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Gene gun-mediated DNA vaccination enhances antigen-specific immunotherapy at a late preclinical stage of type 1 diabetes in nonobese diabetic mice

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Abstract

Type 1 diabetes (T1D) is characterized by the T cell mediated destruction of the insulin producing β cells. Antigen-specific immunotherapies are used to selectively tolerize β cell-specific pathogenic T cells either directly, or indirectly through the induction of immunoregulatory T cells. A key concern of antigen-specific immunotherapy is exacerbating autoimmunity. We compared the T cell reactivity and efficacy induced by plasmid DNA (pDNA) encoding glutamic acid decarboxylase 65 (GAD65) administered via intramuscular versus gene gun vaccination in NOD mice at a late preclinical stage of T1D. Whereas intramuscular injection of pGAD65 promoted a predominant type 1 $CD4^+$ T cell response and failed to suppress ongoing β cell autoimmunity, gene gun vaccination preferentially induced IL-4 secreting $CD4^+$ T cells and significantly delayed the onset of diabetes. These findings demonstrate that gene gun delivery of autoantigen-encoding pDNA preferentially elicits immunoregulatory T cells and offers a safe, effective mode of pDNA vaccination for the treatment of T1D and other autoimmune diseases.

Keywords

GAD65; autoimmunity; immunoregulatory T cells

INTRODUCTION

Type 1 diabetes (T1D) is caused by the autoimmune-mediated destruction of the insulin-producing β cells of the islets of Langerhans [1]. Based on studies in the nonobese diabetic (NOD) mouse, a spontaneous model of T1D, the primary effectors of β cell destruction are $CD4^+$ and $CD8^+$ T cells [2;3]. Pathogenic effector T cells typically exhibit a type 1 phenotype, and target a number of β cell autoantigens including proinsulin, insulin, islet-specific glucose-6-phosphatase catalytic subunit related protein (IGRP), and glutamic acid decarboxylase 65 (GAD65) [4;5;6;7;8]. The critical events associated with the development and expansion of these type 1 β cell-specific effector T cells remain ill-defined, although aberrant peripheral tolerance is believed to contribute. For example, insufficient numbers of IL-4 secreting type 2 T cells or IL-10 secreting Tr1 cells, and defective development and/or

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function of FoxP3-expressing immunoregulatory T cells (Treg) have been reported in NOD mice and diabetic patients [9;10;11;12;13].

A variety of immunotherapies have been developed to reestablish the functional balance between pathogenic and immunoregulatory T cells and prevent and/or treat T1D [14;15]. Antigen-specific immunotherapy is one such strategy to induce β cell-specific immunoregulatory T cells and selectively block the initiation and/or progression of β cell autoimmunity [16;17;18;19]. The efficacy of the approach is largely dependent on the frequency of immunoregulatory T cells induced upon administration of a given β cell autoantigen versus induction and/or expansion of pathogenic T cells. The conditions to preferentially elicit immunoregulatory T cells in an antigen-specific manner become more stringent at late preclinical or clinical stages of T1D when β cell autoimmunity is well established [20;21].

We and others have successfully used plasmid DNA (pDNA)-based vaccines to induce β cell-specific immunoregulatory $CD4^+$ T cells, and in turn prevent and suppress β cell autoimmunity in NOD mice [22;23;24;25;26;27;28;29;30;31]. In general, pDNA offer: i) a relatively facile approach for vaccination, overcoming the need to produce and store antigen, and ii) an opportunity to readily manipulate the nature of the induced immune response [32]. Studies using NOD mice have delivered β cell autoantigen-encoding pDNA prepared in saline via intramuscular (i.m.) [26;28;29;30] and intradermal (i.d.) needle injection [25], or via oral administration [25;31]. Typically, effective prevention of diabetes has been dependent on co-injection of pDNA encoding anti-inflammatory cytokines such as IL-4 and/or IL-10, especially once β cell autoimmunity is well established [26;28;29;30;31]. Indeed, i.m. injection of pDNA encoding antigen-only has had either no immunotherapeutic effect or worse, elicited β cell-specific type 1 $CD4^+$ T effectors [29;30]. The current study was initiated to determine how the mode of delivery affects the efficacy of pDNA to prevent diabetes at a late preclinical stage in NOD mice. Specifically, the nature of the T cell response and immunotherapeutic efficacy of pDNA encoding a fragment of murine GAD65 fused to human immunoglobulin Fc (pGAD65) administered by i.m. versus gene gun vaccination were compared. Gene gun vaccination entails bombardment of the upper most layers of the skin with pDNA-coated particles[32;33]. Notably, studies have shown that gene gun immunization preferentially induces type 2 T effectors [34;35]. With this in mind, we tested the hypothesis that gene gun vaccination of pGAD65-only is more effective at eliciting GAD65-specific immunoregulatory T cells and suppressing ongoing β cell autoimmunity than i.m. injection of pGAD65 prepared in saline.

