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Regulatory T cell phenotype and function 4 years after GAD–alum treatment in children with type 1 diabetes

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Summary

Glutamic acid decarboxylase (GAD)$_{65}$ formulated with aluminium hydroxide (GAD-alum) was effective in preserving insulin secretion in a Phase II clinical trial in children and adolescents with recent-onset type 1 diabetes. In addition, GAD-alum treated patients increased CD4$^+$CD25^{hi} forkhead box protein 3$^+$ (FoxP3$^+$) cell numbers in response to *in-vitro* GAD$_{65}$ stimulation. We have carried out a 4-year follow-up study of 59 of the original 70 patients to investigate long-term effects on the frequency and function of regulatory T cells after GAD-alum treatment. Peripheral blood mononuclear cells were stimulated *in vitro* with GAD$_{65}$ for 7 days and expression of regulatory T cell markers was measured by flow cytometry. Regulatory T cells (CD4$^+$CD25^{hi}CD127^{lo}) and effector T cells (CD4$^+$CD25$^-$CD127$^+$) were further sorted, expanded and used in suppression assays to assess regulatory T cell function after GAD-alum treatment. GAD-alum-treated patients displayed higher frequencies of *in-vitro* GAD$_{65}$-induced CD4$^+$CD25$^+$CD127$^+$ as well as CD4$^+$CD25^{hi}CD127^{lo} and CD4$^+$FoxP3$^+$ cells compared to placebo. Moreover, GAD$_{65}$ stimulation induced a population of CD4^{hi} cells consisting mainly of CD25$^+$CD127$^+$, which was specific of GAD-alum-treated patients (16 of 25 *versus* one of 25 in placebo). Assessment of suppressive function in expanded regulatory T cells revealed no difference between GAD-alum- and placebo-treated individuals. Regulatory T cell frequency did not correlate with C-peptide secretion throughout the study. In conclusion, GAD-alum treatment induced both GAD$_{65}$-reactive CD25$^+$CD127$^+$ and CD25^{hi}CD127^{lo} cells, but no difference in regulatory T cell function 4 years after GAD-alum treatment.

Keywords: CD4 T cells (T helper, Th0, Th1, Th2, Th3, Th17), diabetes, immune regulation, regulatory T cells (Treg), therapy/immunotherapy

Introduction

Type 1 diabetes (T1D) is a consequence of an autoimmune reaction towards insulin-producing β cells of the pancreas. Immunomodulatory approaches to prevent or treat T1D have been developed and tested, with variable results [1–4]. Autoantigens may be used to induce immunologic tolerance as an alternative to immunosuppression [5]. Glutamic acid decarboxylase 65 (GAD$_{65}$) is one of the main antigens to which patients with T1D mount a destructive immune response, and autoantibodies directed against GAD$_{65}$ (GADA) and T cells specific for GAD$_{65}$ epitopes are common in T1D patients [6–8]. We have shown previously preservation of residual insulin secretion by GAD-alum treatment in a Phase II clinical trial in children with recent-onset T1D [3]. In addition, trial participants treated with GAD-alum up-regulated CD4$^+$CD25^{hi}forkhead box P3$^+$ (FoxP3$^+$) cells in response to GAD$_{65}$ stimulation *in vitro* and had a predominant T helper type 2 (Th2) immune response [9,10]. Preservation of C-peptide secretion was still detectable after 4 years in patients with <6 months T1D duration at baseline in the same trial [11], and the residual C-peptide secretion was accompanied by sustained high levels of GADA, increased memory T cell frequencies and T cell activation upon *in-vitro* GAD$_{65}$ stimulation [12]. Recently, additional Phases II and III clinical trials of GAD-alum have

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been conducted both in Europe and the United States, neither finding an effect on preservation of insulin secretion [13,14]. The present Phase II trial included patients with a T1D duration of <18 months, whereas the European Phase III trial included patients with a duration of <3 months, which may contribute to the discrepancy in outcome.

Self-tolerance is maintained physiologically by regulatory T cells (T_{reg}) in the periphery [15], and defects in T_{reg} function have been hypothesized to be involved in the pathogenesis of autoimmune disease [16]. Because tolerance in the periphery is maintained by T_{regs}, induction of active tolerance has long been a proposed mechanism of action of antigen-based therapies such as GAD-alum treatment [17]. T_{regs} typically express high levels of the interleukin (IL)-2 receptor α-chain CD25, the transcription factor FoxP3 and low levels of the IL-7 receptor CD127 [18–22]. However, both FoxP3 and CD25 can also be expressed by activated non-regulatory T cells. CD39 has also been suggested to be involved in T_{reg} function through the removal of adenosine triphosphate (ATP) and has thus been used to identify subsets of T_{regs} [23]. T_{regs} can suppress proliferation and cytokine secretion in a broad range of cell types, including $CD4^+$ and $CD8^+$ T cells, and their dysfunction leads to immunopathology [24]. It has been reported recently that rather than there being a deficiency in T_{reg} numbers, effector T cells (T_{eff}) from patients with T1D are resistant to T_{reg}-mediated suppression [25,26].

