Sofie Robert,[1] Conny Gysemans,[1] Tatiana Takiishi,[1] Hannelie Korf,[1] Isabella Spagnuolo,[2] Guido Sebastiani,[2] Karolien Van Huynegem,[3] Lothar Steidler,[3] Silvia Caluwaerts,[3] Pieter Demetter,[4] Clive H. Wasserfall,[5] Mark A. Atkinson,[5] Francesco Dotta,[2] Pieter Rottiers,[3] Tom L. Van Belle,[1] and Chantal Mathieu[1]

Oral Delivery of Glutamic Acid Decarboxylase (GAD)-65 and IL10 by *Lactococcus lactis* Reverses Diabetes in Recent-Onset NOD Mice

Diabetes 2014;63:2876–2887 | DOI: 10.2337/db13-1236

Growing insight into the pathogenesis of type 1 diabetes (T1D) and numerous studies in preclinical models highlight the potential of antigen-specific approaches to restore tolerance efficiently and safely. Oral administration of protein antigens is a preferred method for tolerance induction, but degradation during gastrointestinal passage can impede such protein-based therapies, reducing their efficacy and making them cost-ineffective. To overcome these limitations, we generated a tolerogenic bacterial delivery technology based on live *Lactococcus lactis* (LL) bacteria for controlled secretion of the T1D autoantigen GAD65$_{370–575}$ and the anti-inflammatory cytokine interleukin-10 in the gut. In combination with short-course low-dose anti-CD3, this treatment stabilized insulitis, preserved functional β-cell mass, and restored normoglycemia in recent-onset NOD mice, even when hyperglycemia was severe at diagnosis. Combination therapy did not eliminate pathogenic effector T cells, but increased the presence of functional CD4$^+$Foxp3$^+$CD25$^+$ regulatory T cells. These preclinical data indicate a great therapeutic potential of orally administered autoantigen-secreting LL for tolerance induction in T1D.

Type 1 diabetes (T1D) is a chronic autoimmune disease characterized by the destruction of insulin-producing β-cells in the islets of Langerhans in the pancreas (1). Past and current attempts to cure this immune-mediated disease included antigen-specific therapies and systemic immunomodulatory and immunosuppressive agents (1–4), but many of these trials suffered from a lack of efficacy, as reflected by only transient β-cell preservation, or adverse effects due to immune suppression. Autoantigen-specific therapies remain appealing to restore tolerance in T1D patients because they hold promise in targeting only β-cell–reactive T cells without impairing normal immune responses to diabetes-unrelated antigens.

Studies in man and NOD mice, the most widely used preclinical model of T1D, have identified glutamic acid decarboxylase (GAD65) as one of the major autoantigens in this disease (5,6). GAD65 is expressed not only in the central nervous system but also in the β-cell (7), and peptide sequences within GAD65 behave as autoantigens in T1D both in man and in NOD mice (5,8). The majority of prediabetic and newly diagnosed T1D patients test positive for anti-GAD65 autoantibodies (6,9) or GAD65-reactive T cells (10). In NOD mice, GAD65-specific T-cell responses can be detected as early as 4 weeks of age (5). Although its role in initiating human T1D remains controversial, preclinical studies in NOD mice have shown that vaccines based on GAD65

[1]Clinical and Experimental Endocrinology, Katholieke Universiteit Leuven, Leuven, Belgium
[2]Diabetes Unit, Department of Internal Medicine, Endocrine and Metabolic Sciences and Biochemistry, University of Siena and Fondazione Umberto Di Mario ONLUS, Siena, Italy
[3]ActoGeniX NV, Zwijnaarde, Belgium
[4]Department of Pathology, Université Libre de Bruxelles, Brussels, Belgium
[5]Department of Pathology, Immunology and Laboratory Medicine, College of Medicine, University of Florida, Gainesville, FL
Corresponding author: Chantal Mathieu, chantal.mathieu@med.kuleuven.be.

