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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}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 (16/25 vs. 1/25 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.

Introduction

Type 1 diabetes (T1D) is a consequence of an autoimmune reaction toward 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 previously shown 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 $CD4^+CD25^{hi}FOXP3^+$ cells in response to GAD_{65} stimulation *in vitro* and had a predominant T helper (Th) 2 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 phase II and III clinical trials of GAD-alum have 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 physiologically maintained by regulatory T cells (Treg) in the periphery [15], and defects in Treg function have been hypothesized to be involved in the pathogenesis of autoimmune disease [16]. Since tolerance in the periphery is maintained by Tregs, induction of active tolerance has long been a proposed mechanism of action of antigen-based therapies like GAD-alum treatment [17]. Tregs 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 Treg function through the removal of ATP and has thus been used to identify subsets of Tregs [23]. Tregs 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 recently been reported that rather than there being a deficiency in Treg numbers, effector T cells (Teff) from patients with T1D are resistant to Treg-mediated suppression [25-26].

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

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 <18 years old 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 less than 18 months of disease duration were recruited at 8 Swedish pediatric centers. 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), 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), Alexa 700- and Pacific Blue-conjugated anti-CD4 (clone RPA-T4), APC-Cy7-conjugated anti-CD25 (clone M-A251; BD Pharmingen), 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) density gradient centrifugation within 24 h after drawing. Three of the GAD-alum treated patients were classified as responders, 14 as intermediate responders and 7 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) with β-mercaptoethanol at 37 °C, 5% CO_2 for 7 days, with or without 5 µg/ml recombinant GAD_{65} (Diamyd Medical). One million cells were washed in 2ml Phosphate Buffered Saline (PBS) containing 0.1% bovine serum albumin (BSA; Sigma-Aldrich) 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 (BD FACSAria) and analyzed using Kaluza software version 1.1 (Beckman Coulter). The FOXP3^{+} gate was set using the negative population, since the negative population had a higher 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 Treg and Teff subsets based on $CD4^+CD25^{hi}CD127^{lo}$ and $CD4^+CD25^-CD127^+$ phenotype, respectively. After sorting, cells were pelleted by centrifugation at 400g for 10 minutes, 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 Teff contaminant in sorted Tregs was 0.1%. PBMC from one single freshly drawn healthy donor were stained, sorted as above and stored frozen to serve as inter-assay control.

Teff and Treg expansion

Tregs were distributed at $4x10^4$ cells per well in 125μl AIM-V 10% HS into 96-well U-bottom plates, and stimulated with anti-CD3/CD28 Dynabeads (Invitrogen) at a 1:1 bead-to-cell ratio. Teffs were plated at $5x10^5$ cells per 500μl medium, into 96-well flat-bottom plates pre-coated over night with 10μg/ml anti-CD3 (OKT3, eBioscience) at 4°C. Cultures also contained 1μg/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) were added to Teff and Treg cultures respectively. Tregs were washed and supplemented with fresh IL-2 every two days. Tregs and Teffs were re-stimulated as above on the ninth day of culture, and frozen down after 15 days of expansion. To verify post-

expansion phenotype, cryopreserved Tregs and Teffs were cultured for 24h in AIM-V 10% HS and 5U/ml IL-2, and subsequently stained and acquired as described above.

Suppression assay

Treg mediated suppression was assessed using expanded Tregs and Teffs from placebo- (n=3) and GAD-alum (n=4) treated patients. Expanded Tregs and Teffs were thawed and incubated in AIM-V 10% HS at 37°C, 5% CO_2 over night, then re-suspended at 0.5×10^5 cells/ml. Teffs were plated into 96-well U-bottom plates at a density of 5×10^4 cells per well, while Tregs were plated into Teff-containing wells at Treg-to-Teff ratios of 1:1, 1:2, 1:4, 1:8 and 1:16. Treg/Teff cultures were stimulated with 5μg/ml soluble anti-CD3 and 1 μg/ml soluble anti-CD28 antibodies. Unstimulated wells were included as negative controls; both from patients and inter-assay control healthy Teffs. 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) for 18h 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^{TM} C-peptide kit, Wallac), 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 Mann-Whitney two-tailed U test for unpaired observations, since data was determined to be significantly different from a Gaussian distribution. Wilcoxon 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.).