MATERIALS AND METHODS

Mice

NOD/LtJ, NOD.*il4*^{tm1Cgn} (NOD.IL-4^{null}), and NOD.*scid* mice were purchased from Jackson Laboratory (Bar Harbor, MA) and bred under specific pathogen-free conditions. All experiments with mice were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

pDNA vaccination

pDNA encoding human immunoglobulin Fc (IgFc) fusion molecules consisting of a fragment of murine GAD65 (pGAD65) or hen egg lysozyme (pHEL) spanning nucleotides 656–1070 and 175–270, respectively, have previously been described [29]. The Jw4303 vector backbone contains two CpG motifs [30]. pDNA was prepared from DH5. *Escherichia coli* using a Qiagen endotoxin-free kit (Qiagen, Valencia, CA). For i.m. injection, pDNA was resuspended at 1.0 mg/ml in calcium-free PBS, and NOD mice received four i.m. injections of 50 ul (50 μg) of pDNA in each hind quadricep muscle over 28 days with an insulin syringe. Biolistic delivery

of pDNA was carried out using a Helios gene gun (Bio-Rad, Hercules, CA) and 1.6 μM gold particles (Bio-Rad). Gold particles (0.5 mg) were coated with 1 μg of pDNA using 1 mM $CaCl_2$ and 0.05 mM spermidine (Sigma, St. Louis, MO). The efficiency of coating was assessed by eluting pDNA off the gold particles in Tris-EDTA buffer and then quantitating pDNA via agarose gel electrophoresis and spectrophotometry (Beckman Coulter, Fullerton, CA). NOD mice with shaven abdomens received four shots at 1-week intervals at a helium pulse of 425 pounds per square inch.

In other experiments pDNA encoding a luciferase gene (pLUC) driven by a CMV/CB promoter [36] was administered via gene gun or i.m. injection as described above. *In vivo* bioluminescence was measured by injecting mice intraperitoneally (i.p.) with 5 mg of luciferin (Promega, Madison, WI) and using a two-photon Xenogen imaging system (Xenogen, Alameda, CA) while mice were under isofluorane anesthesia. The intensity of bioluminescence was measured at the site of injection as the number of photons per second per centimeter squared per steradian as determined by the Xenogen software and reperesented as relative optic intensity (ROI) units.

GAD65-IgFc ELISA

Serum levels of GAD65-IgFc were measured using a human IgG ELISA kit as per the manufacturer's recommendations (Bethyl Laboratories, Montgomery, TX). Briefly, the capture antibody was incubated for 45 minutes in PBS on a 96-well high-binding plate (Corning, Lowell, MA) at room temperature. The plate was blocked with 1% bovine serum albumin (BSA)/PBS, washed, and 25 ul of serum diluted 1:3 with 1% bovine serum albumin/PBS was added to each well in duplicate. Samples were incubated at room temperature for 2 hours followed by washing three times. Detection antibody was then added and incubated at room temperature for 1 hour. The plate was washed and developed using substrate A and substrate B (BD Pharmingen, San Francisco, CA); the reaction was stopped using 4N phosphoric acid (Sigma, St. Louis, MO). Optical density values were determined at 450 nm wavelength using an ELISA plate reader (Molecular Devices, Sunnyvale, CA.)

Assessment of diabetes and insulitis

NOD mice were monitored weekly for the development of glycosuria via Diastix (Ames, Elkhart, IN). Diabetes was diagnosed by 2 consecutive positive measurements over 48 hours. Insulitis was assessed by histology. Pancreata were prepared for histology by fixation in neutral buffered formalin followed by paraffin embedding. A minimum of five sections, 90 μm apart, were cut from each block, stained with hematoxylin and eosin (H&E), and viewed by light microscopy. A minimum of 40 islets were scored for each animal. The severity of insulitis was scored as either peri-insulitis (islets surrounded by an infiltrate) or intra-insulitis (< or >50% infiltration of the islets).

Flow cytometry

Antibodies specific for murine CD3 (2c11), CD4 (GK1.5), and CD25 (PC61) (eBiosience, San Diego, CA) were used to stain splenocytes, inguinal lymph nodes and pancreatic lymph nodes at 1:500 dilution. Staining with anti-FoxP3 (FJK-16s) and isotype control (IgG2a) antibody (eBioscience, San Diego, CA) was done according to manufacturer's recommendations at a 1:400 dilution. Acquisition of samples was performed on a 3-laser Cyan and analyzed using Summit software build 4.3 (DAKO, Ft. Collins, CO).

Peptides

Peptides were synthesized using standard F-moc chemistry on a Rainin Symphony (Rainin Instruments, Woburn, MA) at the peptide synthesis facility of University of North Carolina. The purity of the peptides was verified by reverse phase HPLC and mass spectroscopy.

ELISPOT

ELISPOT was carried out as previously described [26]. Briefly, ImmunoSpot M200 plates (Millipore, Billerica, MA) were coated overnight at 4°C with either 2 μg/ml anti-IL-5, -IL-10, or -IFNγ and 1 μg/ml anti-IL-4 (BD PharMingen, San Jose, CA) prepared in PBS, and then blocked with 1% BSA-PBS for a minimum of 1 hour at room temperature. Splenocytes suspensions prepared from individual mice were plated at 1×10^6 cells/well. Inguinal and pancreatic lymph nodes of 5 mice from each treatment group were harvested, pooled and cultured overnight in the presence of 4 ng/ml of mIL-2 (Peprotech, Rocky Hill, NJ) then washed and plated at 2×10^5 with an equal number of irradiated splenocytes. Peptide was added in duplicate to wells at a final concentration of 40 μg/ml. The plates were incubated for 48 hours at 37°C in 5.5% CO_2 and then washed. Biotinylated anti-IL-4, -IL-5, -IL-10 or -IFNγ (BD PharMingen, San Jose, CA) were added at 2 μg/ml, in 1% BSA-PBS and plates incubated overnight at 4°C. Plates were washed and incubated with streptavidin-HRP (BD PharMingen; 1/1000) for 2 hours at room temperature. This was followed by three washes with 0.025% Tween 20-PBS and three washes with PBS only. Development solution consisted of 0.8 ml of 3-amino-9-ethyl-carbazole (Sigma, St. Louis, MO) added to 24 ml of 0.1 M sodium acetate (pH 5.0), plus 0.12 ml of 3.0% hydrogen peroxide; 0.2 ml was added per well.