Received 14 August 2013 and accepted 24 March 2014.
This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db13-1236/-/DC1.
T.L.V.B. and C.M. share senior authorship.

See accompanying article, p. 2603.

protein or peptides can prevent diabetes (11–15) but lose efficacy after diabetes onset (16), even when combined with anti-CD3 (17). Similarly, phase II/III clinical trials in recent-onset T1D patients treated with GAD65 protein in an alum formulation also failed to show efficacy (18,19), despite earlier encouraging results in a small-scale study (4).

Formulation and route of administration play important roles in inducing antigen-specific tolerance and imparting clinical efficacy (20). A preferred route for tolerance induction exploits the gut-associated lymphoid tissue, the largest immune system in the body, with the physiological role to digest dietary antigens in a manner that does not result in untoward immune reactions. Oral tolerance is associated with induction of regulatory T cells (Tregs) and bystander suppression (21,22). However, actual delivery of compounds to this primarily tolerogenic environment is impeded by degradation during gastric passage (23).

We hypothesized that a major reason for the failure of studies involving oral antigen administration is that the delivery to the tolerizing microenvironment of the gut mucosa is ineffective and that the regulatory responses induced by antigen-based therapy peak too late to be effective by themselves. We have previously demonstrated that a novel system for antigen delivery in the distal gut, based on genetically modified *Lactococcus lactis* (LL) (ActoBiotics), offers a safe and effective means to deliver autoantigens to the immune system and induce tolerance in NOD mice (24). Here, we tested whether oral administration of genetically modified LL-expressing $GAD65_{370–575}$ and interleukin-10 (IL10) can reverse diabetes in recent-onset diabetic NOD mice when combined with short-course low-dose anti-CD3 (termed "combi-GAD therapy"). We also analyzed metabolic parameters and the phenotype and function of T-cell subsets to shed light on the mechanism of action.

RESEARCH DESIGN AND METHODS

Genetically Modified LL Strains

LL strains secreting human $GAD65_{370–575}$, having 98% amino acid identity to mouse $GAD_{370–575}$ (Supplementary Fig. 1*A* and *B*), with or without human IL10 were generated by transformation of the parental MG1363 strain or the IL10-secreting sAGX0037 strain with $GAD65_{370–575}$-encoding plasmid (24,25). The amino acid sequence for GAD65 was retrieved from the National Center for Biotechnology Information (NP_000809.1).

Bacteria and Media

Bacterial strains were grown and harvested as previously described (24).

Animals

NOD mice were housed and inbred as previously described (24). NOD mice were monitored three times weekly for glycosuria (Clinistix; Bayer Diagnostics, Tarrytown, NY) and considered diabetic when blood glucose concentrations exceeded 200 mg/dL for 2 consecutive days (Accu-Chek; Roche, Almere, the Netherlands).

Treatments and Follow-up

Hamster anti-mouse CD3 monoclonal antibody (mAb) 2.5 μg/day i.v. (145-2C11; Bio X Cell, West Lebanon, NH) was administered for 5 consecutive days. LL-pT1NX, LL-IL10, LL-OVA+IL10, LL-$GAD65_{370–575}$, or LL-$GAD65_{370–575}$+IL10 were given by intragastric inoculation (2×10^9 cfu/day) five times a week for 6 weeks. Weight and glycemia were measured three times a week. Diabetes remission was defined as the absence of glycosuria and glycemia values $<$200 mg/dL on 2 consecutive days.

β-Cell Parameters

Pancreata were harvested for histological analyses and/or insulin content determination as described (24). Intraperitoneal glucose tolerance tests (IPGTTs) were performed 1-2 weeks before killing the mice. Mice were fasted for 16 h, injected intraperitoneally with glucose (2 g/kg), and glycemia was measured at 15, 30, 60, 90, and 120 min postinjection. Serum C-peptide levels were measured by ELISA (Millipore, Billerica, MA) (24). Insulin autoantibodies were measured as published (26).

