

## The clinical and immunological significance of GAD-specific autoantibody and T-cell responses in type 1 diabetes



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### ABSTRACT

Antigen-specific interventions are desirable approaches in Type 1 Diabetes (T1D) as they can alter islet-specific autoimmunity without systemic side effects. Glutamic acid decarboxylase of 65 kDa (GAD65) is a major autoantigen in type 1 diabetes (T1D) and GAD-specific autoimmunity is a common feature of T1D in humans but also in mouse models of the disease. In humans, administration of the GAD65 protein in an alum formulation has been shown to reduce C-peptide decline in recently diagnosed patients, however, these observations were not confirmed in subsequent phase II/III clinical trials. As GAD-based immune interventions in different formulations have successfully been employed to prevent the establishment of T1D in mouse models of T1D, we sought to analyze the efficacy of GAD-alum treatment and the effects on the GAD-specific immune response in two different mouse models of T1D. Consistent with the latest clinical trials, mice treated with GAD-alum were not protected from diabetes, although GAD-alum induced a GAD-specific Th2-deviated immune response in transgenic rat insulin promoter-glycoprotein (RIP-GP) mice. These observations underline the importance of a thorough, preclinical evaluation of potential drugs before the initiation of clinical trials.

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### 1. Introduction

Type 1 diabetes (T1D) is an autoimmune disease leading to destruction of the insulin producing pancreatic beta ( $\beta$ )-cells, permanent dysregulation of blood glucose homeostasis, a lifetime of dependence on insulin injections or infusions, and often inevitable and severe microvascular complications [1,2]. It is without question that a cure is needed for T1D, but past and current efforts in this direction have been met by many challenges. In attempts to preserve  $\beta$ -cell function in patients with recent-onset diabetes, intervention trials have included antigen-specific therapies and systemic immunosuppressants as well as anti-inflammatory drugs [3–10].

Many of these trials suffered from lack of efficacy, and those that initially showed some promise resulted in only transient preservation of  $\beta$ -cell or serious adverse effects due to immunosuppression [11]. However,  $\beta$ -cell antigen-specific therapies remain particularly appealing as they have the potential to restore immunological tolerance to  $\beta$ -cell antigens without systemic immunosuppression.

Glutamic acid decarboxylase of 65 kDa (GAD65) is one of the major autoantigens in human T1D. GAD is found in the central nervous system where it catalyzes the conversion of the neurotransmitter glutamate to GABA. It is also found in pancreatic  $\beta$ -cells where its functional role remains largely unknown [12–14]. The emergence of GAD-specific autoantibodies occurs prior to  $\beta$ -cell destruction in most cases [15,16], and while GAD autoantibodies have not been causally linked to the T-cell mediated autoimmune attack, they serve as a clear predictor and marker of the disease process [17,18]. Previous preclinical studies have shown that GAD vaccines have the potential to halt the progression of diabetes in the NOD mouse models of T1D [19,20]. However, Phase II/III clinical trials in recent-onset T1D patients treated with GAD-protein in an alum formulation have shown discordant results. A 70-patient human trial conducted in Sweden, showed that treatment with two doses of 20  $\mu$ g alum-formulated GAD induces tolerance to GAD resulting in preservation of  $\beta$ -cell insulin

**Abbreviations:** GAD65, Glutamic acid decarboxylase of 65 kDa; T1D, type 1 diabetes; NOD, non-obese diabetic; LCMV, lymphocytic choriomeningitis virus; RIP-GP, rat insulin promoter-glycoprotein; TNF, tumor necrosis factor; IFN- $\gamma$ , interferon- $\gamma$ ; PBMC, peripheral blood mononuclear cells; IL, interleukin; Th, T-helper; PFU, plaque-forming units; GABA,  $\gamma$ -aminobutyric acid.

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secretion in a sub-group of 11 patients with recent-onset (<6 months) T1D [21]. Unfortunately, these effects could not be reproduced by subsequent clinical trials with the same drug [22,23]. The reasons for these discrepancies are not entirely understood but may lie in the relatively low number of patients in the initial promising sub-group, or influenza vaccination campaigns concurrent with the enrollment of the patients in the Phase III trial that may have impaired patients' immune responses [22].

As GAD-based interventions have previously shown efficacy in mouse models of T1D but alum-formulated GAD has never been tested in murine disease, we revisited the preclinical realm by testing its efficacy with several doses, frequencies and mouse models of the disease. In the present study, we aimed to elucidate whether prevention of diabetes could be achieved using GAD-alum at several doses and frequencies in two mouse models, the non-obese diabetic (NOD) mouse, which spontaneously develops T1D, and the transgenic RIP-LCMV-GP mouse, in which  $\beta$ -cells express the glycoprotein (GP) from lymphocytic choriomeningitis virus (LCMV) on their surface and the disease is induced upon LCMV infection. We also examined the cytokine and autoantibody profiles using ELISA and phenotype of T-cell subsets through flow cytometry in order to shed light on GAD's mechanism of action.

## 2. Materials and methods

### 2.1. Mice

NOD/ShiLt mice were purchased from Jackson Laboratories and housed in specific-pathogen free conditions at La Jolla Institute for Allergy and Immunology (LIAI). Transgenic rat insulin promoter (RIP)-lymphocytic choriomeningitis virus (LCMV)- glycoprotein (GP) mice, as previously described [24], were bred in-house under the same conditions and transferred to an infectious suite prior to treatments and infection. A single dose of  $1 \times 10^4$  plaque-forming units (PFU) of LCMV Armstrong was injected intraperitoneally in order to induce diabetes (usually by day 8–12 post-infection). In both mouse models, blood glucose values (BGVs) were recorded 1–2 times a week with an AccuCheck Glucometer (LifeScan Inc., Milpitas, CA, USA) and mice were considered diabetic with BGVs above 250 mg/dl. For mechanistic studies, mice were euthanized by CO<sub>2</sub> asphyxiation and cervical dislocation per animal protocol.