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 previously demonstrated 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 analyzed CD25^{hi}CD127^{lo} cells and used FOXP3 and CD39 as additional markers to better discriminate Tregs from activated T cells. Thus, the expression of CD25, CD127, FOXP3 and CD39 on CD4^{+} lymphocytes was analyzed 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 Figure 1A-B.

The frequency of CD25^{hi}CD127^{lo} cells in the CD4^{+} population was significantly increased 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 (Figure 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; Figure 1D). In contrast, GAD$_{65}$ stimulation did not induce expression of CD25^{hi}CD127^{lo} or CD25^{+}CD127^{+} compared to resting cells in the placebo group (Figure 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} (Figure 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 $FSC^{hi}SSC^{hi}$ cells, consisting mainly of $CD4^+$ memory T cells, as we have previously reported [12] . These $FSC^{hi}SSC^{hi}$ cells are illustrated in Figure 1A-B and are characterized by high CD4 expression (Figure 1E). The $FSC^{hi}SSC^{hi}$ population was observed in 16/24 GAD-alum patients and in 1/25 placebo individuals. In line with GAD_{65} recall response induced in GAD-immunized individuals, GAD_{65} stimulation induced higher CD4 MFI (Figure 1E) and higher percentages of $FSC^{hi}SSC^{hi}$ cells (Figure 1F) among $CD4^+$ cells from GAD-alum patients compared to the placebo group. Next, we analyzed expression of Treg 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 $FOXP3^+$ (Figure 1G).

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

FOXP3 expression on $CD4^+$ and $CD4^+FSC^{hi}SSC^{hi}$ cells was significantly enhanced by GAD_{65} stimulation in the GAD-alum group (Figure 2A-C), while GAD_{65} stimulation did not induce any change compared to resting cells in the placebo group (Figure 2C). To further define whether the increased $CD25^+CD127^{lo}$ population in GAD_{65} stimulated PBMC from GAD-alum treated patients corresponded to a Treg population, CD39 and FOXP3 were added as additional Treg 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 (Figure 2D). Thus, *in vitro* GAD recall leads to expansion of both Tregs and activated $CD25^+CD127^+$ T effector cells, which is only observed in patients previously treated with GAD-alum. There were no significant differences in expression of any measured marker on resting cells between the two treatment arms (Figure 1-2).

The post-expansion phenotype of Tregs and Teffs remains lineage-specific.

Tregs (CD4$^+$CD25^{hi}CD127^{lo}) from GAD-alum treated patients expanded approximately 900-fold, to a similar extent as Tregs from placebo treated patients (800-fold; Table 1). Teffs (CD4$^+$CD25$^-$CD127$^+$) from both GAD-alum and placebo treated patients expanded approximately 100-fold. To verify the phenotype of sorted and expanded Tregs and Teffs after cryopreservation, we analyzed the expression of Treg markers on thawed cells by flow cytometry. Tregs maintained predominant expression of CD25, FOXP3, 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 Teff, but the majority expressed CD127 and expressed CD39 in the same proportion as Tregs. Measurement of cell viability by 7-AAD staining 24h after thawing demonstrated that Teffs had a viability of 90% whereas 70% of Tregs were viable (data not shown).

Expression of Treg 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 Treg markers used in this study and changes in stimulated C-peptide measured as ΔAUC or 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 Teffs is not affected by GAD-alum treatment.

To test whether the function of Tregs in T1D children included in the Phase II trial was affected by GAD-alum or placebo administration, suppression assays using sorted and expanded Tregs (CD4$^+$CD25^{hi}CD127^{lo}) and Teffs (CD4$^+$CD25$^-$CD127$^+$) were performed. Gates used to sort Tregs and Teffs are illustrated in Figure 3A. Expanded Tregs from patients treated with GAD-alum suppressed proliferation of autologous Teffs to the same extent as Tregs from placebo patients (Figure 3B). Tregs