Flow Cytometry

Single-cell suspensions were prepared from lymphoid organs by mechanical disruption. Pancreata were digested in prewarmed digestion medium (RPMI medium + 5% FCS, 2 mmol/L L-glutamine, 0.05 mmol/L β-mercaptoethanol, 100 units/mL penicillin, 100 μg/mL streptomycin, 1 mg/mL collagenase NB8, and 0.02 mg/mL DNase I) for 30 min at 37°C. Lamina propria of small intestine and colon was isolated as previously described (27). Cells were stained for $CD4^+CD25^+Foxp3^+$ Treg phenotyping as previously described (24). Intracellular staining for interferon-γ (IFN-γ) was performed after in vitro restimulation with 50 ng/mL phorbol myristic acid and 1 μg/mL ionomycin for 6 h in the presence of monensin and brefeldin A 1:1,000 (eBioscience) for the last 4 h. Samples were acquired on a Gallios flow cytometer, and data were analyzed with Kaluza software (Beckman-Coulter).

Antigen-Specific Proliferation Assay

Total splenocytes (5×10^5) and pancreatic lymph node (PLN) cells ($1–2 \times 10^5$) were cultured with irrelevant ovalbumin peptide ($OVA_{323–339}$ peptide; Innovagen) or GAD p524 (524–543 peptide; Eurogentec, Seraing, Belgium) for 72 h. PLN cells were cultured with mitomycin-treated splenocytes ($1–2 \times 10^5$) isolated from normoglycemic NOD mice (10 weeks). ^{3}H-thymidine (Amersham; Buckinghamshire, U.K.) was added for the last 18 h (1 μCi). Cells were harvested on glass filter paper and counts per minute (cpm) were determined in a liquid scintillation counter. Cells isolated from mice primed with GAD p524 were used as positive control.

Laser Capture Microdissection and Real-Time PCR

Tissue slides were processed as previously described (28). Lymphocytic infiltrates were captured using high specificity cap support. Real-time PCR analyses were performed with a Verity Thermal Cycler and ViiA 7 system for real-time

PCR according to the manufacturer's instructions. Data analysis was performed using ViiA 7 research use only software to collect data and Expression suite 2.1 software to evaluate amplification plot efficiency and to export Ct values. Analysis was performed by 2^-dCt method following normalization to CD45.

Cell Isolations

$CD4^+CD25^-$ T cells were isolated by negative selection using antibodies to CD25, CD8, B220, CD11c, CD11b, major histocompatibility complex class II, and sheep anti-rat IgG Dynabeads (Invitrogen, Merelbeke, Belgium). $CD4^+CD25^+$ T cells were isolated by positive selection from $CD4^+$ T cells using anti-mouse CD25 biotin (eBioscience) and antibiotin MicroBeads (Miltenyi Biotec).

In Vitro Polyclonal Suppression Assay and IFN-γ Detection

Suppressive function of $CD4^+CD25^+$ Tregs was assessed in an in vitro polyclonal suppression assay conducted as previously described (24). IFN-γ was measured in cell-free supernatants as previously described (24).

In Vivo Suppression Assay

$CD4^+CD25^+$ T cells from cured anti-CD3– or combi-GAD–treated mice were coinjected with CD25-depleted splenocytes from diabetic mice in 8–12-week-old NOD/SCID mice as previously described (24).

In Vitro Antigen-Specific Suppression Assay

GAD- or OVA-specific responder cells were generated by immunizing mice in the rear footpads with 100 μg GAD65 p524 or $OVA_{323–339}$ emulsified 1:1 in complete Freund adjuvant (Difco Laboratories, Detroit, MI). Control mice were injected with PBS:complete Freund adjuvant emulsion. After 11 days, popliteal lymph nodes were dispersed into single-cell suspension. GAD- or OVA-specific responders (50×10^3) were cultured in round-bottom 96-well plates for 4 days with GAD p524 or $OVA_{323–339}$ (1 μg/mL), respectively, and $CD4^+CD25^+$ Tregs (50×10^3) isolated from spleen and lymph nodes of cured combi-GAD–treated mice. ^{3}H-thymidine addition was as in the antigen-specific proliferation assay.