Adoptive transfer

$CD4^+$ T cells were isolated from splenocytes of gene gun treated NOD and NOD.IL-4^{null} female mice using a CD4 enrichment kit (BD Pharmingen, San Jose, CA), and resuspended at 20×10^6/ml in PBS. In addition, splenocytes were harvested from diabetic NOD female mice, red blood cells lysed and the cells resuspended in PBS at 20×10^6/ml. NOD.*scid* male mice then received i.p. injections of 8×10^6 diabetogenic splenocytes alone or in combination with 2×10^6 $CD4^+$ T cells isolated from treated NOD or NOD.IL-4^{null} donor mice.

RESULTS

Gene gun or i.m. delivery of pGAD65 yield high systemic levels of transgene expression

The type of immune response induced by pDNA vaccination is dependent on both the site and mode of delivery [27;32;33]. The first set of experiments was aimed to determine the levels and localization of transgene expression of pDNA delivered to the skin via gene gun and the muscle via needle injection. Under the gene gun parameters employed, the 1.6 μm gold particles were delivered to the dermal layer of the skin (Fig. 1A). To initially evaluate the duration and level of transgene expression, pLUC which encodes luciferase was delivered once to the abdomen (1 μg) or quadriceps (50 μg) of NOD mice via gene gun or i.m. injection, respectively, and bioluminescence measured at the site of delivery over a 2 week period. The kinetics of bioluminescence after a single vaccination were similar between the 2 treatment groups; luciferase expression was readily detected after 24 hours and persisted up to 14 days post-treatment (Fig. 1B). However, despite a 50-fold difference in the amount of pLUC administered, up to an ~3-fold increase in the intensity of bioluminescence was detected in NOD mice receiving pLUC delivered via gene gun versus i.m. injection (Fig. 1B).

The serum level of GAD65-IgFc protein in NOD mice receiving pGAD65 via gene gun versus i.m. injection was compared. Here, pGAD65 was administered under treatment conditions used to prevent diabetes (see below). Namely, NOD mice received a total of 4 injections over 4

weeks of: i) 50 μg of pGAD65 delivered i.m. to each quadricep, or ii) gold-particles coated with a total of 1 μg of pGAD65 to the abdomen. Note that the treatment protocol for i.m. injection of pGAD65 was established in a previous study [29].The concentration of GAD65-IgFc in serum was then measured over time by ELISA specific for human IgFc. Significant levels of GAD65-IgFc (e.g. 2–3 ng/ml) were detected 7 days after the initial treatment in both experimental groups (Fig. 1C). Notably, an ~3-fold increase in GAD65-IgFc was detected at later time points in NOD mice receiving pGAD65 via gene gun versus i.m. injection (Fig. 1C). Together these findings demonstrate that although robust and persistent transgene expression is induced by i.m. injected pDNA, gene gun delivered pDNA results in enhanced and more efficient transgene expression.

Gene gun but not i.m. delivered pGAD65 prevents diabetes in NOD mice with ongoing β cell autoimmunity

The immunotherapeutic efficacy of gene gun versus i.m. vaccination of pGAD65 was assessed. Ten week-old NOD female mice were treated as above and diabetes monitored up to 36 weeks of age. At 10 weeks of age, NOD female mice are typically euglycemic yet β cell autoimmunity is well established. The majority of NOD female mice receiving control pHEL via gene gun (8/10) and i.m. injection (7/10) or left untreated (9/10) developed overt diabetes with no significant difference in the time of diabetes onset (Fig. 2A). Furthermore, all NOD female mice treated with pGAD65 via i.m. injection (10/10) developed diabetes (Fig. 2A). On the other hand, significant protection was detected in NOD female mice receiving gene gun delivered pGAD65 versus i.m. injected pGAD65 or the respective control groups ($P \leq 0.005$; Kaplan-Meier Log Rank Test). In addition, a significant reduction in the frequency of diabetes was detected in NOD female mice receiving gene gun (5/10) versus i.m. (10/10) delivered pGAD65 ($P=0.039$; Chi Square) (Fig. 2A). Histological analysis of the nondiabetic 36 week-old NOD female mice demonstrated that the majority of islets were infiltrated in the pGAD65 gene gun delivered group, but that the frequency of “severe” intra-insulitis (e.g. >50% intra-insulitis) was significantly reduced compared to pHEL gene gun vaccinated or untreated NOD mice ($P<10^{-3}$, Chi-Square) (Fig. 2B). No correlation was observed between the serum levels of GAD65-IgFc measured over time and protection against diabetes in the NOD female mice treated with gene gun delivered pGAD65 (Fig. 2C,D). In summary, these results demonstrate that gene gun but not i.m. vaccination of pGAD65 prevents diabetes at a late preclinical stage of T1D, and that protection is independent of systemic levels of GAD65-IgFc.