from both groups of patients displayed dose-dependent suppression of proliferation. As previously reported [25, 27-28], we further found that suppression in autologous cultures of Tregs and Teffs was reduced in all patients (n=7, placebo and GAD-alum combined) compared to a healthy control (7 repeated measurements, Figure 3C, p<0.0001). To determine whether this attenuated suppression was intrinsic to Tregs or Teffs, we tested the suppression of Tregs from T1D patients (either GAD-alum or placebo treated, with similar results; Figure 3B & D), and from a healthy control in autologous and cross-over culture suppression assays. As shown in Figure 3C, T1D Tregs exerted the same level of suppression on Teffs coming from either T1D or healthy subjects. In the reverse experiment, healthy Tregs were able to suppress Teffs from healthy or T1D subjects to a similar degree. Taken together, these results suggest that attenuated suppression from Tregs of T1D patients is due to reduced Treg efficacy rather than to increased Teff resistance to suppression.

To determine whether there was a difference in reduced Treg-mediated suppression due to treatment, we tested if the suppression exerted in cross-over cultures of T1D Tregs vs healthy Teffs and healthy Tregs vs T1D Teffs was different between treatment arms. There was no difference in suppression exerted by Tregs from GAD-alum treated patients compared to placebo Tregs in cross-over cultures with healthy Teffs, nor in the suppression exerted by healthy Tregs cultured with Teffs from GAD-alum treated patients and Teffs from placebo subjects (Figure 3D).

Discussion

Previous findings from the phase II GAD-alum trial indicated an ability of GAD-alum to upregulate 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 years 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 Tregs. 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 Treg and activated-non-Treg phenotype increased following GAD$_{65}$ stimulation.

Expression of CD39, an ectonucleotidase expressed on a subset of Tregs which hydrolyzes ATP into 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 Tregs. In a recent study, CD39$^+$ but not CD39$^-$ CD4$^+$CD25^{hi} cells were able to suppress IL-17 production [30]. Since the levels of IL-17 were undetectable in the supernatants of both expanded Teffs and Teff/Treg cultures, we cannot make any conclusion on the ability of Tregs to suppress production of this cytokine in our settings. However we have previously shown that secretion of IL-17, along with that of several other cytokines, was increased by GAD$_{65}$ stimulation in PBMC supernatants [12]. Though 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} Tregs were suppressive and retained their phenotype after expansion and cryopreservation. Though 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 Tregs between placebo and GAD-alum treated patients 4 years after

administration of the treatment. Cross-over culture experiments revealed that Tregs isolated from patients with T1D participating in the GAD-alum trial had an impaired suppressive effect on autologous Teffs and also on Teffs from a healthy individual. In addition, Tregs from T1D patients were not capable of more readily suppressing the proliferation of Teffs from the healthy control than what they did with their own Teffs. Further, Teffs from T1D patients were suppressed to a greater extent by Tregs from the healthy control than by their own Tregs. Taken together, these findings suggests that the reduced regulation observed in autologous co-cultures of cells isolated from T1D patients was due to reduced Treg-mediated suppression intrinsic to the Treg 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 definition of cellular phenotypes and expansion conditions. While Schneider *et al* used adaptive Tregs generated *in vitro* from CD4$^+$CD25$^-$ cells [25], the Tregs 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 Tregs [26]. Though we have identified a deficient Treg-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 Tregs. It should be kept in mind that samples included in the current study have been drawn four 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 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. Since 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 Treg phenotype during the trial have only been observed upon GAD$_{65}$ stimulation [9], while the frequency of Tregs 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 previously reported [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 $CD4^+CD25^{hi}CD127^{lo}$ Tregs as well as $FSC^{hi}SSC^{hi}CD4^+CD25^+CD127^+$ activated T cells, detectable 4 years after treatment. A deficiency in Treg-mediated suppression detected in T1D patients was intrinsic to the Treg population, but was not affected by GAD-alum treatment. Overall, these results suggest that higher numbers of Treg cells readily expandable upon GAD_{65} stimulation rather than increased Treg function may be responsible for the persistent clinical benefit of GAD-alum treatment after 4 years [11].