The aim of this work was to investigate whether an increase in cells with a T_{reg} phenotype persisted at 4 years after GAD-alum treatment. In addition, we tested whether GAD-alum treatment affected the suppressive capacity of T_{regs}.

Materials and methods

Ethics statement

This study was approved by the Research Ethics Committee at the Faculty of Health Sciences, Linköping University, Sweden. Written informed consent was obtained from participating individuals, and for those aged <18 years also their parents, in accordance with the Declaration of Helsinki.

Population

The design and characteristics of the Phase II trial have been described elsewhere [3]. Briefly, 70 T1D children between 10 and 18 years of age with fewer than 18 months of disease duration were recruited at eight Swedish paediatric centres. Participants had a fasting serum C-peptide level above 0·1 nmol/l and detectable GADA at inclusion. They were randomized to subcutaneous injections of 20 μg GAD-alum ($n = 35$) or placebo ($n = 35$) at day 0 and a booster injection 4 weeks later in a double-blind setting. After 4 years, patients and their parents were asked whether they were willing to participate in a follow-up study. Fifty-nine patients, of whom 29 had been treated with GAD-alum and 30 received placebo, agreed to participate.

Antibodies

Fluorescein isothiocyanate (FITC)-conjugated anti-CD39 (clone A1; Biolegend, San Diego, CA, USA), phycoerythrin (PE)-conjugated anti-FoxP3 (clone PCH101), allophycocyanin (APC)-conjugated anti-CD25 (clone BC96) and FITC- and PE-cyanine 7 (PE-Cy7)-conjugated anti-CD127 (clone eBioRDR5; eBioscience, San Diego, CA, USA), Alexa 700- and Pacific Blue-conjugated anti-CD4 (clone RPA-T4), APC-Cy7-conjugated anti-CD25 (clone M-A251; BD Pharmingen, Franklin Lakes, NJ, USA), and relevant isotype- and fluorochrome-matched control antibodies were used in this study. In addition, 7-amino-actinomycin D (7-AAD; BD Pharmingen) was used to measure cell viability.

Flow cytometry

Peripheral blood mononuclear cells (PBMC) from GAD-alum-treated ($n = 24$) and placebo-treated ($n = 25$) patients were isolated from whole blood by Ficoll-Paque (Pharmacia Biotech, Piscataway, NJ, USA) density gradient centrifugation within 24 h after drawing. Three of the GAD-alum-treated patients were classified as responders, 14 as intermediate responders and seven were non-responders, while the placebo group contained 10 intermediate responders and 14 non-responders (one patient unclassified due to missing serum sample). PBMC were incubated in AIM-V medium (Invitrogen, Carlsbad, CA, USA) with β-mercaptoethanol at 37°C, 5% CO_2 for 7 days, with or without 5 μg/ml recombinant GAD_{65} (Diamyd Medical, Stockholm, Sweden). One million cells were washed in 2 ml phosphate-buffered saline (PBS) containing 0·1% bovine serum albumin (BSA; Sigma-Aldrich, St Louis, MO, USA) and subsequently stained with anti-CD4, CD39, CD127 and CD25 antibodies. Cells were then fixed and permeabilized using a FoxP3 staining kit (eBioscience), according to the manufacturer's instructions. After washing, cells were stained with PE anti-FoxP3, reconstituted in PBS, acquired on a fluorescence activated cell sorter (FACS) (BD FACSAria) and analysed using Kaluza software version 1·1 (Beckman Coulter, Indianapolis, IN, USA). The $FoxP3^+$ gate was set using the negative population, as the negative population had a higher median fluorescence intensity (MFI) than the isotype control.

Cell sorting

Cells were sorted and expanded when sufficient cell numbers were available. Cryopreserved cells from GAD-

alum- ($n = 4$) and placebo- ($n = 3$) treated patients were stained with Pacific Blue conjugated anti-CD4, FITC-conjugated anti-CD127 and APC-conjugated anti-CD25 and sorted into T_{reg} and T_{eff} subsets based on $CD4^+CD25^{hi}CD127^{lo}$ and $CD4^+CD25^-CD127^+$ phenotype, respectively. After sorting, cells were pelleted by centrifugation at 400 ***g*** for 10 min, resuspended in AIM-V 10% human serum (HS) and allowed to rest for 2 h at 37°C, 5% CO_2 before expansion was initiated. Aliquots of sorted cells were re-acquired to assess purity. The average T_{eff} contaminant in sorted T_{regs} was 0·1%. PBMC from one single freshly drawn healthy donor were stained, sorted as above and stored frozen to serve as interassay control.