Protection mediated by gene gun delivery of pGAD65 correlates with the induction of type 2 GAD65-specific T cells

The magnitude and nature of GAD65-specific T cell reactivity elicited by gene gun versus i.m. injection of pGAD65 were compared. Groups of five 10-week old NOD female mice were vaccinated with pGAD65 as described above or left untreated, and 3 weeks after the final treatment the frequency of IL-4, IL-5, IL-10 and IFNγ secreting T cells in response to the IA^{g7}-restricted GAD65 peptides spanning amino acid residues 206–220 (p206), p217–236 (p217) and p290–309 (p290) was measured via ELISPOT. These three peptides are the immunodominant epitopes found within the fragment of GAD65 encoded by pGAD65. T cell reactivity was assessed in the: i) pancreatic lymph nodes (PLN) which drain the pancreas, ii) inguinal lymph nodes (ILN) which drain the abdominal site of gene gun delivered pGAD65, and iii) the spleen. A typical type 1-like response was detected in NOD female mice treated i.m. with pGAD65 (Fig. 3). Namely a significant increase in the frequency of IFNγ secreting T cells in response to the GAD65-specific peptides was detected in the PLN, ILN and spleen of NOD mice vaccinated i.m. with pGAD65 versus untreated animals (Fig. 3). Furthermore no significant increase in the frequency of IL-4 and IL-5 secreting T cells in the PLN or ILN was detected in the pGAD65 i.m. group compared to untreated animals (Fig. 3). Interestingly, a small but significant frequency of IL-4 secreting GAD65 peptide-specific T cells was also

detected in the spleen of the i.m. pGAD65 vaccinated NOD mice; this response, however, was markedly reduced compared to NOD mice treated with gene gun delivered pGAD65 (Fig. 3). A predominate type 2-like GAD65 peptide-specific T cell response was detected in NOD mice receiving gene gun delivered pGAD65. The frequency of IL-4 and IL-5 secreting T cells was significantly increased in the PLN, ILN and spleen of the pGAD65 gene gun treated group versus pGAD65 i.m. vaccinated and untreated NOD mice (Fig. 3). GAD65-specific T cells secreting IFNγ were also induced via gene gun delivery of pGAD65, however, the frequency of these type 1 effectors was reduced compared to the frequency of IL-4 (and to a lesser extent IL-5) secreting T cells detected within a given tissue (Fig. 3). No significant increase was detected in the frequency of GAD65 peptide-specific IL-10 secreting T cells relative to medium-only controls in either of the pGAD65 treatment groups or in untreated NOD mice, regardless of the tissue examined. Furthermore, GAD65-specific T cell reactivity was similar between untreated NOD female mice and animals receiving pHEL by gene gun or i.m. delivery (data not shown).

No significant increase was detected in the frequency of FoxP3-expressing $CD4^+CD25^+$ T cells residing in the PLN, ILN and spleen of the two pGAD65 treatment groups compared to untreated NOD mice (Fig. 4). In conclusion, these results demonstrate that whereas i.m. injection of pGAD65 induces a type 1-like T cell response, gene gun delivery of pGAD65 elicits a predominately type 2-like response marked by a high frequency of GAD65 peptide-specific IL-4 secreting T cells.

GAD65-specific IL-4 secreting $CD4^+$ T cells are necessary to mediate the protective effect of gene gun delivered pGAD65

The above findings suggested that suppression of β cell autoimmunity via gene gun delivered pGAD65 was mediated by GAD65-specific $CD4^+$ T cells secreting IL-4. To directly examine this possibility, a co-adoptive transfer model of T1D was employed exploiting the use of NOD mice lacking IL-4 expression (NOD.$IL4^{null}$). Here 10 week-old wild-type NOD or NOD.$IL4^{null}$ female mice received pGAD65 via gene gun. Three weeks after the final treatment, $CD4^+$ T cells were isolated from the pGAD65 treatment groups, and co-adoptively transferred with diabetogenic splenocytes into groups of 5 NOD.*scid* mice. As demonstrated in Fig. 5, the transfer of diabetes was significantly blocked by $CD4^+$ T cells prepared from pGAD65 gene gun vaccinated wild-type NOD mice compared to mice receiving diabetogenic splenocytes-only ($P<0.0053$; Kaplan Meier Log Rank Test) (Fig. 5). In contrast, $CD4^+$ T cells prepared from pGAD65 gene gun vaccinated NOD.$IL4^{null}$ mice failed to block the transfer of diabetes; no significant difference in the frequency and time of onset of diabetes was detected in this group compared to NOD.*scid* mice receiving diabetogenic splenocytes-only (Fig. 5). These data demonstrate that suppression of β cell autoimmunity by gene gun delivered pGAD65 is dependent on GAD65-specific IL-4 secreting $CD4^+$ T cells.

DISCUSSION

The key finding made in this study is that despite ongoing β cell autoimmunity, gene gun delivered pGAD65 preferentially promotes a robust type 2 $CD4^+$ T cell response that significantly prevents the development of diabetes in NOD mice. This is in marked contrast to i.m. injection of pGAD65 which induced a dominant type 1 GAD65-specific $CD4^+$ T cell response and failed to prevent diabetes in 10 week-old NOD female mice.

Our findings are consistent with previous studies demonstrating that antigen-encoding pDNA delivered via gene gun typically induces type 2 immunity, whereas i.m. needle injection of soluble pDNA promotes type 1 immunity [34;35]. Currently, the mechanisms which drive these “default” T cell responses remain ill-defined. Interestingly, both gene gun and i.m. vaccination of pGAD65 elicited high systemic levels of GAD65-IgFc protein based on serum

levels in treated NOD mice (Fig. 1C). Although serum levels of GAD65Ig were greater in gene gun treated NOD mice, protection did not correlate with systemic GAD65-IgFc concentrations (Fig. 2C,D). These observations argue that the nature of the T cell response and suppression of β cell autoimmunity are largely dependent on the site and mode of pDNA delivery. Indeed, work by Escher and colleagues demonstrated that the onset of diabetes was delayed when 4 week-old NOD female mice received i.d. versus i.m. needle injections of pDNA encoding human GAD65 [25]. In this study, however, i.d. needle injection failed to induce a significant increase in GAD65-specific IL-4 secreting T cells despite the relatively non-stringent conditions (e.g. lack of ongoing β cell autoimmunity) [25]. Our results in which a significant frequency of GAD65-specific type 2 T cell reactivity was induced by gene gun delivery at a late preclinical stage of T1D further underscores the potent effects of particle-delivery of pDNA to the skin. The latter in part may be explained by the tolergenic properties of epidermal Langerhans cells [37] that have been transfected by pDNA or which have endocytosed the transgene encoded protein. Although not assessed in this study, the effect of gene gun-delivered pGAD65 on islet-specific autoantibodies is of interest. Islet-specific autoantibodies are used in the clinic to define the progression of β cell autoimmunity in at risk individuals [1]. Future efforts will determine whether the delay in diabetes onset in NOD mice treated with gene gun-delivered pGAD65 is reflected by changes in the level and/or isotype of serum islet-specific autoantibodies.

