Combination Therapy With an Anti–IL-1β Antibody and GAD65 DNA Vaccine Can Reverse Recent-Onset Diabetes in the RIP-GP Mouse Model

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Type 1 diabetes is thought to be an autoimmune condition in which self-reactive T cells attack insulin-secreting pancreatic β-cells. As a proinflammatory cytokine produced by β-cells or macrophages, interleukin-1β (IL-1β) represents a potential therapeutic target in diabetes. We reasoned IL-1β blockade could be combined with islet antigen-specific approaches involving GAD of 65 kDa (GAD65)-expressing plasmids, as previously shown in combination therapies (CTs) with anti-CD3. Thus, we investigated whether anti–IL-1β antibody alone or combined with GAD65 vaccine could reverse diabetes development in a virus-induced mouse model. Given alone, anti–IL-1β had no effect on diabetes, while GAD65 plasmid resulted in 33% disease reversal after a 5-week observation. However, CTs cured 53% of animals and prevented worsening of glycemic control in nonprotected individuals for up to 12 weeks. While the GAD65 vaccine arm of the CT was associated with increased forkhead box p3δ regulatory T-cell frequency in pancreatic lymph nodes, islet infiltration by CD11b+high cells was less frequent upon CT, and its extent correlated with treatment success or failure. Altogether, our CTs provided prolonged improvement of clinical and immunological features. Despite unsuccessful clinical trials using anti–IL-1β monotherapy, these data hold promise for treatment of type 1 diabetic patients with IL-1β blockade combined with antigen-specific vaccines.

Inflammation of the pancreatic islets is observed in patients with type 1 and type 2 diabetes (1–3). Islets are sensitive to proinflammatory cytokines and the combination of interferon-γ (IFN-γ), tumor necrosis factor (TNF), and interleukin-1β (IL-1β) can act synergistically to lead to β-cell demise (4–6). IL-1β can be produced by cells such as monocytes, macrophages, dendritic cells, or neurons (1,7). In β-cells, IL-1β secretion in conditions of high glucose concentrations can have cytotoxic effects such as altered insulin secretion and β-cell apoptosis (8–10). Therefore, IL-1β blockade as a means to prevent or reverse type 1 and type 2 diabetes onset has become a potential therapeutic target.

In type 2 diabetes, neutralization of IL-1β signaling has resulted in improved glycemic control and insulin secretion in murine (11) and human studies (12,13). Microarray analyses have shown that compared with healthy control subjects, IL-1β gene expression was increased threefold in peripheral blood mononuclear cells (PBMCs) from both type 1 and type 2 recent-onset diabetic patients but significantly declined upon a 4-month course of insulin therapy and adjusted glycemic control (14). In type 1 diabetic patients, Pfleger et al. (15) found a positive association at 1 and 6 months postdiagnosis between levels of C-peptide and that of IL-1 receptor antagonist (IL-1Ra), the natural antagonist to proinflammatory cytokines IL-1α/β and the footprint of earlier IL-1
action. Moreover, short-term neutralization of IL-1β resulted in reduced chemokine receptor expression on CD11b+ circulating monocytes in people with type 1 diabetes. More recently, two randomized, placebo-controlled phase 2a trials both enrolling 69 type 1 diabetic patients have evaluated whether IL-1 blockade could improve β-cell function in recent-onset type 1 diabetes (17). One used a human monoclonal anti–IL-1β antibody (canakinumab), whereas the other used a recombinant human IL-1Ra (anakinra). Despite no severe safety issues, neither trial met its primary or secondary end points. Thus, despite good scientific rationale, the effect of IL-1β blockade alone in overt type 1 diabetes is, if any, minor, and combination with other agents will be needed. Antigen-specific therapies, due to their lack of inducing systemic side effects, are preferable to using additional systemic immunosuppressants.