### 2.2. Treatments

Stock solutions of 40  $\mu$ g/ml GAD-Alum and placebo containing only alum were provided by Diamyd Medical AB (Sweden) and injected subcutaneously in anesthetized mice.

### 2.3. Enzyme-linked immunosorbent assays (ELISAs)

#### 2.3.1. Cytokines

Detection of interleukin (IL)-5 and tumor necrosis factor (TNF) in culture supernatants were performed with ELISA kits purchased from eBioscience (San Diego, CA) according to the manufacturer's instructions. Other cytokines were detected with Bio-Plex Pro™ Mouse Cytokine 23-plex Assay (Bio-Rad, cat. # M60-009RDPD) according to the manufacturer's instructions.

#### 2.3.2. Autoantibodies

GAD-specific autoantibodies were measured in Maxisorp Nunc 96-well plates by coating with 2  $\mu$ g GAD-Alum or Alum alone as a negative control overnight at +4C. After 3 washes in PBS 0.05% Tween and a 1-h blocking step with PBS 10% fetal bovine serum (FBS) at room temperature (RT), serially diluted serum samples were added and incubated at RT with agitation for 2 h. As capture

antibodies, goat anti-mouse IgG antibodies coupled with horseradish peroxidase (LifeTech, cat. # M30107) were diluted 1/1000 in blocking buffer and incubated with agitation for 1.5 h. Enzymatic reactions were revealed with the addition of 100  $\mu$ L/well of TMB substrate (cat # 421101, Biolegend, San Diego, CA) for 5–10 min. Reactions were stopped with 100  $\mu$ L/well of 1 N HCl. Plates were read with a spectrophotometer measuring absorbance at 450 nm.

### 2.4. Flow cytometry staining for intracellular Foxp3 and cytokines

At 4 weeks post-infection, spleen and pancreatic lymph nodes (pLN) were collected from euthanized mice and processed for further staining. Splenocytes were incubated with 2 mL ACK lysis buffer for 2 min to lyse red blood cells. Thereafter, pLN cells and splenocytes were resuspended at  $10 \times 10^6$  cells/mL in complete RPMI (Gibco) containing 10% FBS, 50  $\mu$ M  $\beta$ -mercaptoethanol, Pen/Strep, Glutamine and Hepes (all from Gibco). Cells were cultured in the presence of Brefeldin A (cat. # B7651, Sigma, St. Louis, USA) and 5  $\mu$ g/mL GP<sub>33–41</sub> peptide from LCMV (Abgent Inc, San Diego, USA) and incubated at 37 °C for 4 h. Cells were then stained in FACS buffer (PBS 0.05% bovine serum albumin) for surface antibodies (CD8, CD4, CD25) and fixed for 30 min at 4 °C using the fixation reagent from Foxp3 detection kit (cat. # 88-8115-40, eBioscience, San Diego, USA). Finally, cells were permeabilized using the same kit and stained for IFN- $\gamma$ , IL-17, TNF (BD Biosciences) as well as Foxp3, CD107a (eBioscience) and granzyme B (Life Technologies). All the antibodies were diluted 1/100. Samples were recorded on an LSRII cytometer (Beckton Dickinson) and analyzed with FlowJo software (Tree Star).

### 2.5. In vitro culture with GAD65 peptide libraries

Total splenocytes were collected, teased out and resuspended at  $10 \times 10^6$  cells/mL in complete RPMI. Cells were then stimulated with 11 overlapping pools of peptides from a human GAD65 peptide library. Amino acid sequences and peptide pools have been previously published [25]. Upon 72-h incubation, supernatants were collected and frozen until use for the detection of cytokines by standard ELISA or Bio-Plex assay. In other experiments, cultured cells were stained with Treg-specific markers (CD4, CD25, CD127, Foxp3) as described above.

### 2.6. Statistics

Data are expressed as mean  $\pm$  standard error of the mean. Differences in the incidence of diabetes were assessed using the Log-rank test. Graphs were plotted and statistics calculated with GraphPad Prism® (versions 4 and 5). Comparisons of GAD-Alum versus placebo groups were performed with two-tailed unpaired *t*-tests. *p*-values <0.05 were considered statistically significant.

### 2.7. Ethics

All the studies were performed in the La Jolla Institute for Allergy and Immunology upon approval of LIAI's Animal Care and Use Committee.

## 3. Results

### 3.1. Preventive treatment with different doses of GAD-alum did not protect NOD mice from T1D

A dose response study was performed in non-obese diabetic (NOD) mice known for spontaneously developing T1D [26]. NOD mice were assigned to receive two subcutaneous injections of

either 1  $\mu\text{g}$ , 5  $\mu\text{g}$  or 20  $\mu\text{g}$  of GAD-alum or placebo at 8 and 10 weeks of age. Mice were subsequently monitored for diabetes onset. We observed that 1  $\mu\text{g}$  GAD-alum (cumulative dose 2  $\mu\text{g}$ ) treated mice presented similar T1D incidence compared to alum treated controls (87% versus 71%,  $p = 0.806$ ) (Fig. 1, left panel). Mice that received 5 times more GAD-alum also had comparable incidence in relation to their respective placebo controls (80% versus 93%,  $p = 0.363$ ) (Fig. 1, middle panel). Finally, there was a slight tendency toward protection from T1D when administering mice 20  $\mu\text{g}$  GAD-alum (73% versus 93% for controls). However, this trend failed to reach statistical significance ( $p = 0.35$ ) (Fig. 1, right panel). Collectively, increasing doses of GAD-alum did not protect NOD mice from T1D.