T_{eff} and T_{reg} expansion

T_{regs} were distributed at 4×10^4 cells per well in 125 µl AIM-V 10% HS into 96-well U-bottomed plates, and stimulated with anti-CD3/CD28 Dynabeads (Invitrogen) at a 1:1 bead-to-cell ratio. T_{effs} were plated at 5×10^5 cells per 500 µl medium, into 96-well flat-bottomed plates precoated overnight with 10 µg/ml anti-CD3 (OKT3; eBioscience) at 4°C. Cultures also contained 1 µg/ml soluble anti-CD28 antibody (CD28·2; eBioscience). Culture volume was doubled the following day, and 30 and 300 U/ml of recombinant human IL-2 (R&D Systems, Abingdon, UK) were added to T_{eff} and T_{reg} cultures, respectively. T_{regs} were washed and supplemented with fresh IL-2 every 2 days. T_{regs} and T_{effs} were restimulated as above on the ninth day of culture, and frozen down after 15 days of expansion. To verify post-expansion phenotype, cryopreserved T_{regs} and T_{effs} were cultured for 24 h in AIM-V 10% HS and 5 U/ml IL-2, and subsequently stained and acquired as described above.

Suppression assay

T_{reg}-mediated suppression was assessed using expanded T_{regs} and T_{effs} from placebo- ($n = 3$) and GAD-alum- ($n = 4$) treated patients. Expanded T_{regs} and T_{effs} were thawed and incubated in AIM-V 10% HS at 37°C, 5% CO_2 overnight, then resuspended at $0{\cdot}5 \times 10^5$ cells/ml. T_{effs} were plated into 96-well U-bottomed plates at a density of 5×10^4 cells per well, while T_{regs} were plated into T_{eff}-containing wells at T_{reg}-to-T_{eff} ratios of 1:1, 1:2, 1:4, 1:8 and 1:16. T_{reg}/T_{eff} cultures were stimulated with 5 µg/ml soluble anti-CD3 and 1 µg/ml soluble anti-CD28 antibodies. Unstimulated wells were included as negative controls, both from patients and interassay control healthy T_{effs}. IL-2 (1 U/ml) was added to all wells. Supernatants were collected after 3 days of culture and cells were incubated with 0·2 µCi [^{3}H]-thymidine (PerkinElmer, Waltham, MA, USA) for 18 h before harvesting. Thymidine incorporation was measured using a 1450 Wallac MicroBeta counter (PerkinElmer).

C-peptide measurements

C-peptide levels were measured in serum samples with a time-resolved fluoroimmunoassay (AutoDELFIA™ C-peptide kit, Wallac; PerkinElmer), as described [3]. Stimulated C-peptide was measured during a mixed meal tolerance test (MMTT) in GAD-alum- ($n = 21$) and placebo- ($n = 10$) treated patients who had a maximal C-peptide response of >0·20 nmol/l at the 30-month follow-up. Clinical effect of treatment was defined by changes in stimulated C-peptide measured as area under the curve (AUC) from baseline to 48 months.

Statistical analysis

Statistically significant differences were determined using the Mann–Whitney two-tailed *U*-test for unpaired observations, as data were determined to be significantly different from a Gaussian distribution. Wilcoxon's signed-rank test was used to compare paired samples. Linear regression was used to compare slope and *Y*-intercept of suppression curves, and correlations were determined with Spearman's rank correlation coefficient test. A probability level of <0·05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism software, version 5·04 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

GAD_{65} stimulation increases the proportion of both $CD25^{hi}CD127^{lo}$ and $CD25^+CD127^{+/hi}$ cells in GAD-alum-treated patients

We have demonstrated previously that *in-vitro* stimulation with GAD_{65} induced $CD4^+CD25^{hi}$ $FoxP3^+$ cells in PBMC from GAD-alum-treated patients [9]. To determine whether this effect persisted 4 years after treatment, we analysed $CD25^{hi}CD127^{lo}$ cells and used FoxP3 and CD39 as additional markers to discriminate T_{regs} from activated T cells more accurately. Thus, the expression of CD25, CD127, FoxP3 and CD39 on $CD4^+$ lymphocytes was analysed in PBMC after 7 days of incubation with or without GAD_{65}. Gates used for analysis and representative PBMC samples describing the expression of CD4, CD25 and CD127 are shown in Fig. 1a,b.

The frequency of $CD25^{hi}CD127^{lo}$ cells in the $CD4^+$ population was increased significantly upon GAD_{65} stimulation in GAD-alum-treated patients compared to unstimulated cells (7·4% and 4·5%, respectively), but not in the placebo group (Fig. 1c). The frequency of $CD4^+CD25^+CD127^+$ cells was also increased following GAD_{65} stimulation in the GAD-alum-treated patients compared to resting cells (13·1% and 2·6%, respectively; Fig. 1d). In contrast, GAD_{65} stimulation did not induce expression of $CD25^{hi}CD127^{lo}$ or