Statistics

Differences in diabetes incidence were assessed using the Mantel-Cox log-rank test. Statistical significance of other comparisons was tested using two-tailed unpaired *t* test (data with normal distribution) or Mann-Whitney *t* test, as indicated. $P < 0.05$ was considered statistically significant. Graphs were plotted and statistics calculated with GraphPad Prism software.

RESULTS

Diabetes Reversal by Combi-GAD Therapy

We constructed various bacterial strains secreting full human GAD65 protein or fragments thereof. For in vivo validation, we selected LL-secreting human $GAD65_{370–575}$ (LL-$GAD65_{370–575}$) or secreting human $GAD65_{370–575}$ in combination with human IL10 (LL-$GAD65_{370–575}$+IL10) based on secretion capacity verified by Western blotting and ELISA, bacterial growth profile, and the presence of relevant $CD4^+$ T-cell epitopes (8) (data not shown). We examined whether oral administration of LL-$GAD65_{370–575}$ could reverse autoimmune diabetes by treating recent-onset diabetic NOD mice with LL-$GAD65_{370–575}$+IL10, LL-$GAD65_{370–575}$, LL-IL10, or LL-OVA+IL10 (2×10^9 cfu) through gavage for 5 days a week for 6 weeks with or without an initial course (days 0–4) of systemic low-dose anti-CD3 mAb (2.5 μg/day) (24). As expected, in the absence of treatment, none of the recent-onset diabetic NOD mice returned to normoglycemia (Fig. 1*A*). Administration of LL-$GAD65_{370–575}$+IL10 alone normalized glycemia in 20% of mice by the end of the treatment. Treatment with anti-CD3 alone or anti-CD3 plus empty vector control LL-pT1NX restored normoglycemia in 32% and 34% of treated mice, respectively, indicating that the anti-CD3 dose used is indeed suboptimal. Combination treatments of anti-CD3 with LL-$GAD65_{370–575}$ alone or anti-CD3 with LL-IL10 alone resulted in 50% and 39% diabetes remission, respectively. Importantly, we found synergistic effects of combining LL-$GAD65_{370–575}$+IL10 with low-dose anti-CD3 (combi-GAD therapy) as shown by diabetes reversal in 67% of treated mice. Inclusion of a diabetes-related autoantigen was necessary because combination treatment with anti-CD3 and LL-OVA+IL10 restored normoglycemia in only 40% of the mice (Fig. 1*A*) (i.e., similar to anti-CD3 plus LL-IL10 alone).

Combi-GAD therapy induced long-lasting diabetes remission for at least an additional 8 weeks of follow-up after treatment termination (Fig. 1*A*). Additionally, glycemia values of cured combi-GAD–treated mice remained stable once normalized in contrast to the more oscillating glycemia values of cured anti-CD3–treated mice (Fig. 1*B*).

Important to potential clinical application, the combi-GAD therapy cured NOD mice with severe hyperglycemia at diagnosis (>350 mg/mL) with similar efficacy as those with mild hyperglycemia (<350 mg/mL). This was in contrast with anti-CD3 alone, which was not effective in NOD mice with severe hyperglycemia (Fig. 1*C*). In addition, combi-GAD therapy was efficacious in NOD mice regardless of the presence of detectable insulin autoantibody concentrations at the start of treatment in contrast with anti-CD3 alone, which was less efficacious in autoantibody-negative mice (Fig. 1*D*). Finally, combi-GAD therapy reversed diabetes in NOD mice independent of sex and age (Fig. 1*E* and data not shown).

