Title:

The Clinical and Immunological Significance of GAD-Specific Autoantibody and T-Cell Responses in Type 1 Diabetes

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

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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 65kDa (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, pre-clinical evaluation of potential drugs before the initiation of clinical trials.

Keywords

Type 1 diabetes; GAD65; GAD-Alum; Immunotherapy; NOD; RIP-LCMV

1. Introduction

Type 1 diabetes (T1D) is an autoimmune disease leading to destruction of the insulin producing pancreatic beta (β)-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 β-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 β-cell or serious adverse effects due to immunosuppression [11]. However, β-cell antigen-specific therapies remain particularly appealing as they have the potential to restore immunological tolerance to β-cell antigens without systemic immunosuppression.

Glutamic acid decarboxylase of 65kDa (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 β-cells where its functional role remains largely unknown [12-14]. The emergence of GAD-specific autoantibodies occurs prior to β-cell 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 20μg alum-formulated GAD induces tolerance to GAD resulting in preservation of β-cell insulin 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 β-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 $1x10^4$ 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 250mg/dl. For mechanistic studies, mice were euthanized by CO_2 asphyxiation and cervical dislocation per animal protocol.

2.2. Treatments

Stock solutions of 40µg/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)

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.

Autoantibodies. GAD-specific autoantibodies were measured in Maxisorp Nunc 96-well plates by coating with 2µg GAD-Alum or Alum alone as a negative control overnight at +4C. After 3 washes in PBS 0.05% Tween and a 1-hour 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 hours. 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 hours. Enzymatic reactions were revealed with the addition of 100µL/well of TMB substrate (cat #421101, Biolegend, San Diego, CA) for 5-10 minutes. Reactions were stopped with 100µL/well of 1N HCl. Plates were read with a spectrophotometer measuring absorbance at 450nm.

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 2mL ACK lysis buffer for 2 minutes to lyse red blood cells. Thereafter, pLN cells and splenocytes were resuspended at $10x10^6$ cells/mL in complete RPMI (Gibco) containing 10% FBS, 50µM β-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 5µg/mL GP_{33-41} peptide from LCMV (Abgent Inc, San Diego, USA) and incubated at

37C for 4h. Cells were then stained in FACS buffer (PBS 0.05% bovine serum albumin) for surface antibodies (CD8, CD4, CD25) and fixed for 30 minutes at 4C 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-γ and TNF as well as Foxp3. All the antibodies (BD Biosciences) were diluted 1/100. Samples were recorded on a 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 $10x10^6$ 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-hour incubation, supernatants were collected and frozen until use for the detection of TNF and IL-5 by ELISA.

2.6. Statistics

Data are expressed as mean ± 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 1μg, 5μg or 20μg of GAD-alum or placebo at 8 and 10 weeks of age. Mice were subsequently monitored for diabetes onset. We observed that 1μg GAD-alum (cumulative dose 2μg) treated mice presented similar T1D incidence compared to alum treated controls (87% versus 71%, p=0.806) (Figure 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) (Figure 1, middle panel). Finally, there was a slight tendency toward protection from T1D when administering mice 20μg GAD-alum (73% versus 93% for controls). However, this trend failed to reach statistical significance (p=0.35) (Figure 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 2μg or 10μg GAD-alum or placebo on days 2, 8 and 14 post-infection (arrows, Figure 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 (Figure 2A). On a per-individual basis, there was no significant difference in the proportions of protected versus non-protected mice, as illustrated in Figure 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 (3 doses of 0.5μg) or increased (9 doses of 20μg). Figure 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 3 times with 2μg 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 (Figure 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-γ.