### 3.2. Preventive treatment with different doses of GAD-alum did not protect transgenic RIP-GP B6 mice from diabetes development

Previous studies have demonstrated that genetic variations could dictate the success or failure of therapies that include GAD65-specific tolerance induction [25]. Indeed, the combination of non-mitogenic anti-CD3 and GAD65 DNA plasmid injections was able to synergistically reverse the course of T1D in recent-onset diabetic RIP-GP mice on the C57Bl/6 (B6) background, but not in NOD mice. Therefore, we investigated the efficacy of various doses of GAD-alum treatments using the transgenic RIP-GP B6 model for diabetes [27]. To this aim, RIP-GP mice were infected with LCMV Armstrong on day 0 and were administered 2  $\mu\text{g}$  or 10  $\mu\text{g}$  GAD-alum or placebo on days 2, 8 and 14 post-infection (arrows, Fig. 2A). Upon 8 weeks of observation, mouse groups that received either dose of GAD-alum presented a moderate but non-significant reduction in glycemia levels compared to their placebo counterparts (Fig. 2A). On a per-individual basis, there was no significant difference in the proportions of protected versus non-protected mice, as illustrated in Fig. 2C by cumulated BGVs at day 28 post-infection, i.e., prior to mechanistic studies performed below.

A major hurdle that needs to be overcome for proper translation of preclinical data from bench to bedside is the issue of dosing. In this context, our laboratory and others have previously shown in mice that successful antigen-specific therapies with oral insulin did not follow a linear, but rather a bell-shaped, trend when increasing doses [28,29], indicating the need for antigen dose titration whenever possible. This prompted us to perform experiments in RIP-GP mice where GAD-alum doses and/or frequencies of administration were significantly decreased (three doses of 0.5  $\mu\text{g}$ ) or increased (9 doses of 20  $\mu\text{g}$ ). Fig. 2B depicts BGVs in groups of 5–6 mice per regimen and although mice that received decreased and increased doses of GAD-alum displayed a trend toward lower BGVs compared to placebo, these trends were not statistically significant

after 4 weeks of observation. Moreover, the proportion of protected individuals (one in each group) was comparable in all treatment groups (not shown).

### 3.3. RIP-GP mice treated with GAD-alum presented high titers of GAD autoantibodies

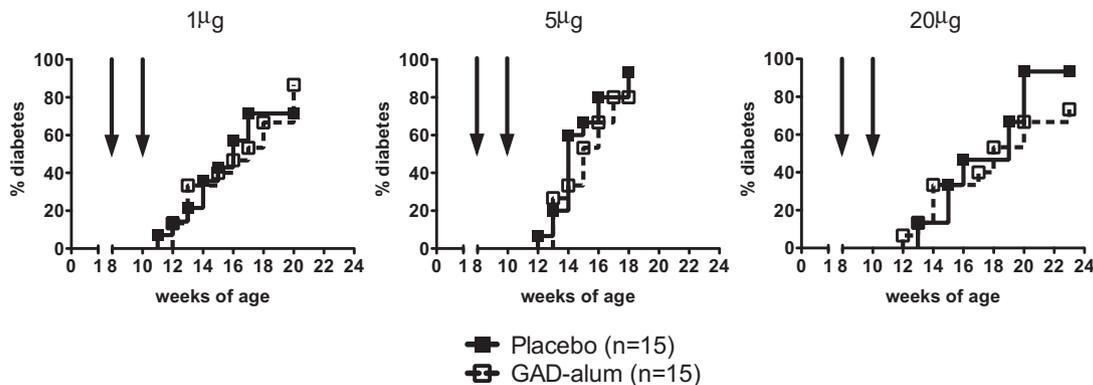
Next, in order to confirm that GAD-alum immunizations induced the production of anti-GAD65 autoantibodies as was shown in human patients [21,23], we performed serum titrations at 4 weeks post-infection in RIP-GP mice that were treated three times with 2  $\mu\text{g}$  GAD-alum or alum alone. Our findings show that GAD-alum treated individuals harbored significantly increased titers of GAD65-specific IgG autoantibodies compared to mice treated with placebo (Fig. 2D), suggesting that our administration regimen effectively led to GAD-specific humoral responses.

### 3.4. pLN from RIP-GP mice treated with GAD-alum have decreased proportions of LCMV-specific CD8 T-cells that produce TNF and interferon- $\gamma$