Acknowledgements

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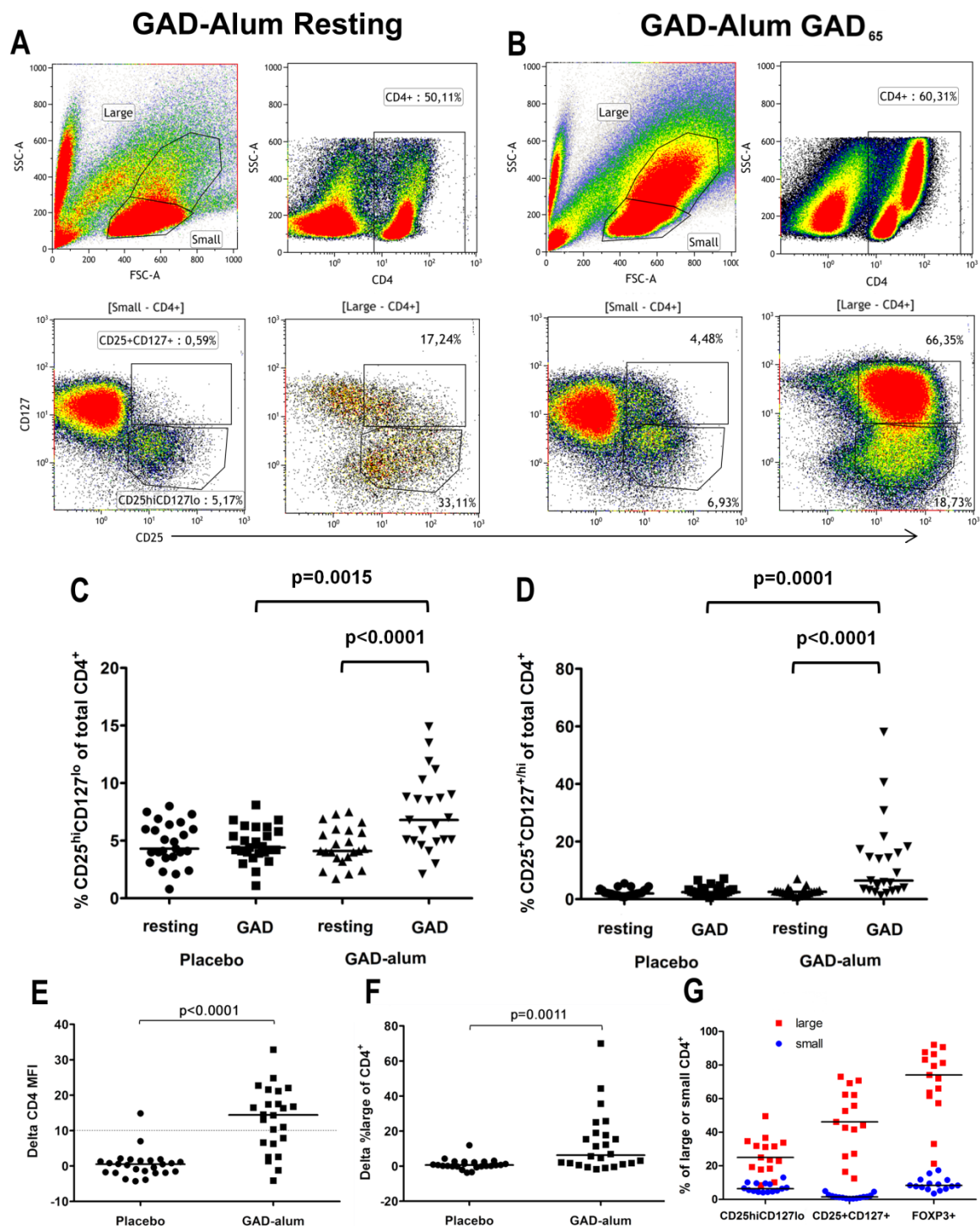
Figure 1

Gating strategy and GAD$_{65}$-induced T cell populations.