In the RIP-GP model, LCMV-GP_{33-41}-specific CD8 T cells are responsible for the immune-mediated demise of pancreatic β-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_{33-41} peptide in the presence of Brefeldin A and stained for TNF and IFN-γ. Our data indicate that mean LCMV-specific CD8 T-cell TNF (Figure 3A, left panel) production was significantly reduced in the pLN of mice administered GAD-alum compared to their counterparts (1.17±0.2% versus 4.98±1.73%, respectively; p=0.040). Similarly, IFN-γ^+TNF^+ double-producing CD8 T cells were found in lower frequencies upon GAD-alum treatment (Figure 3A, right panel). Interestingly, this decrease appeared to be specific to pLN since both TNF and/or IFN-γ levels were found to be similar in the spleen (Figure 3B and not shown).

3.5. pLN from RIP-GP mice treated with GAD-alum have increased proportions of Foxp3-expressing CD4+CD25^{high} T regulatory cells.

Furthermore, we examined in parallel the proportions of Foxp3-expressing T regulatory cells (Tregs) as it has been published that treatment with GAD-alum could result in enhancement of CD4+CD25^{high}Foxp3$^+$ 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) (Figure 3C). 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) (Figure 3D).

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

Mechanistic data presented in Figure 2 and 3 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. Figure 4A depicts one-on-one correlation between the percentages of Tregs and that of TNF-producing CD8 T cells in 2µg 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 (Figure 4B) or Foxp3-expressing Tregs (Figure 4C). We conclude that 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) (Figure 5). Interestingly, stimulation with pool 10 from the hGAD65 library led to the highest IL-5, but no TNF, production. Overall, these data confirm that the TCR repertoire of GAD65-specific CD4 T cells is shifted toward the C-terminal domain of the protein [25] and

further argue for the existence of non-overlapping GAD65-specific CD4 Th2-biased responses.

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 (Figure 1, 2A, 2C). On a per-individual basis, there was no significant difference in the proportions of protected versus non-protected mice (Figure 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 to 4 doses of 20μg 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 β-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 20μg 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 (Figure 2C). Similarly, lower doses of GAD-alum did not result in a relevant protection from T1D (Figure 2C). The broad range of doses tested (cumulative doses ranging from 1.5 to 180μg in RIP-GP mice and 2 to 40μg 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 $Foxp3^+$ Treg and decrease in TNF-producing 'autoreactive' CD8 T-cell populations in the pLN (Figure 3A, C). Of note, these effects were observed locally but

not systemically, although there was a trend toward increased percentage of Tregs in the spleen (Figure 3B, D; 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 β-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.

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 (Figure 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 an 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 and colleagues have recently shown that combination of 100µg GAD-alum and 6mg/ml γ-aminobutyric acid (GABA) was able to prolong survival of β-cells transplanted in recent-onset diabetic NOD mice [40]. Of note, lower doses of either GAD-alum (20µg, maximal dose used both presently

and in human trials) or GABA (2mg/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-1β, as well as combination with 1,25-dihydroxyvitamin D3 [41, 42], or with a 'response modifier' inducing IL-10 or transforming growth factor (TGF)-β 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. In contrast to our observations with GAD-alum, other GAD formulations [19, 20, 25, 43] have shown efficacy in mice, even in recent onset disease. 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

The authors declare none.

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Figure legends

Figure 1: Diabetes incidence in NOD mice treated with GAD alum.

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

Figure 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) 2μg or 10μg of GAD-alum and (B) 0.5μg or 20μg GAD-alum. Three placebo mice received the volume corresponding to 20μg GAD-alum formulation and 3 mice received the volume corresponding to 0.5μg GAD-alum formulation on indicated days. (C) Blood glucose values from placebo and GAD-alum (3x2μg)-treated mice at time point of mechanistic studies, 28 days post infection. Dashed line represents the threshold of 250mg/dl used for diagnosis. (D) GAD autoantibodies in placebo and GAD-alum (3x2μg)-treated mice. Shown is the last positive dilution above background. **$p<0.01$.

Figure 3: Analysis of effector and regulatory T-cell responses in RIP-GP mice treated with GAD alum.