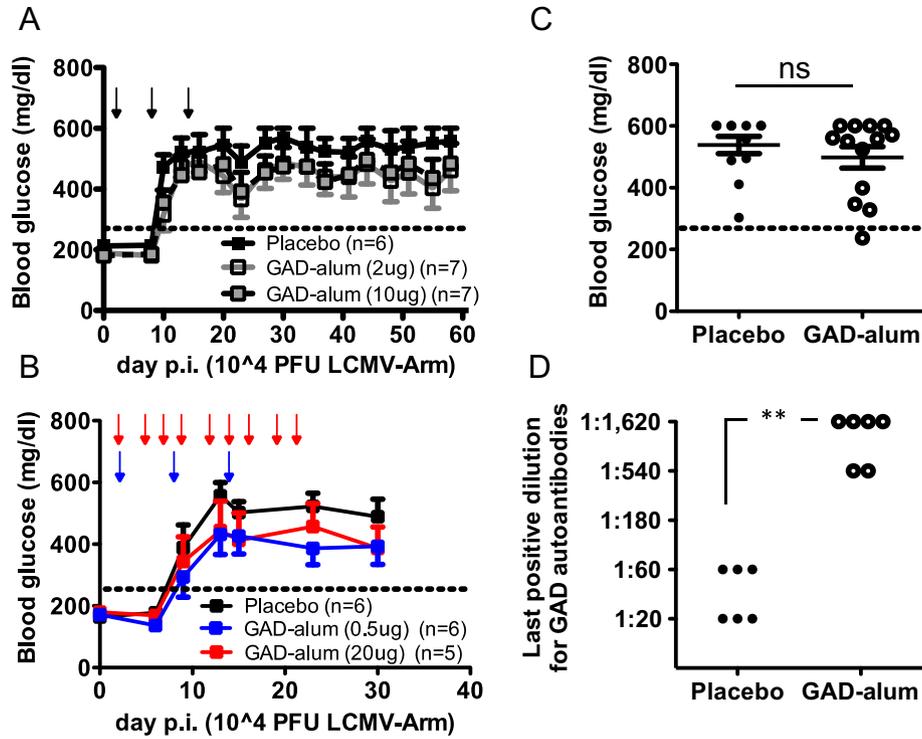
In the RIP-GP model, LCMV-GP<sub>33–41</sub>-specific “autoreactive” CD8 T-cells are responsible for the immune-mediated demise of pancreatic  $\beta$ -cells resulting in diabetes onset [30]. Thus, we analyzed the GP-specific CD8 T-cell responses of treated mice 4 weeks post-infection. Spleen and pancreatic lymph node (pLN) cells were stimulated *in vitro* with GP<sub>33–41</sub> peptide in the presence of Brefeldin A and stained for TNF and IFN- $\gamma$ . Our data indicate that mean LCMV-specific CD8 T-cell TNF (Fig. 3A, left panel) production was significantly reduced in the pLN of mice administered GAD-alum compared to their counterparts (1.17  $\pm$  0.2% versus 4.98  $\pm$  1.73%, respectively;  $p = 0.040$ ). Similarly, IFN- $\gamma$ <sup>+</sup>TNF<sup>+</sup> double-producing CD8 T-cells were found in lower frequencies upon GAD-alum treatment (Fig. 3A, right panel). Interestingly, this decrease appeared to be specific to pLN since both TNF and/or IFN- $\gamma$  levels were found to be similar in the spleen (Fig. 3B and not shown). Nonetheless, after examining the overall cytokine production of CD8 T-cells in pLN, we found no difference between Placebo and GAD-alum treated groups regarding single-producers of IFN- $\gamma$ , IL-17, CD107a (LAMP-1, surrogate marker for cytotoxic T lymphocyte degranulation) and Granzyme B (Fig. 3C).

### 3.5. pLN from RIP-GP mice treated with GAD-alum have increased proportions of Foxp3-expressing CD4<sup>+</sup>CD25<sup>high</sup> T regulatory cells

Furthermore, we examined in parallel the proportions of Foxp3-expressing T regulatory cells (Tregs) as it has been published that



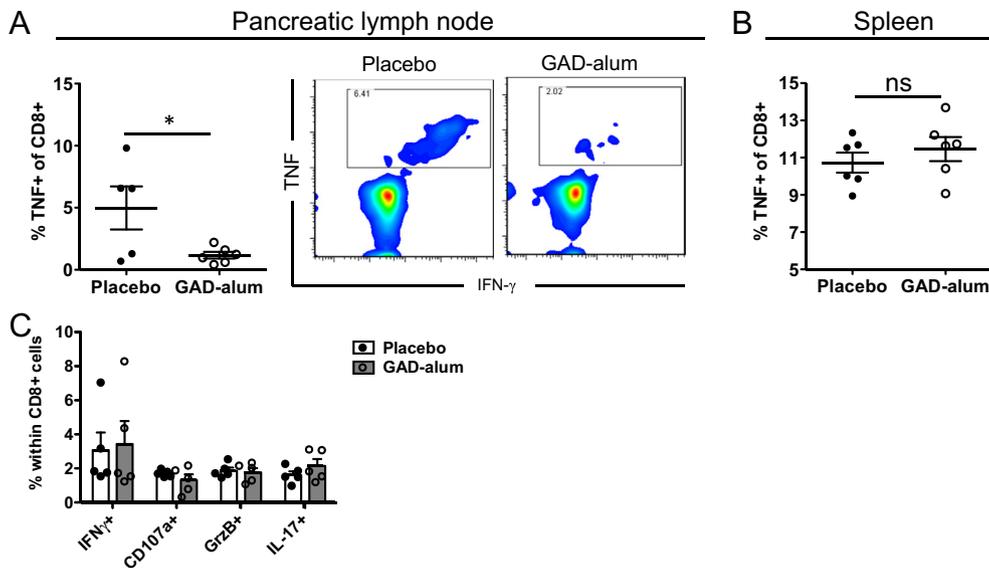
**Fig. 1.** Diabetes incidence in NOD mice treated with GAD-alum. Diabetes incidence (two consecutive BGV readings > 250 mg/dl and eventually full blown diabetes) in NOD mice treated with increasing doses (left panel, 1  $\mu\text{g}$ ; middle panel, 5  $\mu\text{g}$ ; right panel, 20  $\mu\text{g}$ ) of GAD-alum or placebo, namely. Arrows indicate days of treatment.  $N = 15$  for each in treatment group in each experiment.



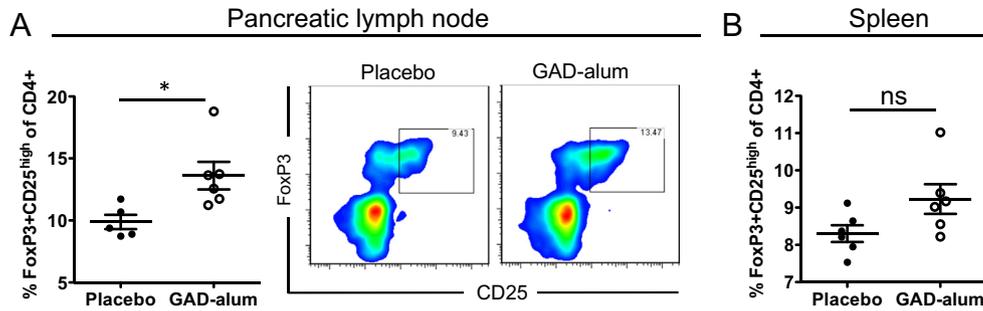
**Fig. 2.** Blood glucose levels and GAD-specific immune responses in RIP-GP mice treated with GAD-alum. (A, B) Blood glucose levels in RIP-GP mice treated with GAD-alum or placebo. Arrows indicate days of treatment, (A) 2 µg or 10 µg of GAD-alum and (B) 0.5 µg or 20 µg GAD-alum formulation and 3 mice received the volume corresponding to 0.5 µg GAD-alum formulation on indicated days. (C) Blood glucose values from placebo and GAD-alum (3 × 2 µg)-treated mice at time point of mechanistic studies, 28 days post-infection. Dashed line represents the threshold of 250 mg/dl used for diagnosis. (D) GAD autoantibodies in placebo and GAD-alum (3 × 2 µg)-treated mice. Shown is the last positive dilution above background. \*\**p* < 0.01.