The increased efficiency of transgene expression detected with gene gun versus i.m. vaccination was another parameter favoring the use of particle-mediated delivery of pDNA (Fig. 1B,C). Levels of protein expressed by pLUC or pGAD65 were increased by gene gun delivery, despite using 50- to 100-fold less pDNA compared to i.m. injection (Fig. 1B,C). This difference is likely attributed to the fact that pDNA coated on particles is directly delivered to the cytoplasm and/or nucleus of cells residing in the skin [33]. Intramuscular vaccination of pDNA in solution results in transfection of cells found at the site of injection as well as systemically, but this process is nevertheless relatively inefficient.

Our results contrast findings made by Joussemet *et al.* [38] in which no significant induction of type 2 T effectors or protection was observed when 3 week-old NOD female mice were treated with gene gun delivered pDNA encoding full-length human GAD65. The major difference between the respective studies is the cellular localization of the pDNA encoded GAD65 protein. Full-length GAD65 is intracellularly expressed and presentation of GAD65 epitopes would be largely limited to professional antigen presenting cells (APC) residing in the dermal region that have been directly transfected. Furthermore, intracellular GAD65 would be preferentially processed and presented via the MHC class I pathway, which would reduce the efficiency of $CD4^+$ T cell activation. We have previously shown for example, that i.m. delivery of pDNA encoding intracellular GAD65 to 4 wk-old NOD female mice had only a minimal effect on GAD65-specific $CD4^+$ T cell reactivity and disease progression [29]. On the other hand, GAD65-IgFc is secreted by the transfected cells so that an increased number of resident APC can endocytose the antigen, and preferentially present the GAD65-specific epitopes via the MHC class II pathway and efficiently stimulate $CD4^+$ T cells. Indeed other studies have demonstrated that the magnitude and nature of T cell immunity induced by pDNA vaccination is in part determined by the cellular localization of the protein (e.g. secreted versus intracellular) [39].

Although gene gun delivered pGAD65 proved to be more effective than i.m. injection, a significant number of NOD female mice nevertheless developed diabetes (Fig. 2A). This is likely due to the frequency and/or type of GAD65-specific immunoregulatory T cells induced following pGAD65 vaccination. For example, effective and long-term suppression of ongoing β cell autoimmunity requires a relatively high frequency of diverse subsets of immunoregulatory T effectors [26]. Protection mediated by gene gun delivered pGAD65 was

dependent and limited to the induction of IL-4 secreting GAD65-specific $CD4^+$ T cells (Fig. 3,Fig. 5). An increased frequency of IL-4 secreting insulin B chain-specific T cells was also detected in the pGAD65 gene gun vaccinated NOD mice (data not shown) indicating "epitope-spread" of type 2 immunoregulatory T effectors. Other types of immunoregulatory T cells such as IL-10 secreting Tr1 cells or FoxP3-expressing $CD4^+CD25^+$ T cells (Fig. 4), however, were not significantly increased following pGAD65 gene gun vaccination. Accordingly, the magnitude and diversity of immunoregulatory T effectors can be effectively enhanced by co-immunization with pDNA encoding other β cell autoantigens (e.g. insulin B chain) and/or anti-inflammatory cytokines such as IL-4, IL-10 and TGFβ. Similarly, gene gun delivery of autoantigen-encoding pDNA in a clinical setting may also require co-vaccination of "adjuvants" to increase the frequency and types of immunoregulatory T effectors [40].

In summary, this study demonstrates that the route and mode of antigen-specific pDNA vaccination are critical parameters for blocking β cell autoimmunity at a late preclinical stage of T1D. Particle-delivered pDNA offers a promising approach to enhance the therapeutic efficacy and safety of autoantigen-specific vaccination. These findings are especially relevant in view of current efforts assessing antigen-specific pDNA vaccination for the treatment of autoimmunity in the clinic [41;42].