(A) Percentage of pancreatic lymph node (pLN) derived CD8+ T cells producing TNF in response to in vitro GP33 stimulation. Two representative FACS plots gated on pLN-derived CD8+ T cells. (B) Percentage of spleen derived CD8+ T cells producing TNF in response to in vitro GP33 stimulation. (C) Percentage of pancreatic lymph node (pLN) derived CD4+ T cells expressing FoxP3 and high levels of CD25. Two representative

FACS plots gated on pLN-derived CD4+ T cells. (D) Percentage of spleen derived CD4+ T cells expressing FoxP3 and high levels of CD25. *p<0.05.

Figure 4: Correlative analyses of effector and regulatory T-cell responses in pLN in relation to mouse glycaemia.

(A) Correlation between the percentages of pLN-derived, GP33-specific CD8+ T cells producing TNF and CD4+CD25^{high}Foxp3+ Treg cells. (B) Correlation between individual mouse BGV and percentage of pLN-derived, GP33-specific CD8+ T cells producing TNF. (C) Correlation between individual mouse BGV and percentage of pLN-derived, CD4+CD25^{high}Foxp3+ 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.

Figure 5: Analyses of GAD-specific immune responses elicited by GAD-alum. C57BL/6 mice were treated with GAD-alum (3x2ug, 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 hours, culture supernatants were analyzed for cytokines by ELISA. Data shown are corrected for background (Unstimulated wells per mouse and stimulated wells from untreated mice).

GAD Treatment and Insulin Secretion in Mouse Models of T1DM Mice: Pursuing a cure for type 1 diabetes

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INTRODUCTION

Type 1 diabetes mellitus (T1DM) is an autoimmune disease with genetic and environmental components that trigger the body's T cells to destroy beta cells of the pancreas that are responsible for producing insulin. The disease is commonly diagnosed during childhood when the beta cell destruction is near complete and the lack of insulin production surfaces with clinical symptoms, such as polyuria, polydipsia, polyphagia, weight loss, and ketoacidosis. T1DM results in a permanent dysregulation of blood glucose homeostasis and a lifetime dependence on insulin injections or infusions for survival. Proper management and control of T1DM can be difficult and commonly results in microvascular complications, such as retinopathy, neuropathy, and nephropathy, leading to blindness, amputation, and renal failure.

The 65-kDa isoform of Glutamic acid decarboxylase (GAD), is one of the major auto-antigens in human T1DM. The slightly larger 67-kDa isoform of GAD is expressed in mice. GAD is the enzyme in the central nervous system responsible for converting the neurotransmitter, glutamate, to GABA, but it is also found in the pancreas where its functional role in pathological and healthy states is still unclear. The body develops auto-antibodies to pancreatic GAD just prior to and during the beta cell destruction that occurs in early T1DM. GAD auto-antibodies have not been causally linked to the T-cell mediated autoimmune attack, but they serve as a clear predictor and marker of the disease process.

It is without question that a cure is needed for T1DM, but past and current efforts in this direction have been met by many challenges. In an attempt to preserve beta cell function in patients with recent onset diabetes, intervention trials have included antigen specific therapies, such as insulin, altered peptide ligand derivative of insulin, and DiaPep277, and systemic immunosupressants, such as cyclosporin-A, anti-CD3, and anti-CD20. Many of these trials suffered from lack of efficacy, and those that initially showed some promise, specifically the immunosuppressants, resulted in only transient preservation of beta cells or serious adverse effects due to immunosupression. Therapies involving pancreas or beta cell transplantation face similar issues. Autoaggressive memory T cells seem to be responsible for the short-lived efficacy of these therapies. This and the adverse effects of immunosupression, among other things, must be circumvented to achieve an ideal cure.