With regard to safety, combi-GAD therapy is well tolerated as indicated by the absence of anaphylactic reactions and inflammation in the colon after combi-GAD therapy (data not shown), similar frequencies of $CD4^+$ and $CD8^+$ T cells in the lamina propria of cured combi-GAD– and anti-CD3–treated mice (data not shown), and stable weight evolution (data not shown).

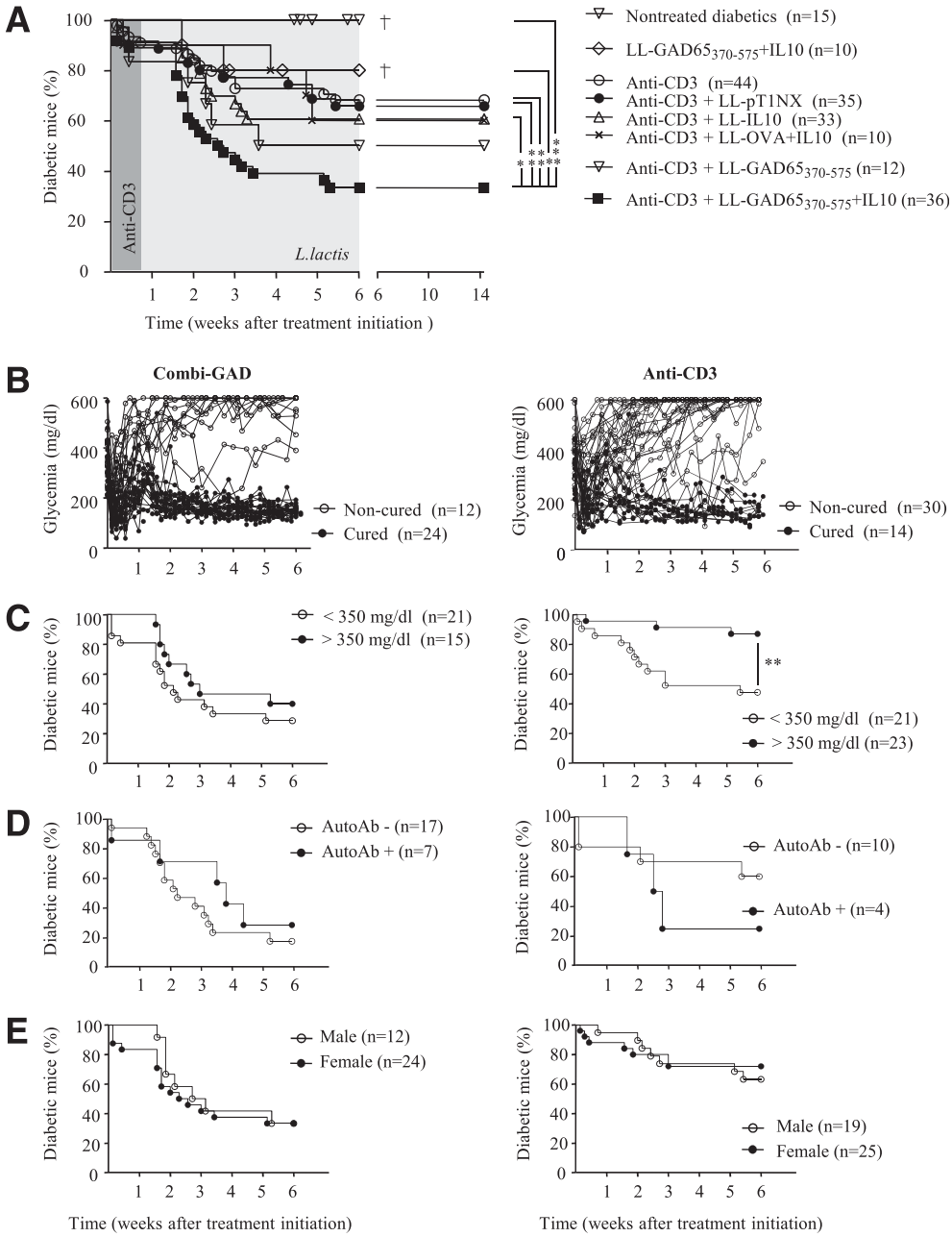

Figure 1— Combi-GAD treatment induces stable diabetes remission in NOD mice. Recent-onset diabetic NOD mice received treatments as indicated. Blood glucose concentrations were monitored until 14 weeks' posttreatment initiation. *A*: Percentage of diabetic mice per treatment group. †Dead or moribund mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. *B*: Individual glycemia values of combi-GAD–treated (*left*) and anti-CD3–treated (*right*) recent-onset diabetic NOD mice. *C–E*: Therapeutic efficacy of combi-GAD–treated (*left*) and anti-CD3–treated (*right*) mice was stratified according to blood glucose concentrations at diabetes diagnosis (*C*), the presence of insulin autoantibodies in the serum at the start of treatment (*D*), or sex (*E*). $^{**}P < 0.01$. Statistical significance between groups was calculated using Mantel-Cox log-rank test. AutoAb, autoantibody.

Combi-GAD Therapy Preserves β-Cell Function and Prevents Progression of Insulitis

Pancreata of cured combi-GAD–treated animals had a similar insulin content as those from age-matched normoglycemic NOD mice (Fig. 2*A*) and more than pancreata of untreated recent-onset, long-standing diabetic mice, or cured anti-CD3–treated mice. In addition, concentrations of random C-peptide in serum of cured combi-GAD– and cured anti-CD3–treated mice were similar to those in recent-onset diabetic mice but significantly higher than those in NOD mice with long-standing diabetes, indicating preservation of functional β-cells in cured mice (Fig. 2*B*). This was supported by IPGTTs performed between week 4 and 6 after the start of treatment, revealing a normal functional β-cell capacity in cured combi-GAD–treated mice (Fig. 2*C*). Histological analysis further showed