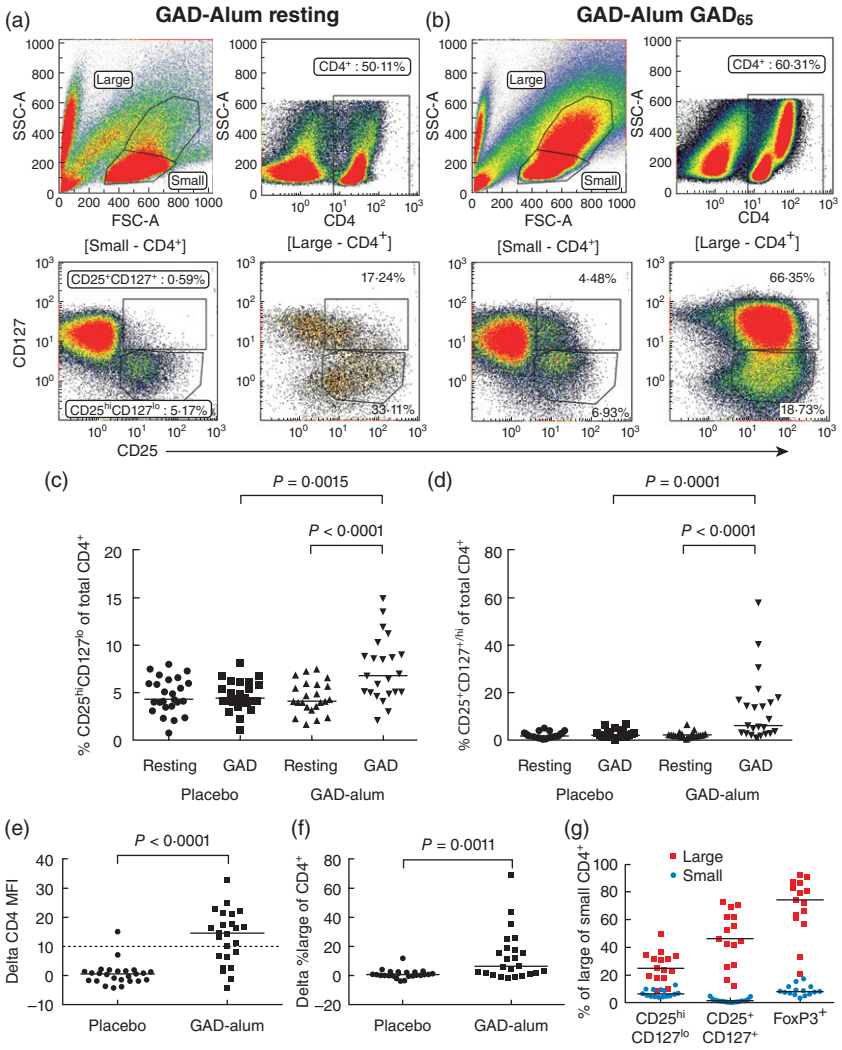

Fig. 1. Gating strategy and glutamic acid decarboxylase (GAD_{65})-induced T cell populations. (a,b) Gating strategy and representative peripheral blood mononuclear cell (PBMC) samples from a GAD-alum-treated subject at resting conditions and after *in-vitro* GAD_{65} stimulation, respectively. Upper left panel describes the division of lymphocytes into small and large. Upper right panel describes CD4 expression. The lower panels describe the distribution of $CD4^+$ cells between the $CD25^{hi}CD127^{lo}$ and the $CD25^+CD127^+$ populations, gates indicated above plots. (c,d) Expression of CD25 and CD127 on $CD4^+$ cells (placebo $n = 25$, GAD-alum $n = 23$). (e) Changes in CD4 median fluorescence intensity (MFI) and (f) frequency of forward-scatter $(FSC)^{hi}$side-scatter $(SSC)^{hi}$ cells were calculated by subtracting MFI and frequencies at resting conditions from those obtained after GAD_{65} stimulation and are represented as delta values (placebo $n = 25$, GAD-alum $n = 24$). The cut-off for high CD4 expression was defined as an increase of 10 units of CD4 MFI in $CD4^+$ cells following GAD_{65} stimulation and is indicated by the dashed line in (e). (g) Frequencies of $CD25^{hi}CD127^{lo}$, $CD25^+CD127^+$ and forkhead box protein 3^+ ($FoxP3^+$) cells among $FSC^{hi}SSC^{hi}$ (red squares) and small (blue circles) $CD4^+$ cells from GAD-alum-treated patients ($n = 15$) are shown as %. Each point represents an individual and median values are indicated with horizontal lines. Statistical significances are shown as *P*-values.

$CD25^+CD127^+$ compared to resting cells in the placebo group (Fig. 1c,d). The frequencies of $CD4^+CD25^{hi}CD127^{lo}$ and $CD4^+CD25^+CD127^+$ cells were also significantly higher in the GAD-alum-treated group compared to placebo individuals after stimulation with GAD_{65} (Fig. 1c,d).