treatment with GAD-alum could result in enhancement of CD4+CD25<sup>high</sup> Foxp3<sup>+</sup> cell frequencies and GAD65-specific Foxp3 mRNA expression [31,32]. Accordingly, proportions of Tregs were significantly elevated in the pLN of mice treated with GAD-alum compared to their placebo controls (13.62 ± 1.1% versus

9.88 ± 0.6%, respectively; *p* = 0.021) (Fig. 4A). It is noteworthy that Treg increase was again specific to local pLN and not confirmed more systemically in the spleen, although approaching statistical significance (*p* = 0.071) (Fig. 4B). However, we cannot rule out that Treg function was not optimal. In this regard, we examined



**Fig. 3.** Analysis of effector T-cell responses in RIP-GP mice treated with GAD-alum. (A) Percentage of pancreatic lymph node (pLN) derived CD8<sup>+</sup> T-cells producing TNF in response to *in vitro* GP33 stimulation. Two representative FACS plots gated on pLN-derived CD8<sup>+</sup> T-cells. (B) Percentage of spleen derived CD8<sup>+</sup> T-cells producing TNF in response to *in vitro* GP33 stimulation. (C) Percentage of pancreatic lymph node (pLN) derived CD8<sup>+</sup> T-cells producing IFN-γ, CD107a, granzyme B and IL-17 in response to *in vitro* GP33 stimulation. Data are representative of two independent experiments. \**p* < 0.05.



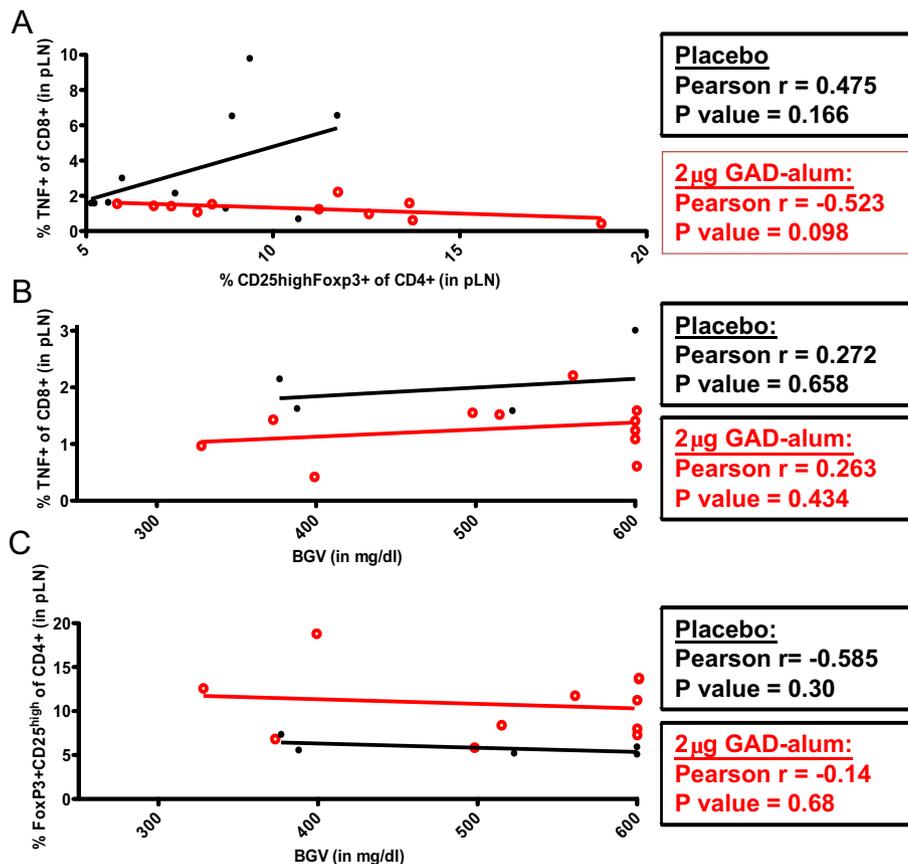
**Fig. 4.** Analysis of regulatory T-cell responses in RIP-GP mice treated with GAD-alum. (A) Percentage of pancreatic lymph node (pLN) derived CD4<sup>+</sup> T-cells expressing FoxP3 and high levels of CD25. Two representative FACS plots gated on pLN-derived CD4<sup>+</sup> T-cells. (B) Percentage of spleen derived CD4<sup>+</sup> T-cells expressing FoxP3 and high levels of CD25. Data are representative of two independent experiments. \**p* < 0.05.

immunomodulatory molecule CTLA-4 and PD-1 expression levels in Tregs presented in Fig. 4A but failed to observe significant differences between both groups on a per-cell basis (data not shown). It is conceivable that this significant elevation in cell frequencies, without any increased suppressive function on a per-cell basis, was not sufficient to tame the ongoing “self-reactive” immune response.