Along these lines, while IL-1R–deficient NOD mice display slowed progression to diabetes but normal incidence (18), Ablamunits et al. (19) have recently shown synergistic reversal of type 1 diabetes after combined anti–IL-1β (using blocking antibody or IL-1Ra) and anti-CD3 antibody treatments. Furthermore, preclinical studies in our laboratory also demonstrated synergistic reversal of type 1 diabetes using similar anti-CD3 antibody combined with islet autoantigen-specific approaches to induce and maintain tolerance to β-cell antigens (20–22). Oral and nasal (pro) insulin were proved efficacious as part of the combination therapy (CT) in the NOD model (20,21), whereas promoter cytomegalovirus (pCMV)-encoded human glutamic acid decarboxylase of 65 kDa (pCMV-human GAD65 [hGAD65]) DNA plasmid was efficacious in the rat insulin promoter (RIP)–lymphocytic choriomeningitis virus (LCMV)–glycoprotein (GP) model (22). RIP-LCMV-GP mice express the GP from LCMV as a transgene under the RIP and turn diabetic within 2 weeks post–LCMV infection (23). Other plasmid DNA vaccines encoding mouse proinsulin II or GAD65–immunoglobulin Fc and IL-4 have also been shown to prevent and/or reverse hyperglycemia (HG) in NOD mice (24,25).

In this study, we investigated whether an IL-1β-neutralizing antibody alone or combined with an islet antigen GAD65-specific vaccine could reverse recent-onset diabetes development in the RIP-GP model. For this, we chose two main time points for our studies: 1) 5 weeks posttreatment (i.e., 1 week after the last administration of anti–IL-1β antibody); and 2) 12 weeks posttreatment (i.e., up to 2 months after the treatments have been stopped).

**RESEARCH DESIGN AND METHODS**

**Mice**

Transgenic RIP-LCMV-GP mice on the C57Bl/6 background have been described (26). Mice were bred in-house under specific pathogen-free conditions at the La Jolla Institute for Allergy and Immunology. A dose of 1 × 10⁶ plaque-forming units of LCMV Armstrong was injected intraperitoneally. Blood glucose values (BGVs) were recorded with an AccuCheck Glucometer (LifeScan Inc., Milpitas, CA).

**Treatments**

From day 7 postinfection, BGV was assessed for all mice, and when >250 mg/dL, mice were included in one treatment group. Novartis (Basel, Switzerland) kindly provided us with a monoclonal anti-mouse IL-1β antibody and its corresponding immunoglobulin G2a isotype (anticyclosporin B) control antibody. Both kinds of antibodies were administered intraperitoneally (200 μg) similar to Osborn et al. (11), once a week for 4 weeks, and GAD65 DNA vaccine was given as described previously in our laboratory (22) (i.e., intramuscularly, 100 μg/leg) at days 0, 4, and 11 post onset. At the end of different periods of observation, mice were killed, and blood, spleen, pancreatic lymph nodes (PLNs), as well as pancreata were collected and processed for analyses (27).

**Histology**

Mouse pancreata were embedded in optimum cutting temperature compound (VWR, Radnor, PA) and stored at −80°C. Six-micron–thick sections were stained for insulin and surface markers. Staining for CD4, CD8, and CD11b was performed using biotinylated primary antibodies (1/100; BD Biosciences) followed by the addition of streptavidin-coupled horseradish peroxidase (1/1,000; Vector Laboratories) and developed using the NovaRed Peroxidase substrate kit (Vector Laboratories). Sections were simultaneously stained with guinea pig anti-insulin primary antibody (DakoCytomation) followed by staining with goat anti-guinea pig alkaline phosphatase antibody (Sigma-Aldrich) and developed using Vector Blue AP III (Vector Laboratories). Sections were mounted with aqueous mounting solution (Lerner Laboratory). Pictures were taken with a Nikon 80i fluorescence microscope (Nikon). The criteria for scoring islet infiltration were as follows: 1) no insulitis; 2) peri-insulitis with no islet destruction or a few (<10) infiltrating cells; and 3) infiltrating insulitis with extensive islet destruction (or islet destroyed). The readings were performed in a blinded fashion by three different coauthors (S.F., A.B., and N.A.) at 1, 5, and 12 weeks post onset, respectively.

**Flow Cytometry Staining**

After various periods of observations post–treatment onset, blood, spleen, and PLNs were collected from killed mice and processed for further staining as previously described (27). Db/GP33 and Db/NP396 pentamers were purchased from Prolimmune (Sarasota, FL). Isolated cells were submitted to an Fc block incubation for 10 min at 4°C, followed by pentamer staining (3 μL per 50-μL reaction) at room temperature for at least 20 min. Last, surface antibodies were added and incubated for 20 min at 4°C. After acquisition of data on an LSRII cytometer (BD Biosciences), events were analyzed with FlowJo software (Tree Star), gating on Aqua Live/Dead dye–negative (Life Technologies) cells as well as CD19−CD8+ T cells.
Statistics
Data are expressed as mean ± SEM. Diabetes incidences were assessed using the Gehan-Breslow and log-rank tests. Graphs were plotted and statistics calculated with GraphPad Prism (versions 4 and 5; GraphPad). Comparisons between the different groups were performed with two-tailed, unpaired Mann-Whitney tests. A two-way ANOVA test was used for histological data analyses. The Wilcoxon signed-rank test was used to determine statistical significance for paired values: *P < 0.05, **P < 0.01, and ***P < 0.001.