GAD_{65} stimulation induces a population of activated T cells in GAD-alum-treated subjects

Stimulation with GAD_{65} in GAD-alum-treated patients induced a population of forward-scatter $(FSC)^{hi}$side-scatter $(SSC)^{hi}$ cells, consisting mainly of $CD4^+$ memory T cells, as we have reported previously [12]. These $FSC^{hi}SSC^{hi}$ cells are illustrated in Fig. 1a,b and are characterized by high CD4 expression (Fig. 1e). The $FSC^{hi}SSC^{hi}$ population was observed in 16/24 GAD-alum patients and in one of 25 placebo individuals. In line with the GAD_{65} recall response induced in GAD-immunized individuals, GAD_{65} stimulation induced higher CD4 MFI (Fig. 1e) and higher percentages of $FSC^{hi}SSC^{hi}$ cells (Fig. 1f) among $CD4^+$ cells from GAD-alum patients compared to the placebo group. Next, we analysed the expression of T_{reg}-associated markers among $FSC^{hi}SSC^{hi}$ $CD4^+$ cells from the GAD-alum group, and found that 25% were $CD25^{hi}CD127^{lo}$, 46·2% were $CD25^+CD127^{+/hi}$ and 74% were $FoxP3^+$ (Fig. 1g).

GAD_{65} stimulation induces cells with a T_{reg} phenotype in GAD-alum-treated subjects

FoxP3 expression on $CD4^+$ and $CD4^+FSC^{hi}SSC^{hi}$ cells was enhanced significantly by GAD_{65} stimulation in the GAD-alum group (Fig. 2a–c), while GAD_{65} stimulation did not induce any change compared to resting cells in the placebo group (Fig. 2c). To define further whether the increased $CD25^+CD127^{lo}$ population in GAD_{65} stimulated PBMC from GAD-alum-treated patients corresponded to a T_{reg} population, CD39 and FoxP3 were added as additional T_{reg} markers. Indeed, $CD4^+CD25^{hi}CD127^{lo}$ $FoxP3^+CD39^+$ cells were also found to be increased selectively in these patients following *in-vitro* GAD_{65} stimulation (Fig. 2d). Thus,

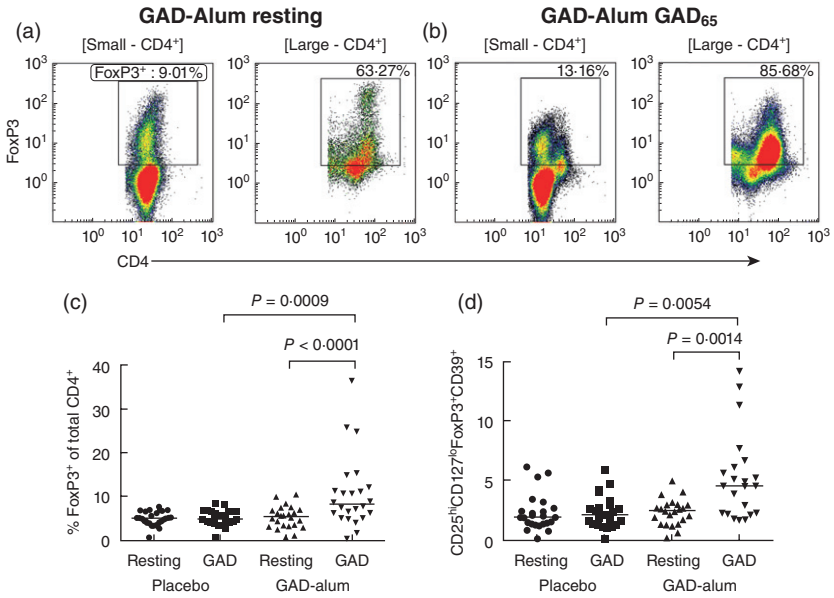

Fig. 2. Expression of regulatory T cell (T_{reg})-associated markers. (a) Expression of forkhead box protein 3 (FoxP3) on $CD4^+$ resting cells from a representative glutamic acid decarboxylase (GAD)-alum-treated subject; (b) expression of FoxP3 in GAD-stimulated cells, gates indicated above plots. (c) Expression of FoxP3 on $CD4^+$ cells (placebo $n = 25$, GAD-alum $n = 24$); (d) the fraction of $CD4^+$ cells co-expressing $CD25^{hi}CD127^{lo}FoxP3^+CD39^+$ from placebo ($n = 24$) and GAD-alum ($n = 22$) patients. Each point represents an individual and median values are indicated with horizontal lines. Statistical significances are shown as *P*-values.

in-vitro GAD recall leads to expansion of both T_{regs} and activated $CD25^+CD127^+$ T effector cells, which is observed only in patients treated previously with GAD-alum. There were no significant differences in expression of any measured marker on resting cells between the two treatment arms (Figs 1 and 2).

The post-expansion phenotype of T_{regs} and T_{effs} remains lineage-specific

T_{regs} ($CD4^+CD25^{hi}CD127^{lo}$) from GAD-alum-treated patients expanded approximately 900-fold, to a similar extent as T_{regs} from placebo-treated patients (800-fold; Table 1). T_{effs} ($CD4^+CD25^-CD127^+$) from both GAD-alum- and placebo-treated patients expanded approximately 100-fold. To verify the phenotype of sorted and expanded T_{regs} and T_{effs} after cryopreservation, we analysed the expression of T_{reg} markers on thawed cells by flow cytometry. T_{regs} maintained predominant expression of CD25, FoxP3, cytotoxic T lymphocyte antigen-4 (CTLA-4) and low expression of CD127 and CD45RA, and roughly 50% were $CD39^+$. Meanwhile, expression of FoxP3, CTLA-4, CD45RA and CD25 was scarce on T_{eff}, but the majority expressed CD127 and expressed CD39 in the same proportion as T_{regs}. Measurement of cell viability by 7-AAD staining 24 h after thawing demonstrated that T_{effs} had a viability of 90%, whereas 70% of T_{regs} were viable (data not shown).