Some success has been reported in a recent trial using GAD as an antigen specific therapy to achieve possible long-term tolerance and regulation of the immune system without involving systemic immunosupression. This 70-patient human trial conducted in Sweden by Diamyd, showed that treatment with an alum-formulated GAD vaccine induces tolerance to GAD resulting in preservation of beta cell insulin secretion in patients with recent onset (<6 months) T1DM. While it did not lessen the insulin requirement of the treated patients, the protective effects of GAD on insulin secretion (measured as change in fasting and stimulated C-peptide levels vs. placebo) can lead to a significant reduction in long-term complications of the disease. The GAD vaccine was shown to be safe and advantageous over other therapies of similar efficacy in that it was not associated with any treatment-related adverse effects. Furthermore, other studies have shown that GAD can prevent and stop progression of diabetes in the NOD mouse models of T1DM. GAD's mechanism of action has not yet been proven, but it is suspected that the vaccine induces GAD-specific regulatory T cells (Tregs).

HYPOTHESIS

Inspired by the success of the Diamyd study above, this project explores GAD's effect on insulin secretion in type 1 diabetic mouse models, so that a GAD vaccine can possibly move forward as part of a cure for the disease.

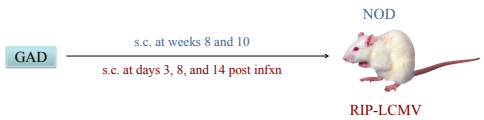
Goal: Define GAD's efficacy and mechanism of action and characterize the cytokine profile to explain how it contributes to preservation of insulin secretion.

Hypothesis: GAD will be efficacious in mouse models of T1DM and vaccination with GAD will protect mice from developing diabetes by inducing GAD-specific regulatory T cells and/or cytokines that promote autoimmune regulation.

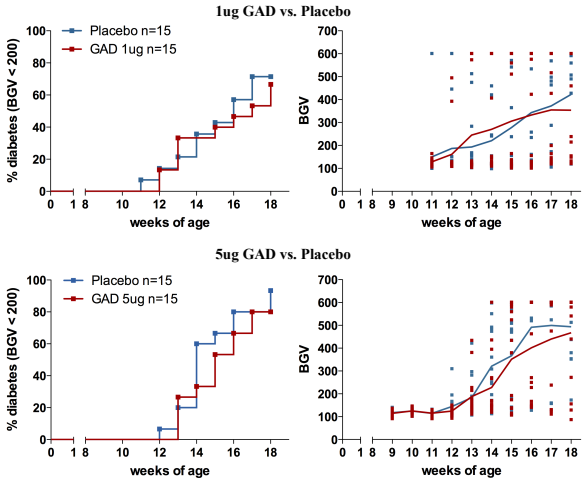
METHODS

Evaluation of efficacy:
GAD's efficacy is being evaluated in preventing T1DM in several mouse models of diabetes, the Non-Obese Diabetic (NOD) Mouse Model and the B6.RIP-LCMV Mouse Model. In each mouse model, there is a treatment group with either GAD or placebo (no-treatment). Due to the different time-course of disease development in the two mouse models, the NOD mice are dosed at weeks 8 and 10, while the RIP-LCMV mice are dosed on days 3, 8, and 14 post LCMV infection. The blood glucose values of the mice are monitored 1-2 times per week using the standard tail nick and glucometer procedure, and mice with blood glucose values 200mg/dl+ are considered diabetic for NOD mice (300mg/dl+ for RIP-LCMV mice). Post-treatment blood glucose values are compared between the GAD treated mice and vehicle to evaluate prevention of diabetes. The initial GAD dosage of 5ug was lowered to 1ug and then raised to 20ug to explore change in efficacy at a new dose.