Ethics
These studies were performed at La Jolla Institute for Allergy and Immunology upon approval of the Institutional Animal Care and Use Committee.

RESULTS
Anti–IL-1β/GAD65 DNA Vaccine Combination, but Not Anti–IL-1β Treatment Alone, Can Reverse HG in Diabetic RIP-GP Mice
To determine the efficacy of IL-1β neutralization as a monotherapy in the RIP-LCMV-GP diabetes model, we treated diabetic mice once weekly for 4 weeks with an anti–IL-1β antibody or an isotype control. After 5 weeks, all isotype-treated animals remained hyperglycemic, whereas 16.7% (1 of 6) of anti–IL-1β-treated mice reverted to euglycemia (Fig. 1A, left panels, and B). This difference did not reach statistical significance, suggesting that monotherapy based upon IL-1β blockade was ineffectual in the rapid-onset RIP-GP mouse model, paralleling the recent clinical trial experience (17).

Previous studies by our laboratory had shown success in curing diabetic RIP-GP mice combining anti-CD3 and pCMV-GAD65 DNA vaccines (22). Thus, we questioned whether the same DNA vaccine could be associated with IL-1β blocking antibody to induce immunological tolerance to GAD65. In accordance with Bresson et al. (22) and reflecting the initial success of DNA vaccines in preserving β-cell mass in a trial in recent-onset diabetes (28), 33% (4 of 12) of mice treated with GAD65 DNA plasmid and isotype antibody (below referred to as the isot/GAD group) reverted to euglycemia after 5 weeks, providing some limited protection compared with isotype-treated counterparts. However, when using a CT with both the GAD65 DNA vaccine and anti–IL-1β antibody (also referred to as the anti–IL-1β/GAD group), we found a significantly higher proportion of cured mice (9 of 17, 53%) (Fig. 1A, right panels, and B). In addition, we observed a significantly faster reversal in the anti–IL-1β/GAD group compared with the isot/GAD group (Fig. 1C), as reflected by the Gehan-Breslow statistical test that gives more weight to earlier events (Fig. 1B).

Overall, the average glycemia levels at entry were comparable among all groups, therefore excluding a bias in this regard (Supplementary Fig. 1B). Moreover, the combination of anti–IL-1β antibody with either empty pCMV plasmid or pCMV expressing the B chain of insulin resulted in reversal rates similar to anti–IL-1β-treated mice (Supplementary Fig. 1C), excluding nonspecific effects as observed previously (20,21) and indicating major histocompatibility complex (MHC) restriction of GAD65-based induction of tolerance. Supporting the point that antigenic vaccines will operate in an MHC-restricted manner, earlier studies have shown that DNA vaccines expressing proinsulin but not GAD65 can protect NOD and Balb/c, but not C57Bl/6, mice from diabetes due to differences in insulin B chain presentation on the H-2b background (20,22,29).

Anti–IL-1β/GAD65 DNA Vaccine Combination Prevents Worsening of Glycemic Control in Nonprotected Mice
Glycemic control is evaluated in diabetic individuals by measuring the percentage of glycated hemoglobin. Importantly, uncontrolled, severe HG steeply increases the risk to develop complications such as retinopathy or neuropathy (30). Moreover, there is evidence for decreased responsiveness to anti-CD3-induced decline in C-peptide after 2 years in type 1 diabetic patients with poorly controlled glycemia (Protégé Trial) (31). Therefore, we examined closely the glycemic status of nonprotected mice, distinguishing mice with mild and severe HG (i.e., with final BGVs comprised between 251 and 400 and 401 and 600 mg/dL, respectively). The isot/GAD and CT groups both presented eight nonprotected mice, out of which 12.5% (one of eight) vs. 75% (six of eight) mice had mild HG, respectively (Fig. 1D and B). These data demonstrate that in cases in which IL-1β blockade as an add-on to GAD65-based tolerance induction does not cure mice from diabetes, it can nevertheless stabilize the extent of HG in RIP-GP mice.