### 3.6. Correlative analyses of effector and regulatory T-cell responses in relation to mouse glycemia

Mechanistic data presented in Figs. 2–4 were analyzed on a per-mouse basis to assess linear correlations between pLN-derived CD8

T-cell and Foxp3-expressing Treg responses in relation to mouse glycemia at euthanasia. Fig. 5A depicts one-on-one correlation between the percentages of Tregs and that of TNF-producing CD8 T-cells in 2 μg GAD-alum treated mice and placebo treated counterparts. In GAD-alum treated animals, there was a trend toward negative correlation (Pearson  $r = -0.523$ ) that however, did not reach statistical significance with 10–11 mice per group ( $p = 0.098$ ). In contrast, in placebo treated mice, there was a trend toward positive correlation (Pearson  $r = +0.475$ ) that was not significant either ( $p = 0.16$ ). No association was found when correlating mouse glycemia to pLN-derived TNF-producing CD8 T-cells (Fig. 5B) or Foxp3-expressing Tregs (Fig. 5C). We conclude that



**Fig. 5.** Correlative analyses of effector and regulatory T-cell responses in pLN in relation to mouse glycemia. (A) Correlation between the percentages of pLN-derived, GP33-specific CD8<sup>+</sup> T-cells producing TNF and CD4<sup>+</sup>CD25<sup>high</sup> Foxp3<sup>+</sup> Treg cells. (B) Correlation between individual mouse BGV and percentage of pLN-derived, GP33-specific CD8<sup>+</sup> T-cells producing TNF. (C) Correlation between individual mouse BGV and percentage of pLN-derived, CD4<sup>+</sup>CD25<sup>high</sup> Foxp3<sup>+</sup> Treg cells. Note that using Pearson correlation test, *r* values between 0.3 and 0.7 (0.3 and -0.7) indicate a moderate positive (negative) linear relationship.

on a per-mouse basis, the effector and regulatory T-cell parameters analyzed in the pLN of GAD-alum and placebo treated mice presented a moderate linear relationship.

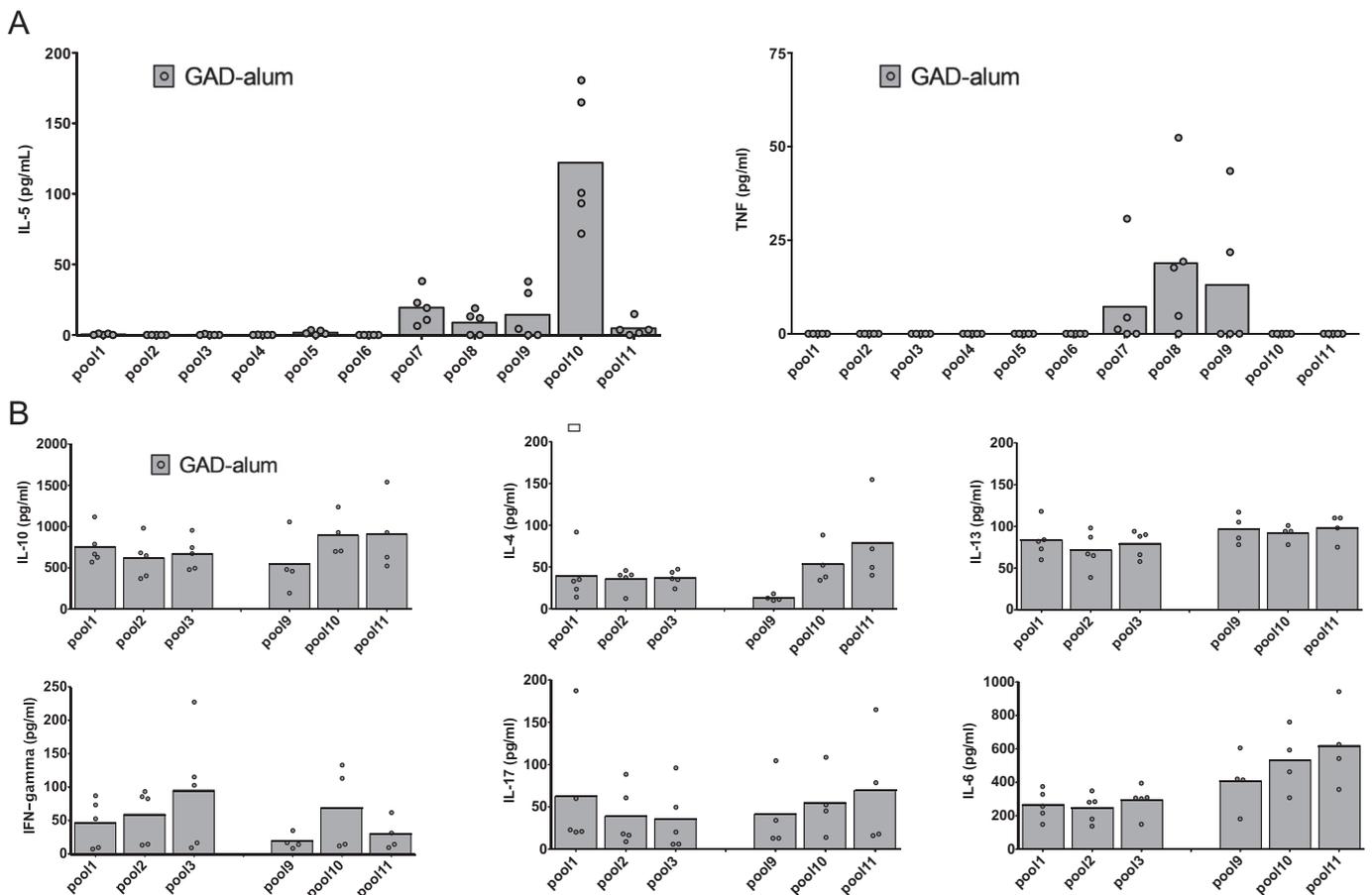
### 3.7. C57Bl/6 mice treated with GAD-alum presented GAD65-specific Th2-deviated immune responses

