgG4 in the 4D group drastically increased in parallel to a decrease of IgG1, supporting the notion of an enhanced humoral Th2-like response by additional GAD-alum doses. Previous studies have shown that higher levels of GADA IgG4 were associated with slower progression to clinical onset of disease in at-risk individuals (24,25). Furthermore, immunization with insulin promoted IgG4 in type 1 diabetic patients, an event interpreted to be associated with a Th2-like response (26). In the previous phase II trial, a transient IgG1 decrease together with an increase of IgG3 and IgG4 was detected in the 2D group compared with placebo (13). In the current study, a similar effect was observed only in the 4D group, but not in 2D patients. This inconsistency might be due to considerably shorter disease duration at inclusion of the patients in the phase III trial, and perhaps additional GAD-alum injections are required to affect the transforming

humoral immune response observed close to disease onset (24,27). Another difference was the significant increase of the GADA titers after receiving the first GAD-alum injection both in the 2D and 4D groups during the phase III trial, whereas in the previous phase II trial, enhancement of GADA was first detected after the second GAD-alum injection (8).

During the current study, four doses of GAD-alum were administered to one of the arms, aiming to reinforce the effect of treatment. Even though absolute GADA titers followed a similar pattern in the 4D and 2D groups, the higher GADA fold change from baseline to 15 and 21 months observed in the 4D group suggests that additional doses amplify the GADA response. Another effect of additional doses was an increased proliferative response to GAD_{65} in 4D compared with 2D at 15 months. In addition, cytokine levels were also higher in 4D at 9 months, i.e., 6 months after their third injection, and the levels continued to increase after the fourth dose. However, even though the levels of GAD_{65}-induced cytokines were increased by extra injections of GAD-alum, the relative proportion of each cytokine was similar in the 2D and 4D patients. Thus, although additional doses of GAD-alum resulted in a stronger immune response, the phenotype of the immune response was similar in the 2D and 4D groups, and increased cytokine secretion was not related to clinical outcome. Further, the persistence in these GAD_{65}-specific immune responses needs to be validated through future studies. Interestingly, a correlation between GADA fold change and the in vitro GAD_{65}-stimulated cytokine secretion at 15 and 21 months in the 2D group suggests an association between GAD-alum–induced humoral and cellular responses. However, this correlation was only observed in the 2D group, which might indicate that the two extra doses administered to 4D patients enhanced the GAD_{65}-specific humoral immune response without simultaneously affecting the specific cellular response.

Although the European phase III study failed to reach primary end point, the treatment showed efficacy in some prespecified subgroups, including the non-Nordic countries, but not in the Nordic patients, completely dominated by Swedes (11). Immunological studies within the trial were, for practical reasons, only performed in the Swedes. However, since baseline characteristics of the Swedish patients did not differ from the complete cohort, results from this group should be representative for the entire study population.

In conclusion, treatment of recent-onset type 1 diabetic children and adolescents with two and four injections of GAD-alum had a specific effect on the immune response, including enhancement of GADA levels and a Th2-associated deviation of the cytokine profile. No specific immune marker associated with clinical efficacy could be identified.

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S.A. and M.C. designed the study, performed the experiments, and wrote the manuscript. L.Å., M.P., and J.L. designed the study. R.C. designed the study and wrote the manuscript. All authors contributed to data interpretation and critically reviewed and approved the final manuscript. S.A., M.C., and R.C. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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References