Effector CD8 T-Cell Responses Are Reduced and Regulatory T-Cell Responses Are Improved After GAD65 DNA Vaccine and CT
Next, we assessed immunological parameters systemically (in the spleen) and locally (in PLNs) in mice. Cells were isolated and restimulated with LCMV-immunodominant CD8 T-cell epitope GP33–41 peptide in order to evaluate cytokine production. No significant difference was observed between isotype- and anti–IL-1β–treated animals, consistent with the absence of efficacy of IL-1β monotherapy (Fig. 2A and B). In comparison with isotype antibody treatment, the isot/GAD group presented diminished frequencies of IFN-γ– and TNF-secreting cells in the spleen (Fig. 2A), but not in PLNs (Fig. 2B). Likewise, compared with anti–IL-1β treatment, we observed reduced fractions of cytokine-secreting cells in the spleen of the CT group (Fig. 2A) and also in PLNs regarding TNF (Fig. 2B).

The effect of IL-1β on the development and/or function of forkhead box p3 (Foxp3)-expressing regulatory T cells (Tregs) is not completely understood. Indeed, while some argued for a role of IL-1β in breaking immunological tolerance via expansion of CD25+ effector T cells (32) and in differentiating T helper 17 cells at the expense of Tregs
(33), others argued for a costimulatory effect of IL-1β on the expansion of Tregs and their maintenance of Foxp3 expression (34,35). In this study, the frequencies of CD4+CD25+Foxp3+ Tregs were comparable between isotype- and anti–IL-1β–treated animals in the spleen and PLNs (Fig. 2C and D). Of note, the CT group displayed a higher percentage of Foxp3-expressing Tregs in PLNs (Fig. 2D), but not in the spleen (Fig. 2C). Interestingly, effector T-cell (Fig. 2A and B) and Treg (Fig. 2C and D) responses were similar upon GAD65 plasmid treatment with or without anti–IL-1β antibody. This indicates that the beneficial neutralization of IL-1β in the CT does not act through the inhibition of cytokine production by GP33-specific (autoreactive in the RIP-LCMV model) CD8 T cells nor increased Foxp3+ Treg fractions.

Anti–IL-1β/GAD65 DNA CT Can Reverse HG Up to 8 Weeks After the Treatment Has Been Stopped

From a translational point of view, one important issue is to know whether the beneficial effect provided by the CT is durable for extended periods of time, notably several weeks after the treatment has been stopped. Thus, we followed BGVs in mice up to 12 weeks post onset (i.e., up to 8 weeks after the last antibody administration). Given our data at
5 weeks post onset, monotherapies with anti–IL-1β or isotype antibody alone were not tested in this study. We observed a higher frequency of mice cured by the CT (6 of 14, 43%) than by the isot/GAD treatment (2 of 11, 18%). This difference was not significant by log-rank test analysis ($P = 0.059$) (Fig. 3A and Supplementary Fig. 1E), but mean BGVs in CT-treated mice were significantly lower than those in their isot/GAD-treated counterparts ($P < 0.001$) (Fig. 3B). Then, we performed analyses on nonprotected mice and found that CT reduced the frequencies of mice with severe HG at the end of the study (Fig. 3C), reminiscent of our findings at 5 weeks post onset. Likewise, 75% (six of eight) of mice that were not cured by the CT at 12 weeks post onset nonetheless maintained glycemia levels comparable to those measured at entry (Fig. 3D). In isot/GAD-treated mice, this proportion was lower (33%, three of nine), and BGVs at euthanasia were significantly higher than at entry (Fig. 3D). Finally, we assessed how many nonprotected mice presented one or several episodes of transient reversal to euglycemia, defined by one BGV reading $<250$ mg/dL during the course of our observation. Compared with the isot/GAD group, CT induced transient reversal to euglycemia more frequently (six of eight, 75%, vs. three of nine, 33%) and more durably (4.16 vs. 1.67 weeks on average; $P = 0.049$) (Fig. 3E).