A and B illustrates gating strategy and representative 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 FSC^{hi}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 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.
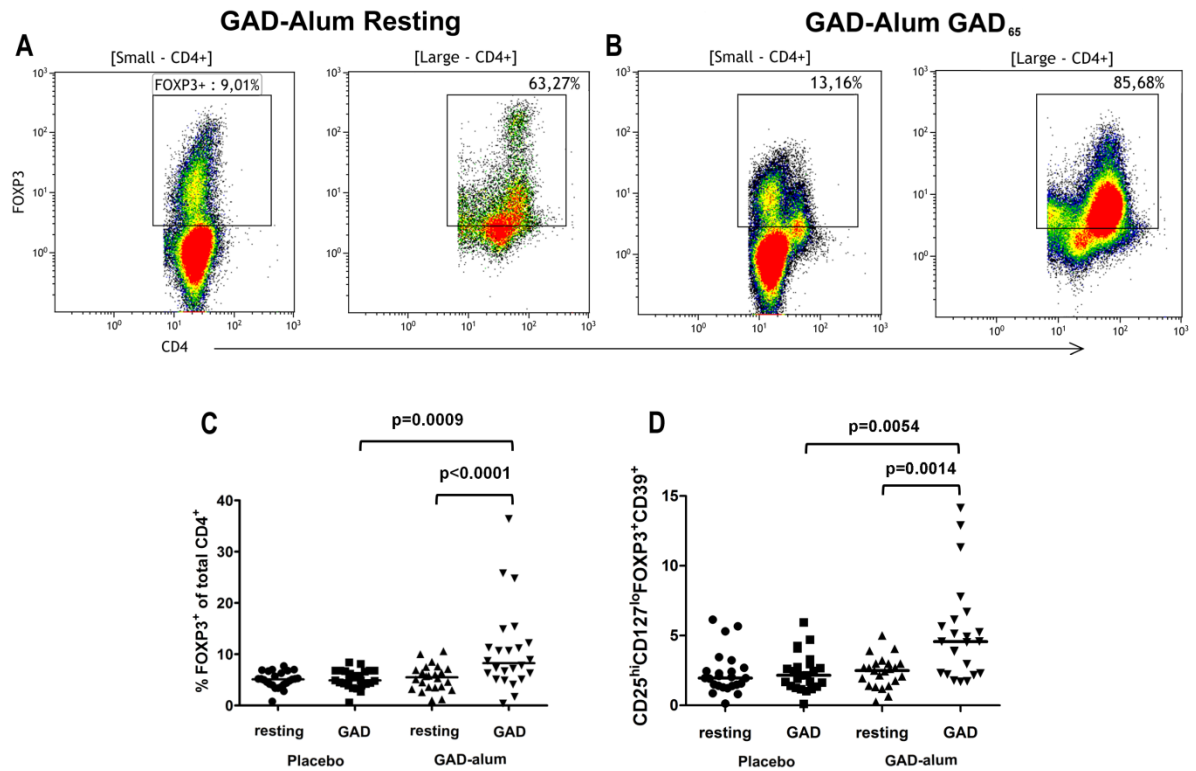
Figure 2

Expression of Treg-associated markers.