Group (n = 6-15)	Treatment	Dosing & Timecourse
NOD untreated	placebo	1, 5, 20ug – weeks 8, 10
NOD protected	GAD	1, 5, 20ug – weeks 8, 10
RIP-LCMV untreated	placebo	10ug – days 3, 8, 14 post LCMV infection
RIP-LCMV protected	GAD	2, 10ug – days 3, 8, 14 post LCMV infection



RESULTS

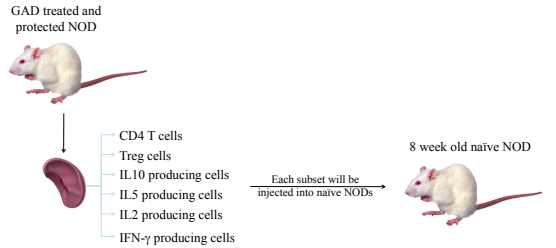


- NOD mice started to become diabetic (BGV 200+) around weeks 11 – 13.
- The 1ug and 5ug doses of GAD (administered on weeks 8 and 10) did not show significant efficacy in prevention of diabetes.
- The NOD mice given 20ug GAD are currently in their 13th week.
- The RIP-LCMV mice are currently around 8 days post LCMV infection.

FUTURE WORK

Mechanism of action:
Once efficacy is established, we will study the mechanism of action to see if regulatory CD4 T cells or specific cytokines mediate GAD's protective effects. This will be done with a series of adoptive transfer experiments with NOD mice, RIP-LCMV mice, or both depending on the results of the efficacy experiments. CD4+ or CD4+FoxP3+ T cells will be transferred from protected, GAD treated mice into naïve, untreated mice. This will reveal whether the CD4+ T cells from protected mice can prevent disease development on their own. The control group of CD4+ T cells transferred from untreated mice into naïve recipients should not prevent disease development. To examine the role of specific cytokines in protected animals, cell transfer experiments will be performed on isolated cytokine producing splenocytes. The splenocytes from GAD treated animals will be stimulated *in vitro* by GAD, then cells that produce the specific cytokines, IL-2, IL-4, IL-10, and IFN-γ, will be detected, isolated via a bead sorting technique, enriched, and transferred into untreated mice.

Group (n = 10-15)	Treatment	Dosing & Timecourse
CD4/Treg donors - untreated	placebo	Xug – weeks 11, 13
CD4/Treg donors - protected	GAD	Xug – weeks 11, 13
Untreated CD4/Treg recipients	1,000,000 CD4/Tregs from untreated donors	Xug – weeks 11, 13
Protected CD4/Treg recipients	1,000,000 CD4/Tregs from protected donors	Xug – weeks 11, 13
IL-2 producing CD4 cell donor	GAD	Xug – weeks 11, 13
IL-2 producing CD4 cell recipient	placebo	Xug – weeks 11, 13
IL-4 producing CD4 cell donor	GAD	Xug – weeks 11, 13
IL-4 producing CD4 cell recipient	placebo	Xug – weeks 11, 13
IL-10 producing CD4 cell donor	GAD	Xug – weeks 11, 13
IL-10 producing CD4 cell recipient	placebo	Xug – weeks 11, 13
IFN-γ producing CD4 cell donor	GAD	Xug – weeks 11, 13
IFN-γ producing CD4 cell recipient	placebo	Xug – weeks 11, 13



Phenotype and function:
Finally, we will characterize the phenotype and function of the T cell subsets. Flow cytometry will be used for phenotypic characterization of T cells from untreated animals versus animals treated with GAD at timepoints before and following GAD treatment. We are most interested in T cells with the regulatory phenotype, such as CD4+CD25+FoxP3+CD127-. Additionally, we will use intracellular cytokine staining to monitor the GAD specific cytokine production of these T cells.

SUMMARY & CONCLUSIONS

- The 1 and 5ug GAD dose did not significantly improve blood glucose values in NOD mice, and the 20ug dose may or may not show efficacy.
- It is too early to determine if GAD will result in protection from diabetes in the RIP-LCMV mouse model.
- This lack of efficacy could be due to not yet finding an optimal dose for GAD in mice. In human studies, doses that were too high or too low showed less efficacy.
- The lack of efficacy could also be due to the nature of these preventive studies. Regardless of GAD's action, insulin is targeted by the immune system first in NOD mice, and later, through antigenic spreading, GAD plays more of a role in the immune response. Therefore, we may see better results in future recent onset studies instead of these preventive studies.