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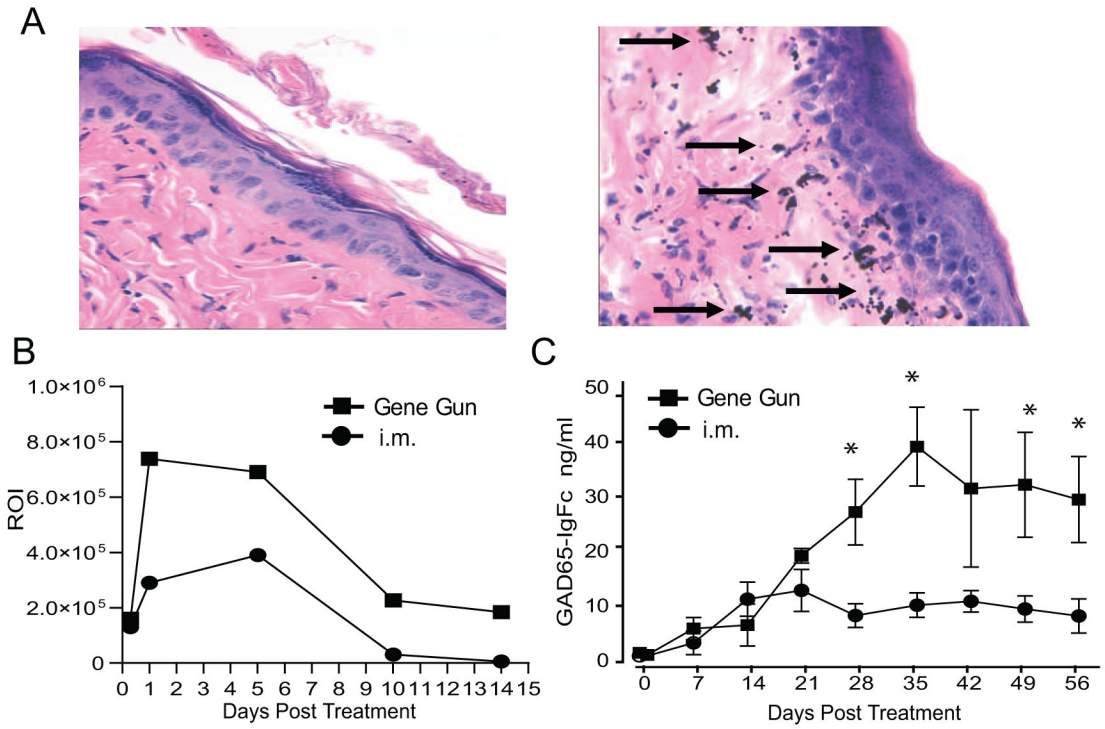

Figure 1. Increased transgene expression detected with gene gun versus i.m. delivery of pDNA

(A) Abdominal skin from untreated (left panel) or gene gun-treated (right panel) mice was harvested, sectioned and stained with H&E. Arrows indicate the site of 1.6 μM gold particles delivered by the gene gun. (B) Bioluminescence of NOD mice treated with pLUC by gene gun or i.m. was measured at the site of injection. (C) Kinetics of systemic expression of GAD65-IgFc transgene was assessed in 10 week-old NOD female mice treated at days 0, 7, 14, and 21 using gene gun or i.m. delivery; data represents the average ± SD of n=5 per group. Data are representative of a minimum of 2 experiments. *P≤0.05, gene gun versus i.m. using Student's T test, n=5 per group.