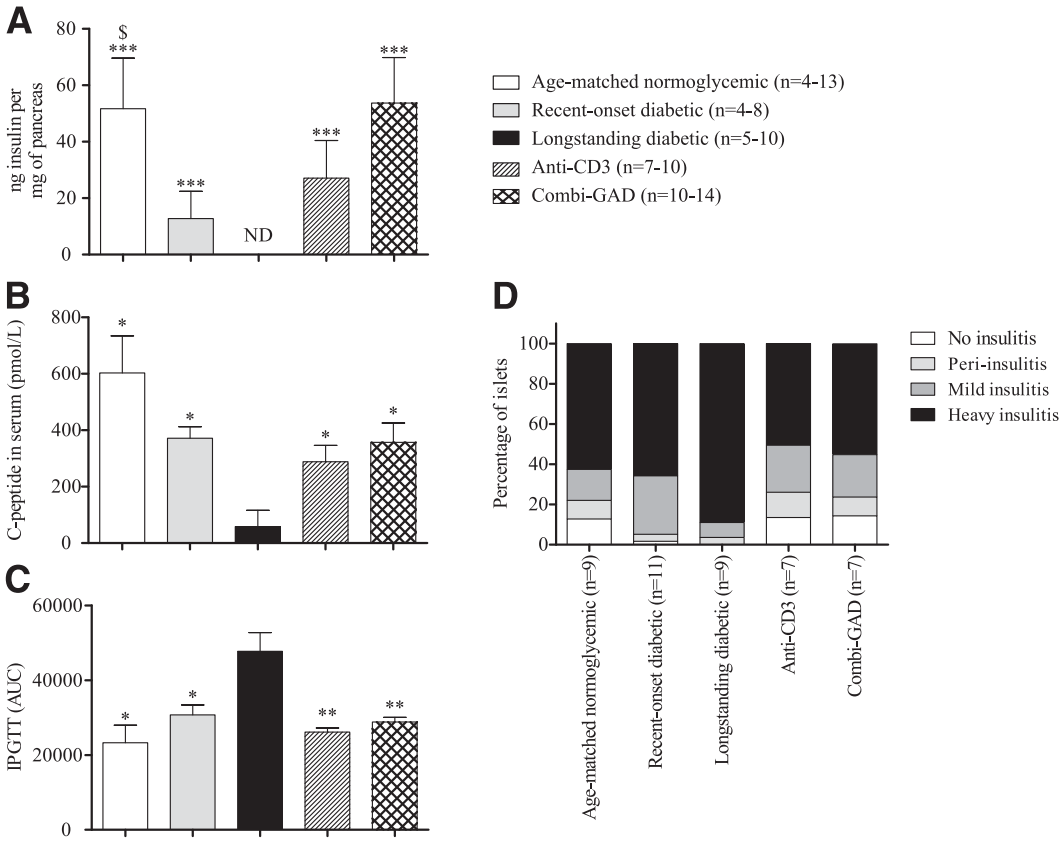

Figure 2—Combi-GAD treatment preserves β-cell function and prevents insulitis progression. *A* and *B*: Pancreatic insulin content (ng insulin/mg pancreas) (*A*) and C-peptide concentration in serum (pmol/L) (*B*) for control groups and cured anti-CD3– and combi-GAD–treated mice at treatment end as measured by insulin and C-peptide ELISA, respectively. *C*: IPGTTs were performed in control groups and cured mice 1–2 weeks before treatment end. Corresponding AUC over 2 h is shown. Data are mean ± SEM. Statistical significance between groups was calculated by Mann-Whitney *t* test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. long-standing diabetes; $ < 0.05 vs. recent-onset diabetes. AUC, area under the curve; ND, not detectable. *D*: Insulitis was blindly scored as indicated in pancreatic sections of control mice and cured anti-CD3– and combi-GAD–treated mice at treatment end.

that combi-GAD therapy not only prevented the worsening of insulitis that is normally observed from recent-onset to long-standing diabetes but also ameliorated insulitis to similar degrees of age-matched normoglycemic mice (Fig. 2*D*).

Combi-GAD Therapy Does Not Eliminate Autoreactive T Cells

Tolerance can be installed through anergy or deletion of pathogenic effector T cells and/or induction of Tregs. We therefore examined the phenotype of T cells present before and after combi-GAD therapy and found that combi-GAD therapy significantly decreased the frequency of $CD4^+$ T cells and increased