**Figure 2**—Effect of CD8 T-cell responses are reduced and Treg responses are improved after GAD65 DNA vaccine and CT. Frequencies of IFN-γ- and TNF-producing GP33-41-specific CD8+ effector T cells in spleen (A) and PLNs (B) of treated mice. After 5-week treatment, spleens and PLNs were mashed onto 70-μm cell strainers to obtain single-cell suspensions. Cells were then plated in round-bottom 96-well plates and left unstimulated or stimulated for 4 to 5 h with 5 μg/mL (CD8 T-cell–specific) GP33 peptide in the presence of brefeldin A (10 μg/mL). Cells were then surface stained with fluorochrome-coupled CD8 antibodies, fixed, permeabilized, and stained with fluorochrome-coupled IFN-γ and TNF antibodies. Data are expressed as percent of gated CD8+ cells. Frequencies of Foxp3-expressing CD4+CD25+ Tregs in spleen (C) and PLNs (D) of treated mice. In parallel to the intracellular cytokine staining, cells were stained for CD4, CD25, and intracellular Foxp3. Data are expressed as percent of gated CD25+Foxp3+ within CD4+ cells. Results from two to three pooled experiments with two to three mice per group are shown. * $P < 0.05$. Ab, antibody.

**Effect CD8 and CD4 T-Cell Responses at 12 Weeks Post Onset Are Largely Unaffected by the CT in Spleen, PLNs, and Blood**

In the perspective of unveiling potential immune biomarkers that would be relevant for human clinical trials, the assessment of the presence of CD8 T cells specific for islet antigens via multimer (e.g., Quantum-dot) flow cytometry staining has gained importance, is now technically achievable, and could thus be considered as a possible secondary end point in the future (28,36,37). In this study, we took advantage of the RIP-GP model to monitor frequencies of CD8 T cells specific for LCMV GP33–41 (Fig. 4A) and nucleoprotein396–404 (Fig. 4B) epitopes via pentamer staining in the blood, as this is the main accessible compartment in human patients. Our results after a 12-week follow-up did not reveal any differences in terms of LCMV-specific CD8 T-cell frequencies for either epitope in the blood (Fig. 4) or spleen (Supplementary Fig. 2). Furthermore, intracellular cytokine staining in CT-treated animals showed decreased frequencies of GP33–41-specific CD8 effector T cells producing TNF in PLNs (Fig. 4B, left panel). However, this effect was limited, as this was not observed regarding IFN-γ (Fig. 4B, right panel) nor was it observed in the spleen. Effector T-cell and Treg CD4 responses in the spleen and PLNs of isot/GAD- and CT-treated mice were also
comparable (Supplementary Fig. 3). Altogether, these results suggest that some, but not all, CD8 and CD4 T-cell responses in the spleen and PLNs are consistent with the improved mean glycemia levels and diabetes incidence in CT-treated mice.

**Islet Infiltration by CD8⁺ and CD11b⁺ Cells Is Reduced After CT at 5 and/or 12 Weeks Post Onset**

Immune cell infiltrates in the pancreas of RIP-LCMV mice contain (CD4 and CD8) T cells as well as innate immune cells such as CD11b⁺ macrophages and CD11c⁺ dendritic cells. It is noteworthy that diabetes onset is preceded by an early MHC class II upregulation and infiltration of macrophages in mouse islets (38), while macrophages also count among the different inflammatory cell infiltrates in human type 1 diabetic patients (39). Thus, we assessed the extent of overall insulitis and islet infiltration by CD11b⁺ cells (presumably macrophages) together with CD4⁺ and CD8⁺ T cells. We found that in accordance with diabetes incidence curves at the 5-week follow up, anti–IL-1β/GAD-treated animals presented significantly reduced insulitis compared with isot/GAD- and anti–IL-1β–treated groups (Supplementary Fig. 1D). In isot/GAD- and CT-treated groups, we found that overall, CD4⁺ and CD8⁺ cell infiltration was similar at 1 and 5 weeks post–treatment onset (Fig. 5A, middle and bottom panels). However, mice treated with anti–IL-1β/GAD65 DNA vaccine displayed less severe infiltration by CD11b⁺ cells compared with isot/GAD65-treated mice at 1, 5, and 12 weeks post–treatment onset (Fig. 5A, top panel). In accordance
with histological data, flow cytometry data (Fig. 5B) revealed that islet CD8+ and CD11bhigh cells were decreased upon CT compared with isot/GAD treatment, although this was not statistically significant for CD11bhigh cells (P = 0.052) but may be explained by the stringency of our gating strategy (Fig. 5B, top panel).