Expression of T_{reg} markers is not related to clinical outcome

We tested whether the expression of any of the markers affected by GAD_{65} stimulation was related to clinical outcome of treatment. We found no significant correlation between expression of T_{reg} markers used in this study and changes in stimulated C-peptide measured as ΔAUC or

Table 1. Characteristics of expanded cells after freeze/thaw.

In-vitro expansion	T_{reg} $CD25^{hi}CD127^{lo}$ Fold increase (range)	T_{eff} $CD25^-CD127^+$ Fold increase (range)
GAD-alum	896 (706–1389)	117 (75–291)
Placebo	800 (667–1039)	80 (45–180)
Protein expression phenotype	% (range)	% (range)
$CD4^+$ out of total $CD3^+$	98·8 (92·3–99·3)	97·9 (67·6–99·3)
$CD25^{hi}CD127^{lo}$ out of total $CD4^+$	94·5 (88·8–98·5)	5·3 (2·7–32·3)
$FoxP3^+$ out of total $CD4^+$	81·4 (51·7–89·7)	9·6 (4·9–16·2)
CTLA-4^+ out of total $CD4^+$	74·7 (30·7–96·5)	14·4 (3·8–26·4)

Expression of regulatory T cell (T_{reg}) markers was measured by flow cytometry 24 h after thawing. Data are pooled from both placebo and glutamic acid decarboxylase (GAD)-alum-treated type 1 diabetes (T1D) patients. CTLA-4: cytotoxic T lymphocyte antigen-4; FoxP3: forkhead box protein 3; T_{eff}: effector T cells.