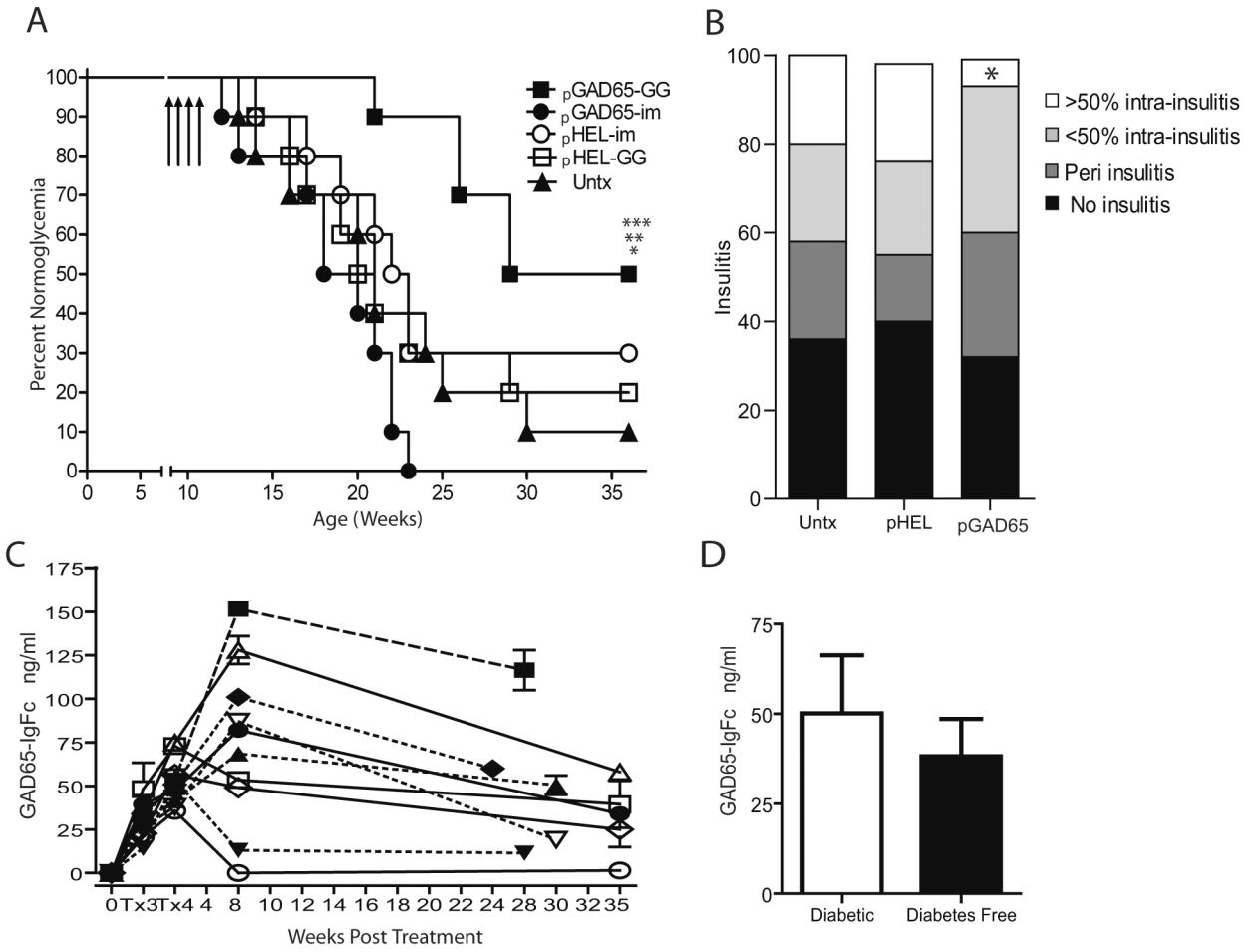

Figure 2. pGAD65 delivery via gene gun protects against diabetes

(A) Groups of 10 female NOD mice 10 weeks of age received 4 gene gun (GG) treatments of pGAD65 or pHEL, or 4 i.m. injections of pGAD65 or pHEL or were left untreated. The treatment groups were monitored for diabetes on a weekly basis. ***P=10^{-4}, pGAD65 gene gun versus pGAD65 i.m.; **P=0.005, pGAD65 gene gun versus untreated; *P=0.02, pGAD65 gene gun versus pHEL gene gun (Kaplan-Meier Log Rank Test). (B) The frequency of insulitis was assessed via H&E staining of pancreatic sections prepared from nondiabetic 36 week-old NOD female mice receiving via gene gun pGAD65 (n=5), or pHEL (n=2), or left untreated (n=1); a minimum of 40 islets was examined per mouse. *P<10^{-3}, >50% intra-insulitis for pGAD65 versus pHEL or untreated mice (Chi Square). (C) Serum levels of GAD65-IgFc were measured from pGAD65 gene gun treated NOD female mice in the above experiment. Dashed and solid lines indicate NOD female mice that became diabetic or remained diabetes-free during the course of the experiment, respectively. (D) The average serum levels of GAD65-IgFc in pGAD65 gene gun treated mice depicted in panel C. Data are representative of a minimum of 2 experiments.