1. Schloot NC, Meierhoff G, Lengyel C, et al. Effect of heat shock protein peptide DiaPep277 on beta-cell function in paediatric and adult patients with recent-onset diabetes mellitus type 1: two prospective, randomized, double-blind phase II trials. Diabetes Metab Res Rev 2007;23:276–285
2. Keymeulen B, Vandemeulebroucke E, Ziegler AG, et al. Insulin needs after CD3-antibody therapy in new-onset type 1 diabetes. N Engl J Med 2005;352:2598–2608
3. Herold KC, Hagopian W, Auger JA, et al. Anti-CD3 monoclonal antibody in new-onset type 1 diabetes mellitus. N Engl J Med 2002;346:1692–1698
4. Pescovitz MD, Greenbaum CJ, Krause-Steinrauf H, et al.; Type 1 Diabetes TrialNet Anti-CD20 Study Group. Rituximab, B-lymphocyte depletion, and preservation of beta-cell function. N Engl J Med 2009;361:2143–2152
5. Orban T, Bundy B, Becker DJ, et al.; Type 1 Diabetes TrialNet Abatacept Study Group. Co-stimulation modulation with abatacept in patients with recent-onset type 1 diabetes: a randomised, double-blind, placebo-controlled trial. Lancet 2011;378:412–419
6. Pozzilli P, Pitocco D, Visalli N, et al.; IMDIAB Group. No effect of oral insulin on residual beta-cell function in recent-onset type I diabetes (the IMDIAB VII). Diabetologia 2000;43:1000–1004
7. Walter M, Philotheou A, Bonnici F, Ziegler AG, Jimenez R; NBI-6024 Study Group. No effect of the altered peptide ligand NBI-6024 on beta-cell residual function and insulin needs in new-onset type 1 diabetes. Diabetes Care 2009;32:2036–2040
8. Ludvigsson J, Faresjö M, Hjorth M, et al. GAD treatment and insulin secretion in recent-onset type 1 diabetes. N Engl J Med 2008;359:1909–1920
9. Ludvigsson J, Hjorth M, Chéramy M, et al. Extended evaluation of the safety and efficacy of GAD treatment of children and adolescents with recent-onset type 1 diabetes: a randomised controlled trial. Diabetologia 2011;54:634–640
10. Wherrett DK, Bundy B, Becker DJ, et al.; Type 1 Diabetes TrialNet GAD Study Group. Antigen-based therapy with glutamic acid decarboxylase (GAD) vaccine in patients with recent-onset type 1 diabetes: a randomised double-blind trial. Lancet 2011;378:319–327
11. Ludvigsson J, Krisky D, Casas R, et al. GAD65 antigen therapy in recently diagnosed type 1 diabetes mellitus. N Engl J Med 2012;366:433–442
12. Chéramy M, Hampe CS, Ludvigsson J, Casas R. Characteristics of in-vitro phenotypes of glutamic acid decarboxylase 65 autoantibodies in high-titre individuals. Clin Exp Immunol 2013;171:247–254
13. Chéramy M, Skoglund C, Johansson I, Ludvigsson J, Hampe CS, Casas R. GAD-alum treatment in patients with type 1 diabetes and the subsequent effect on GADA IgG subclass distribution, GAD65 enzyme activity and humoral response. Clin Immunol 2010;137:31–40
14. Axelsson S, Hjorth M, Akerman L, Ludvigsson J, Casas R. Early induction of GAD(65)-reactive Th2 response in type 1 diabetic children treated with alum-formulated GAD(65). Diabetes Metab Res Rev 2010;26:559–568
15. Axelsson S, Chéramy M, Hjorth M, et al. Long-lasting immune responses 4 years after GAD-alum treatment in children with type 1 diabetes. PLoS ONE 2011;6:e29008
16. Brooking H, Ananieva-Jordanova R, Arnold C, et al. A sensitive non-isotopic assay for GAD65 autoantibodies. Clin Chim Acta 2003;331:55–59
17. DIAPREV-IT study of diabetes therapy Diamyd® receives further funding and will continue despite disappointing phase

III trial results. Immunotherapy 2011;3:923–924
18. Larsson HE, Lernmark A. Does immune-tolerance treatment with Alum-formulated GAD65 protect insulin-production in the pancreatic islet β cells? Hum Vaccin 2011;7:45–49
19. Hjorth M, Axelsson S, Rydén A, Faresjö M, Ludvigsson J, Casas R. GAD-alum treatment induces GAD65-specific CD4+CD25highFOXP3+ cells in type 1 diabetic patients. Clin Immunol 2011;138:117–126
20. Tian J, Atkinson MA, Clare-Salzler M, et al. Nasal administration of glutamate decarboxylase (GAD65) peptides induces Th2 responses and prevents murine insulin-dependent diabetes. J Exp Med 1996;183:1561–1567
21. Tisch R, Liblau RS, Yang XD, Liblau P, McDevitt HO. Induction of GAD65-specific regulatory T-cells inhibits ongoing autoimmune diabetes in nonobese diabetic mice. Diabetes 1998;47:894–899
22. Raz I, Elias D, Avron A, Tamir M, Metzger M, Cohen IR. Beta-cell function in new-onset type 1 diabetes and immunomodulation with a heat-shock protein peptide (DiaPep277): a randomised, double-blind, phase II trial. Lancet 2001;358:1749–1753
23. Alleva DG, Maki RA, Putnam AL, et al. Immunomodulation in type 1 diabetes by NBI-6024, an altered peptide ligand of the insulin B epitope. Scand J Immunol 2006;63:59–69
24. Ronkainen MS, Hoppu S, Korhonen S, et al. Early epitope- and isotype-specific humoral immune responses to GAD65 in young children with genetic susceptibility to type 1 diabetes. Eur J Endocrinol 2006;155:633–642
25. Couper JJ, Harrison LC, Aldis JJ, Colman PG, Honeyman MC, Ferrante A. IgG subclass antibodies to glutamic acid decarboxylase and risk for progression to clinical insulin-dependent diabetes. Hum Immunol 1998;59:493–499
26. Füchtenbusch M, Kredel K, Bonifacio E, Schnell O, Ziegler AG. Exposure to exogenous insulin promotes IgG1 and the T-helper 2-associated IgG4 responses to insulin but not to other islet autoantigens. Diabetes 2000;49:918–925
27. Ronkainen MS, Savola K, Knip M. Antibodies to GAD65 epitopes at diagnosis and over the first 10 years of clinical type 1 diabetes mellitus. Scand J Immunol 2004;59:334–340