The Extent of Islet Infiltration by CD11bhigh but Not T Cells Correlates With Efficacy of the CT

We then assessed the extent of different immune cell infiltrations distinguishing mice that were, or failed to be, cured by their respective treatment. Doing so, we found that CT-treated mice that reverted to euglycemia presented a significantly lower fraction of islet CD11bhigh cells than mice that were also treated with the CT but stayed hyperglycemic (Fig. 6A). Of note, this dichotomy was not observed in the case of isot/GAD treatment, suggesting that this phenotype was likely due to the IL-1β blockade component of the CT. Moreover, we assessed on a per-mouse basis the correlation between pancreas-derived CD11bhigh macrophage infiltration in relation to mouse glycemia. In accordance with our findings in Fig. 6A, the positive linear relationship between these two parameters was strong (Pearson r = 0.78) and significant (P = 0.01) in the CT group, but not in the isot/GAD group (Pearson r = 0.27; P = 0.55) (Fig. 6B). In contrast, no significant association was found when correlating mouse glycemia to pancreas infiltration by CD8 T cells (Fig. 6C and D), CD4 T cells (data not shown), or Foxp3+ Tregs (Fig. 6E and F). We conclude that on a per-mouse
basis, the extent of islet infiltration specifically by CD11b\textsuperscript{high} macrophages, but not T cells, might serve as a biological marker for success or failure of the anti–IL-1\textbeta/GAD65 CT.

**DISCUSSION**

In accordance with the minor effect of IL-1R deficiency on diabetes development in the RIP-LCMV and NOD models (18,40), our present results showed no protective effect of anti–IL-1\textbeta therapy alone. This lack of efficacy is in line with the negative outcome of two recently published placebo-controlled phase IIa trials in recent-onset type 1 diabetic patients (17). Similarly to a recently published trial using DNA vaccines (28), we found that GAD65 plasmid alone provided some efficacy, albeit limited. Thus, the enhanced effect of the CT indicates utility of anti–IL-1\textbeta antibody as an induction agent rather than a monotherapy.

In human trials, it appeared that higher C-peptide levels at study enrollment, reflective of better preserved \(\beta\)-cell function, was associated with increased responsiveness to drugs such as anti-CD3 (31,41). Following the same reasoning, we stratified glycaemia levels at treatment onset and found that CT induced disease reversal in more than half of the treated mice if glycaemia at enrollment was comprised between 250 and 400 mg/dL (Figs. 1 and 3) but not any more if 400 mg/dL (Supplementary Fig. 1A). This suggests that a minimal residual \(\beta\)-cell mass is necessary for protection. Subsequently, we closely analyzed the clinical outcome of nonprotected mice, creating subsets with mild (BGV 251–400 mg/dL) versus severe (BGV 401–600 mg/dL) HG. This refined analysis allowed us to discover that the CT maintained BGVs to a milder HG status compared with the isot/GAD group. It is noteworthy that maintenance of glycemic control was not observed upon monotherapy with anti–IL-1\textbeta antibody alone (Fig. 1A). We showed that compared with anti–IL-1\textbeta-treated mice, CT acted by increasing Treg frequencies in PLNs (Fig. 2). However, in comparison with GAD65 DNA treatment alone, CT did not further increase Treg fractions. We cannot exclude, however, that the Treg pool

\[\text{Figure 5—Islet infiltration by CD8}^+\text{ and CD11b}^+\text{ cells is reduced after CT at 5 and/or 12 weeks post onset. A (left panel):} \text{CD4}^+, \text{CD8}^+, \text{and CD11b}^+\text{ cell infiltration in mice treated with GAD65 DNA with isotype or anti–IL-1\textbeta (Ab). Pancreas sections from treated mice were stained for insulin as well as CD4, CD8, and CD11b in parallel. Classification was performed as follows: 1) no insulitis (white bars); 2) peri-insulitis with no islet destruction or a few (<10) infiltrating cells (gray bars); and 3) infiltrating insulitis with extensive islet destruction (or islet destroyed) (black bars). Two-way ANOVA tests determined statistically significant differences (*}P<0.05). \text{A (right panel): Representative pictures at ×20 original magnification. B: At 12 weeks post onset, the pancreas of mice was processed and cells stained for fluorescence-activated cell sorting analyses to confirm histological analyses (*}P<0.05, Mann-Whitney test). The extent of CD4\textsuperscript{+}, CD8\textsuperscript{+}, or CD11b\textsuperscript{+} cell infiltration was counted as follows: 1 week post onset: 29–134 islets from three mice per group; 5 weeks post onset: 16–111 islets from two to five mice per group; and 12 weeks post onset: 13–39 islets from three mice per group.\]
presented better regulatory properties or a higher fraction of cells with specificity to GAD65. Of note, we have previously shown that when given with anti-CD3, the same pCMV-hGAD65 DNA plasmid promoted the expansion of T cells specific for the COOH-terminal domain of the GAD65 protein (22). Consistent with this, we also observed a similar reactivity to this domain upon treatment of RIP-GP mice with aluminum hydroxide–formulated GAD65 protein (27). Depending on the context, regulatory cells displayed an IL-10–producing or T helper 2–biased, IL-5–secreting phenotype. In this study, we analyzed by enzyme-linked immunsorbent spot whether protection induced by the CT could be associated with increased immunomodulatory cytokine levels (IL-10, transforming growth factor-β, or IL-4), for which elevation can be protective in animal models of diabetes (42–44). However, we did not detect significant differences in the spleen (Supplementary Fig. 3C) or PLNs (Supplementary Fig. 3D).