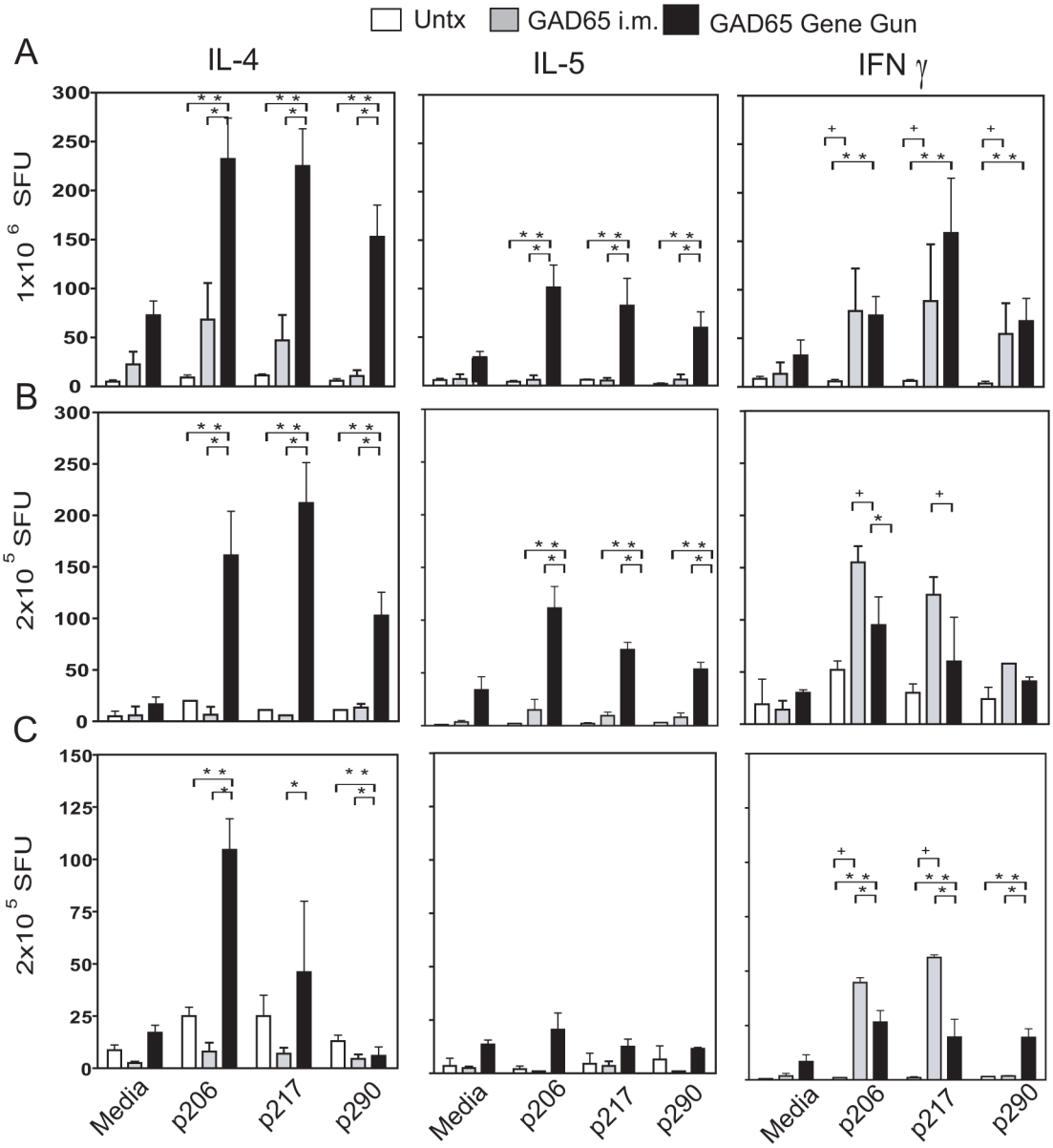

Figure 3. Gene gun induces GAD65-specific type 2 effector cells

Splenocytes (A), ILN (B), and PLN (C) were prepared from NOD female mice treated with pGAD65 delivered via gene gun or i.m. injection or from untreated NOD mice and stimulated with 40 μg/ml peptide. The frequency of GAD65 peptide-specific IL-4, IL-5, and IFN-γ, secreting T cells were measured via ELISPOT. Data are representative of a minimum of 2 experiments. *P≤0.05 pGAD65 gene gun versus pGAD65 i.m.; **P≤0.05 pGAD65 gene gun versus untreated; +P≤0.05 pGAD65 i.m. versus pGAD65 gene gun (One-way ANOVA).

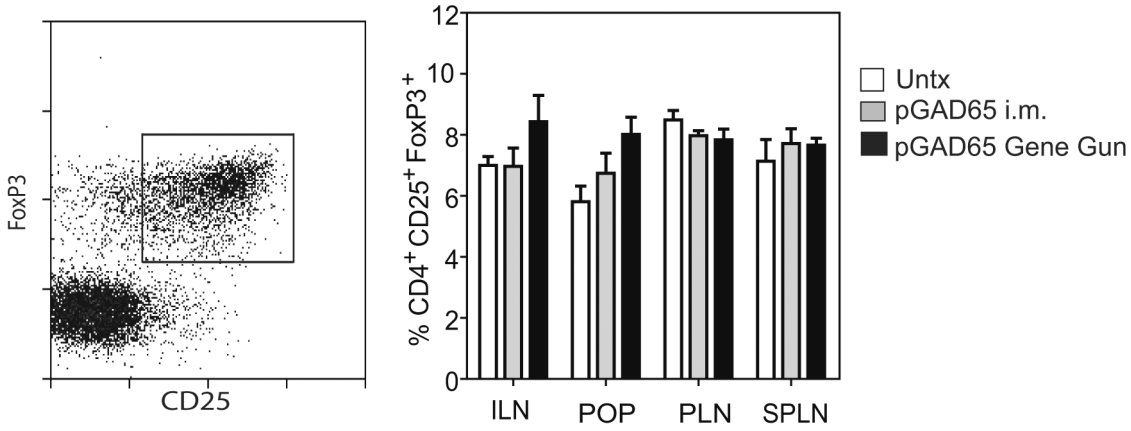

Figure 4. Gene gun and i.m. pGAD65 delivery had no effect on the frequency of FoxP3-expressing CD4$^+$CD25$^+$ T cells

The frequency of FoxP3-expressing CD25$^+$ T cells was determined by gating on CD3$^+$ and CD4$^+$ T cells found in ILN, popliteal lymph nodes (POP), PLN and spleen (SPLN) of NOD female mice treated at 10 weeks of age with pGAD65 delivered via gene gun or i.m. injection or left untreated. Data are representative of 2 experiments.

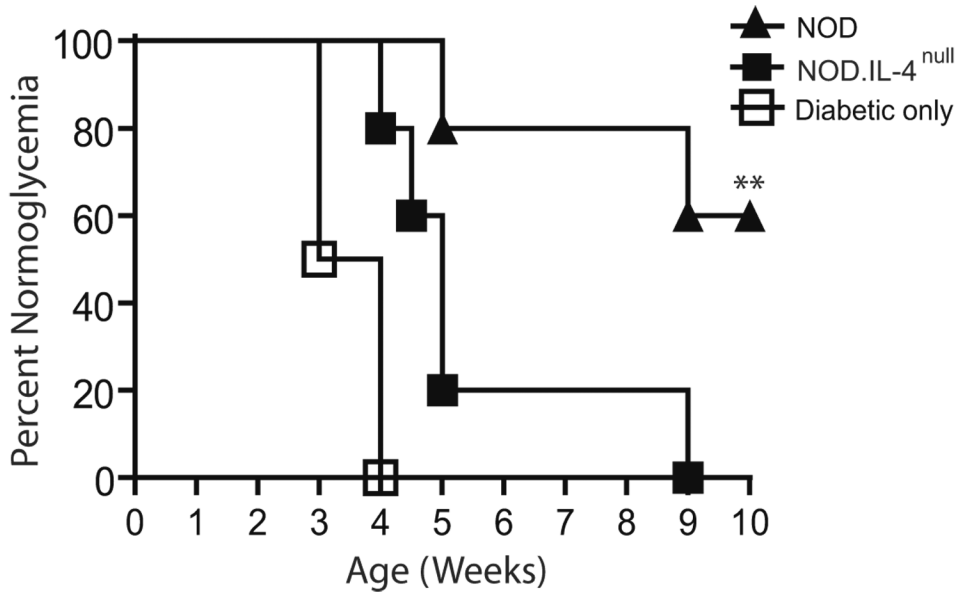

Figure 5. CD4$^+$ T cells from pGAD65 gene gun treated NOD mice prevent diabetes in an IL-4 dependent manner

A mixture of diabetogenic splenocytes and CD4$^+$ T cells purified from the spleens of wild type NOD or NOD.IL-4^{null} female mice treated with gene gun delivered pGAD65 were transferred into groups of 5 NOD.*scid* mice (**P< 0.005, Kaplan Meier Log Rank Test comparing NOD group to the NOD.IL-4^{null} group).