Administration of GAD-alum in recent-onset diabetic patients has been shown to induce the production of cytokines including TNF, IL-10 as well as IL-5 and IL-13 [21,32]. The latter cytokines are associated with Th2-biased responses, which in animal models of diabetes have been shown to be protective [33–36]. Here, we isolated splenocytes from GAD-alum treated mice, stimulated cells *in vitro* with a human (h)GAD65 peptide library covering the entire hGAD65 sequence [25] and assessed cytokine production by ELISA upon 3-day culture. We found that IL-5 and TNF production was elevated in response to peptide pools spanning the C-terminal region of GAD65 (pools 7–10) (Fig. 6A). Interestingly, stimulation with pool 10 from the hGAD65 library led to the highest IL-5, but no TNF, production. Additionally, we used the same supernatants to perform a Bio-Plex assay on samples stimulated with GAD65 peptide pools 1–3 (C-terminal) and pools 9–11 (N-terminal). We first confirmed the increased IL-5 production in samples specifically stimulated with pool 10 (data not shown). However, the production of immunomodulatory cytokine IL-10 was similarly elevated in all samples whereas IL-4 production was somewhat higher in pools 10 and 11 without this being significant (Fig. 6B, upper panels). No

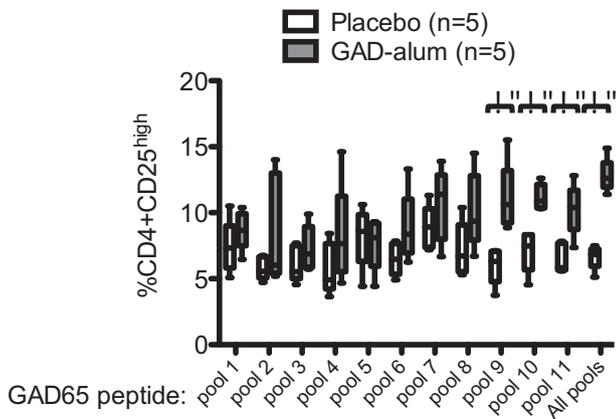
significant difference was found across all samples in terms of proinflammatory cytokine IFN- $\gamma$ , IL-17 and IL-6 production (Fig. 6B, lower panels). Overall, these data confirm that the TCR repertoire of GAD65-specific CD4 T-cells is shifted toward the C-terminal domain of the protein Ref. [25] and further argue for the existence of non-overlapping GAD65-specific CD4 Th2-biased responses.

### 3.8. GAD65-specific Foxp3-expressing CD4 + CD25<sup>high</sup> Treg responses in RIP-GP mice treated with GAD-alum

Despite an increase in the proportions of Foxp3-expressing Tregs in the pLN of GAD-alum treated animals (Fig. 4), mice were not protected and we cannot exclude that the underlying reason is the absence or a lack of specificity toward GAD65 and other islet-specific antigens within the Treg compartment. In order to experimentally address the question of antigen specificity in Tregs, we stimulated *in vitro* splenocytes from placebo or GAD-alum treated RIP-GP mice with an hGAD65 peptide library and measured Treg frequencies upon 3-day culture. When analyzing the percentage of activated CD4+CD25<sup>high</sup> T-cells, we observed a significant increase in GAD-alum treated mice specifically upon *in vitro* re-stimulation with GAD65 peptide pools spanning the C-terminal region of GAD65 sequence (pools 9–11). We saw a similar increase when cells were stimulated with all 11 GAD65 peptide pools together (Fig. 7). However, when analyzing the percentage of CD127<sup>lo</sup>Foxp3<sup>+</sup> cells within CD4+CD25<sup>high</sup> cells, we failed to observe any increase in GAD-alum treated mice (data not shown).



**Fig. 6.** Analyses of GAD-specific immune responses elicited by GAD-alum. C57BL/6 mice were treated with GAD-alum ( $3 \times 2 \mu\text{g}$ , s.c.). Four weeks after the last treatment, splenocyte cultures were stimulated with overlapping peptide pools spanning the entire GAD-protein (11 pools with 10 peptides each). After 72 h, culture supernatants were analyzed for cytokines by ELISA (A) or Bio-plex assay (B). Data shown are corrected for background (Unstimulated wells per mouse and stimulated wells from untreated mice).



**Fig. 7.** Analyses of GAD-specific immune responses elicited by GAD-alum. Four weeks post-LCMV infection and treatments with GAD-alum (2  $\mu$ g) or placebo, RIP-GP mice were euthanized, their spleen cells cultured for three days in the presence of 11 pools of GAD65 peptides spanning the whole sequence of the protein. Upon 3-day culture, the proportion of Treg cells was assessed by flow cytometry. Data are from one experiment with 5 mice per group. \* $P < 0.05$  (unpaired, two-tailed Mann Whitney test).

#### 4. Discussion

In the present study, we demonstrate that GAD-alum treatment administered prior to diabetes onset in NOD or RIP-LCMV-GP mice was unable to protect from clinical disease (Figs. 1, 2A and C). On a per-individual basis, there was no significant difference in the proportions of protected versus non-protected mice (Fig. 2B). This lack of efficacy to prevent T1D in mice is in line with the failure to preserve C-peptide levels that was observed in the latest Phase II/III clinical trials administering diabetic patients with 2–4 doses of 20  $\mu$ g GAD-Alum [22,23]. One potential issue that has been put forward to explain the failure of the latest intervention trial involving GAD-alum treatment [22] is that, compared to the placebo arm of the study, the two arms with GAD-alum treatments enrolled more patients under 15 years old in whom disease is thought to be more aggressive and C-peptide levels decline faster [22]. And while the RIP-GP mouse model is a rather aggressive model as well, the failure of GAD-alum to prevent disease in pre-diabetic NOD mice despite the administration of various doses does not necessarily support this hypothesis.