Upon treatment with anti–IL-1β/anti-CD3, Ablamunits et al. (19) have observed an augmentation in splenic M2-type CD11b+ and CD11c+ cells with increased arginase-1, and decreased nitric oxide, production. In our case, fewer CD11bhigh cells were observed in the pancreas of CT-treated mice, but further phenotypic characterization of these cells will be warranted. Since myeloid dendritic cells can also under some circumstances express the CD11b integrin marker, the exact nature of these CD11bhigh (presumably) macrophages needs to be defined.

Sanda et al. (16) have shown that short-term neutralization of IL-1β in type 1 diabetic patients resulted in a decrease of CD11b expression on monocytes isolated from PBMCs, which is consistent with our observations. Although the phenotype of CD11bhigh cells present in anti–IL-1β/GAD65-treated mice is still unclear, our data rather argue for a diminished cell-infiltration degree.

We found a significant correlation between mouse glycemia and the extent of islet infiltration by CD11bhigh but not T cells upon indicated treatment. Correlation of CD11bhigh (A), CD8 T (C), and CD4+CD25+Foxp3+ Tregs (E) found in the pancreas of mice upon indicated treatment. Correlation between mouse glycemia at 12 weeks posttreatment and the percentage of CD11bhigh (B), CD8 T (D), and CD4+CD25+Foxp3+ Treg (F) infiltration in mouse pancreata. 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. aIL-1β/a-IL-1β, anti–IL-1β; T1D, type 1 diabetes. *P = 0.0159.
glycemic status and a defined immune marker, predictive correlations will yield a major advantage in that these would allow to predict responsiveness to (a) tested drug(s) either before enrollment or early posttreatment. Because of the inaccessibility of the target organ in type 1 diabetic patients, it will be invaluable to identify biomarkers that can be measured in the peripheral blood. In our case, although the mechanism(s) underlying such an impaired recruitment/infiltration of CD11b<sup>+</sup> and CD8<sup>+</sup> cells to the target organ are unclear, it will be interesting to track the behavior of those cells at, or early after, onset in PBMCs. Preliminary data 4 days posttreatment onset in the blood tend to exclude a role for CXCR3 or costimulatory molecule CD40, CD80, and CD86 expression on CD11b<sup>+</sup> cells (Supplementary Fig. 4).

Four recent phase II/III clinical trials, two aiming at IL-1β blockade (17) and two promoting induction of tolerance to GAD65 (45,46), have failed to meet their primary end points. In all cases, later studies in mice models indicated that there would be no effect (19,27). We have shown in this study that compared with isotype/GAD-treated mice, CTs increased the frequency of diabetes reversal, albeit more modestly than other CTs involving synergetic effects of anti-CD3 treatments (19–22). Additionally, however, most nonprotected mice still maintained comparable glycemic levels compared with baseline, suggesting a subtle but discernable effect of the IL-1β blockade arm of the CT. Altogether, this suggests that two such therapies that target IL-1β blockade and tolerance induction to GAD65, yielding suboptimal outcome when given separately, might be promising candidates for combination in future human trials. We believe that such trials could begin soon, since it has been established that DNA vaccines encoding islet antigens such as proinsulin, can preserve C-peptide secretion and are well-tolerated by type 1 diabetic patients (28).

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