A illustrates the expression of FOXP3 on CD4$^+$ resting cells from a representative GAD-alum treated subject, and B the 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) and 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.
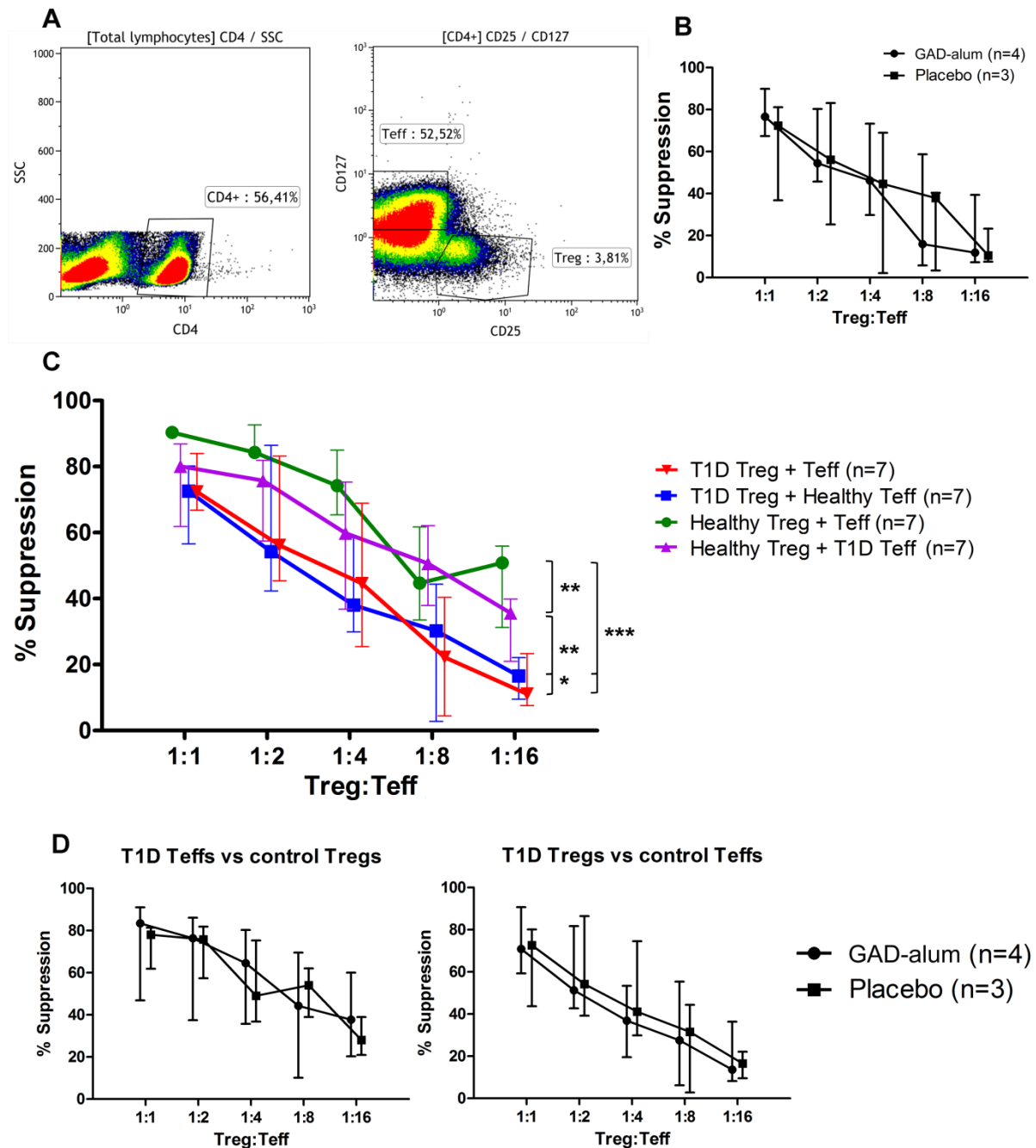
Figure 3

Dose-dependent Treg-mediated suppression of Teff.

Proliferation was assessed by ^{3}H thymidine incorporation. Percentage of suppression of proliferation in co-cultures of Tregs and Teffs was calculated by dividing the mean cpm of co-culture wells by the mean cpm obtained from Teffs cultured alone. A: Left panel illustrates the gate discriminating CD4$^+$ cells and the right panel shows the gates for sorting CD25^{hi}CD127^{lo} (Tregs) and CD25$^-$CD127$^{+/hi}$ cells

(Teffs). B: Suppression exerted by Tregs from patients treated with placebo (n=3, square) and GAD-alum (n=4, circle) is shown as median for each group. C: Suppression exerted by Tregs from T1D patients on autologous Teffs (red downward triangle; n=7), by healthy Tregs on autologous Teffs (green circle), by T1D Tregs in co-culture with healthy Teffs (blue square) and by healthy Tregs in co-culture with T1D Teffs (purple upward triangle). The suppression is shown as percentage of decrease in Teff proliferation in presence of Tregs compared to Teffs cultured in absence of Tregs. Healthy represents repeated measurements (n=7) of one healthy reference. D: Left panel illustrates suppression exerted by healthy Tregs in cross-culture with patient Teffs and the right panel shows suppression exerted by patient Tregs in cross-culture with healthy Teffs. Statistical significances are shown as p-values, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data points indicate medians, error bars designate interquartile range.

Tables

Table 1

Characteristics of expanded cells after freeze/thaw.

	Treg CD25hiCD127lo	Teff CD25-CD127+
In vitro expansion	Fold increase (range)	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)
CD25hiCD127lo 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 Treg markers was measured by flow cytometry 24h after thawing. Data is pooled from

both placebo and GAD-alum treated T1D patients.