Previous publications from our laboratory and others suggest that the therapeutic window for  $\beta$ -cell antigen delivery (e.g., insulin given orally) is rather narrow [28,29]. Thus, we assessed whether higher or lower doses might be more efficacious. In the RIP-GP model, we gave a maximum dose of 20  $\mu$ g GAD-Alum, as was given in humans, up to nine times over the first 3 weeks of follow-up without noting significant changes in mouse glycemia (Fig. 2C). Similarly, lower doses of GAD-alum did not result in a relevant protection from T1D (Fig. 2C). The broad range of doses tested (cumulative doses ranging from 1.5 to 180  $\mu$ g in RIP-GP mice and 2–40  $\mu$ g in NOD mice) suggests that the failure of GAD-alum to prevent T1D was not primarily a question of the dose administered.

Mechanistically, we have shown that treatment with GAD-alum led to concomitant increase in Foxp3<sup>+</sup> Treg and decrease in TNF-producing 'autoreactive' CD8 T-cell populations in the pLN (Figs. 3A and 4A). Of note, these effects were observed locally but not systemically, although there was a trend toward increased percentage of Tregs in the spleen (Figs. 3B and 4B;  $p = 0.07$ ). Taken together, these data are consistent with the concept that induction of immunological tolerance can be achieved through antigen-specific approaches locally without the systemic immune

compromise associated with immunosuppressive drugs [34,37]. In the context of T1D, islet-specific autoreactive memory T-cells that cause  $\beta$ -cell death are virtually impossible to eliminate since they are present in tissues in low numbers and present the same phenotypic features as other, beneficial memory T-cells that help the host combat previously encountered pathogens. Hence, if it were possible to provide patients with a islet-specific 'surgical strike' it would, if not purge, at least dampen the proinflammatory activities of islet-specific autoreactive CD8 and CD4 T-cells. Nonetheless, the only partial (in respect to TNF) inhibition of LCMV-specific CD8 T-cells in pLN (Fig. 3C) is likely responsible for the lack of protection of GAD-alum treated mice. Moreover, our *in vitro* findings suggest that GAD-alum treatment might not favor the induction of Foxp3-expressing regulatory T-cells with a repertoire specific for GAD65 C-terminal region (Fig. 7). Together with the T-cell cytokine profile biased toward the production of IL-5 (but not IL-4 or IL-10), we can conclude that GAD-alum treatment elicited immunomodulatory responses deviated to some extent toward Th2 responses however these induced responses were overall insufficient to provide protection from diabetes in RIP-GP mice.

Due to restricted numbers of protected animals, mechanistic data with adequately powered stratification of responders versus non-responders to GAD-alum could not be assessed in this study. Correlative analyses of effector and regulatory T-cell responses in pLN indicated only a moderate linear relationship that failed to reach statistical significance with 10–11 mice (Fig. 4A,  $p = 0.098$ ). While these results in our transgenic mouse model are encouraging in the perspective of uncovering potential immune biomarkers that would be clinically relevant for future trials, the inaccessibility of the pancreas and pLN in human patients remains a major obstacle [11]. In this regard, studies by Cheramy et al. have shown that the cohort of patients from the 2008 intervention trial [21], presented reduced Th1-associated (IgG1) and augmented Th2-associated (IgG3/4) isotypes of GAD65-specific autoantibodies [38], which is consistent with previous data in mice [39].

In the perspective of refining the experimental strategy for trials to come, another approach would be to combine the positive yet sub-optimal outcome of GAD-alum treatment with another reagent that could either boost pre-existing benefits discussed above or attack the problem from a different angle. Tian et al. have recently shown that combination of 100  $\mu$ g GAD-alum and 6 mg/ml  $\gamma$ -aminobutyric acid (GABA) was able to prolong survival of  $\beta$ -cells transplanted in recent-onset diabetic NOD mice [40]. Of note, lower doses of either GAD-alum (20  $\mu$ g, maximal dose used both presently and in human trials) or GABA (2 mg/ml) failed to provide such a synergistic effect, again underlining the need for ramp-up studies in preclinical and clinical trials whenever this is feasible. Other examples for potential 'partners' may include blockade of proinflammatory cytokines such as IL-6 or IL-1 $\beta$ , as well as combination with 1,25-dihydroxyvitamin D3 [41,42], or with a 'response modifier' inducing IL-10 or transforming growth factor (TGF)- $\beta$  production [37].

Our studies highlight the importance of preclinical studies in the process that leads from translating promising findings at the bench into an efficacious drug in patients. Indeed, although multiple clinical trials have been done in humans, a very limited amount of data has been published in mice to explore the mechanism and immune response to GAD-alum. Further understanding of this vaccine could provide a more solid rationale or targets for secondary end-points that would correlate with drug efficacy and protection from diabetes.

The failure of GAD-alum to prevent T1D in both RIP-GP and NOD mice implies that subcutaneous injections of an alum-formulated GAD may not be the best choice of antigen delivery and adjuvant. Indeed, the choice of the adjuvant and the route of antigen delivery

might have been major issues that culminated in the failure of the GAD-alum trials in humans. By reproducing those negative data in two different mouse models of the disease – in which protection has already been demonstrated by our lab and others using the same antigen but different forms of antigen delivery [19,20,25,43–47] – we believe that we provide good evidence for the importance of thorough preclinical evaluations of antigen-specific vaccines in T1D.

Taken together, this notion underlines the importance of formulation, doses, route, and frequencies of administration in refining GAD-based immunization strategies. Indeed, rather than focusing on different autoantigens, the available data of GAD-based immunization strategies in mice suggest that other forms of GAD-delivery might be able to have a sustained effect on the course of T1D.

## 5. Conclusions

We have attempted to prevent diabetes onset with GAD-alum treatments in two mouse models, namely the spontaneous NOD and the virally induced RIP-GP models. In both cases, we tried a range of at least 3 doses including that used in human clinical trials. Consistent with the observations in the clinical trials, GAD-alum failed to prevent diabetes in the mouse models of T1D. As GAD-based interventions with different forms of antigen delivery have previously shown efficacy in mouse models, our findings suggest that alum-based interventions might not sufficiently support GAD's potential to curb autoimmunity in T1D.

## Conflict of interest statement

None.

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