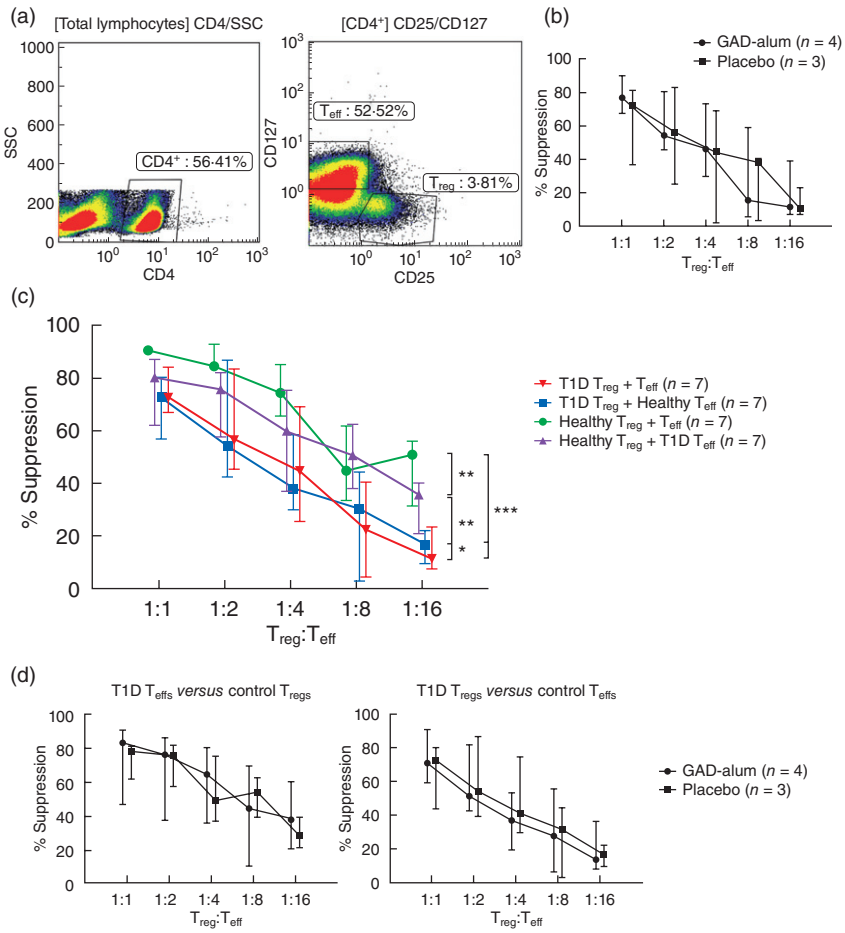

Fig. 3. Dose-dependent regulatory T cell (T_{reg})-mediated suppression of effector T cells (T_{eff}). Proliferation was assessed by [^{3}H]-thymidine incorporation. Percentage of suppression of proliferation in co-cultures of T_{regs} and T_{effs} was calculated by dividing the mean counts per minute (cpm) of co-culture wells by the mean cpm obtained from T_{effs} cultured alone. (a) Left panel illustrates the gate discriminating $CD4^+$ cells and the right panel shows the gates for sorting $CD25^{hi}CD127^{lo}$ (T_{regs}) and $CD25^-CD127^{+/hi}$ cells (T_{effs}). (b) Suppression exerted by T_{regs} from patients treated with placebo ($n = 3$, square) and glutamic acid decarboxylase (GAD)-alum ($n = 4$, circle) is shown as median for each group. (c) Suppression exerted by T_{regs} from type 1 diabetes (T1D) patients on autologous T_{effs} (red downward triangle; $n = 7$), by healthy T_{regs} on autologous T_{effs} (green circle), by T1D T_{regs} in co-culture with healthy T_{effs} (blue square) and by healthy T_{regs} in co-culture with T1D T_{effs} (purple upward triangle). The suppression is shown as percentage of decrease in T_{eff} proliferation in the presence of T_{regs} compared to T_{effs} cultured in the absence of T_{regs}. Healthy represents repeated measurements ($n = 7$) of one healthy reference. (d) Left panel illustrates suppression exerted by healthy T_{regs} in cross-culture with patient T_{effs} and the right panel shows suppression exerted by patient T_{regs} in cross-culture with healthy T_{effs}. Statistical significances are shown as *P*-values: $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$. Data points indicate medians, error bars designate interquartile range.

AUC 4 years after treatment. C-peptide secretion was not significantly different in patients where an $FSC^{hi}SSC^{hi}$ population was induced by GAD_{65} stimulation compared to those who did not respond in this way (data not shown).

Suppression of polyclonally activated T_{effs} is not affected by GAD-alum treatment

To test whether the function of T_{regs} in T1D children included in the Phase II trial was affected by GAD-alum or placebo administration, suppression assays using sorted and expanded T_{regs} ($CD4^+CD25^{hi}CD127^{lo}$) and T_{effs} ($CD4^+CD25^-CD127^+$) were performed. Gates used to sort T_{regs} and T_{effs} are illustrated in Fig. 3a. Expanded T_{regs} from patients treated with GAD-alum suppressed proliferation of autologous T_{effs} to the same extent as T_{regs} from placebo patients (Fig. 3b). T_{regs} from both groups of patients displayed dose-dependent suppression of proliferation. As reported previously [25,27,28], we further found that suppression in autologous cultures of T_{regs} and T_{effs} was reduced in all patients ($n = 7$, placebo and GAD-alum combined) compared to a healthy control (seven repeated measurements, Fig. 3c, $P < 0{\cdot}0001$). To determine whether this attenuated suppression was intrinsic to T_{regs} or T_{effs}, we tested the suppression of T_{regs} from T1D patients (either GAD-alum- or placebo-treated, with similar results; Fig. 3b,d), and from a

healthy control in autologous and cross-over culture suppression assays. As shown in Fig. 3c, T1D T_{regs} exerted the same level of suppression on T_{effs} coming from either T1D or healthy subjects. In the reverse experiment, healthy T_{regs} were able to suppress T_{effs} from healthy or T1D subjects to a similar degree. Taken together, these results suggest that attenuated suppression from T_{regs} of T1D patients is due to reduced T_{reg} efficacy rather than to increased T_{eff} resistance to suppression.

To determine whether there was a difference in reduced T_{reg}-mediated suppression due to treatment, we tested if the suppression exerted in cross-over cultures of T1D T_{regs} *versus* healthy T_{effs} and healthy T_{regs} *versus* T1D T_{effs} was different between treatment arms. There was no difference in suppression exerted by T_{regs} from GAD-alum-treated patients compared to placebo T_{regs} in cross-over cultures with healthy T_{effs}, nor in the suppression exerted by healthy T_{regs} cultured with T_{effs} from GAD-alum-treated patients and T_{effs} from placebo subjects (Fig. 3d).

Discussion

Previous findings from the Phase II GAD-alum trial indicated an ability of GAD-alum to up-regulate CD25 and FoxP3 following *in-vitro* stimulation with GAD_{65}, in addition to increased secretion of several pro- and anti-inflammatory cytokines from PBMC [9,10]. Preservation of C-peptide secretion was still present in a 4-year follow-up to the Phase II trial [11], and induction of a T cell subset with memory phenotype was observed upon GAD_{65} stimulation [12].

Here we demonstrate that a great majority of lymphocytes in this T cell subset with memory phenotype expressed FoxP3 and high levels of CD25. Although some differences in the experimental setup were introduced in the present study, the main difference being that PBMC were cultured for 72 h at 21 and 30 months and for 7 days at the 4-year follow-up, the increased frequencies of $CD25^{hi}$ and $FoxP3^{+}$ cells detected in this 4-year follow-up of the study are in agreement with our previous findings at 21 and 30 months after treatment [9]. In the present study, the CD127 and CD39 markers were included to further define T_{regs}. Both $CD4^{+}CD25^{hi}CD127^{lo}$ and $CD4^{+}CD25^{+}CD127^{+}$ cells were expanded by GAD_{65} stimulation, but a higher proportion of $FSC^{hi}SSC^{hi}$ $CD4^{+}$ cells were $CD127^{+}$ than $CD127^{lo/-}$, suggesting that the frequency of T cells with both T_{reg} and activated-non-T_{reg} phenotype increased following GAD_{65} stimulation.

Expression of CD39, an ectonucleotidase expressed on a subset of T_{regs} which hydrolyzes ATP into adenosine monophosphate (AMP) [23,29], was also increased upon antigen recall in GAD-alum-treated patients. It has been postulated that removal of proinflammatory ATP could be a suppressive mechanism mediated by CD39 on T_{regs}. In a recent study, $CD39^{+}$ but not $CD39^{-}CD4^{+}CD25^{hi}$ cells were able to suppress IL-17 production [30]. As the levels of IL-17 were undetectable in the supernatants of both expanded T_{effs} and T_{eff}/T_{reg} cultures, we cannot draw any conclusion on the ability of T_{regs} to suppress production of this cytokine in our settings. However, we have shown previously that secretion of IL-17, along with that of several other cytokines, was increased by GAD_{65} stimulation in PBMC supernatants [12]. Although the current study does not include healthy subjects, the expression of CD39 on resting $CD4^{+}CD25^{hi}CD127^{lo}$ cells detected by us in these T1D patients seems to be lower than what has been reported in healthy individuals by others using the same anti-CD39 clone and fluorochrome [30].

In line with previous findings [31], expanded $CD25^{+}CD127^{lo}$ T_{regs} were suppressive and retained their phenotype after expansion and cryopreservation. Although we were able to sort, expand and assess suppression in a limited number of individuals, there was no readily evident difference in the suppressive capacity of T_{regs} between placebo and GAD-alum-treated patients 4 years after administration of the treatment. Cross-over culture experiments revealed that T_{regs} isolated from patients with T1D participating in the GAD-alum trial had an impaired suppressive effect on autologous T_{effs} and also on T_{effs} from a healthy individual. In addition, T_{regs} from T1D patients were not capable of suppressing the proliferation of T_{effs} more readily from the healthy control than they did with their own T_{effs}. Further, T_{effs} from T1D patients were suppressed to a greater extent by T_{regs} from the healthy control than by their own T_{regs}. Taken together, these findings suggest that the reduced regulation observed in autologous co-cultures of cells isolated from T1D patients was due to reduced T_{reg}-mediated suppression intrinsic to the T_{reg} population. Our results are in contrast with previous findings, showing that responder T cells from T1D were more resistant to suppression [25,26]. This could be explained by differences in the definition of cellular phenotypes and expansion conditions. While Schneider *et al.* used adaptive T_{regs} generated *in vitro* from $CD4^{+}CD25^{-}$ cells [25], the T_{regs} used by us in this study were expanded from the $CD4^{+}CD25^{hi}CD127^{lo}$ population. In the study by Lawson *et al.*, sorted $CD4^{+}CD25^{hi}$ cells without *in-vitro* expansion from patients with long-standing T1D were used, and CD127 was not included to discriminate T_{regs} [26]. Although we have identified a deficient T_{reg}-mediated suppression of polyclonal T cell stimulation in T1D patients who participated in the GAD-alum Phase II trial, treatment with GAD-alum did not affect the suppressive activity of T_{regs}. It should be kept in mind that samples included in the current study were drawn 4 years after treatment, and that an effect on suppression shortly after treatment cannot be excluded. Furthermore, due to the random selection of patients based on the availability of samples, none of the GAD-alum-treated patients classified as responders to treatment were included in suppression assays [10], and we were thus unable to relate suppression

to clinical outcome. Because our assay measures suppression of polyclonal activation, an effect on the specific suppression in response to GAD_{65} stimulation cannot be excluded. In fact, changes in the frequency of T cells with a T_{reg} phenotype during the trial have been observed only upon GAD_{65} stimulation [9], while the frequency of T_{regs} after culture in medium alone has been similar in GAD-alum and placebo-treated patients throughout the study. Proliferative responses of PBMC from GAD-alum-treated patients in response to GAD_{65} stimulation were significantly stronger compared to placebo in a thymidine incorporation assay, as we have reported previously [12], suggesting that the GAD_{65}-specific responses initiated by *in-vitro* antigen recall are not anergic.

In conclusion, we demonstrate GAD_{65} recall-induced populations of $CD4^+CD25^{hi}CD127^{lo}$ T_{regs} as well as $FSC^{hi}SSC^{hi}CD4^+CD25^+CD127^+$ activated T cells, detectable 4 years after treatment. A deficiency in T_{reg}-mediated suppression detected in T1D patients was intrinsic to the T_{reg} population, but was not affected by GAD-alum treatment. Overall, these results suggest that higher numbers of T_{reg} cells readily expandable upon GAD_{65} stimulation, rather than increased T_{reg} function, may be responsible for the persistent clinical benefit of GAD-alum treatment after 4 years [11].

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Disclosure

The authors declare that they have no conflicts of interest.

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