the frequency of $CD8^+$ T cells in PLNs and mesenteric lymph nodes (MLNs) compared with untreated recent-onset diabetic animals (Supplementary Table 1). As a result, the CD4:CD8 ratio dropped more in combi-GAD–treated mice than in anti-CD3–treated mice compared with recent-onset diabetic NOD mice (Supplementary Table 1).

We then examined whether combi-GAD therapy changed effector T-cell responses and analyzed the fraction of T cells producing the proinflammatory T-helper 1 cytokine IFN-γ. We found increased frequencies of IFN-γ^+, $CD8^+$, and $CD4^+$ T cells upon in vitro restimulation of spleen and PLN cells isolated from cured combi-GAD–treated mice compared with recent-onset diabetic NOD mice (Fig. 3*A*). This finding suggests that combi-GAD therapy does not eliminate pathogenic effector T cells.

To verify that mucosal $GAD65_{370–575}$ delivery did not inadvertently boost or prime GAD-specific responses in combi-GAD–treated animals, we performed an antigen-specific proliferation assay. We cultured total splenocytes and PLNs of recent-onset diabetic and cured anti-CD3– and combi-GAD–treated animals in the presence of irrelevant ovalbumin peptide ($OVA_{323–339}$) or GAD65 p524 peptide for 3 days (Fig. 3*B*). The results showed that combi-GAD therapy did not boost GAD65-specific T-cell responses.

To test whether pathogenic effector T cells were still present after combi-GAD therapy, we adoptively transferred splenocytes from cured combi-GAD–treated NOD mice after depletion of the $CD25^+$ fraction, which

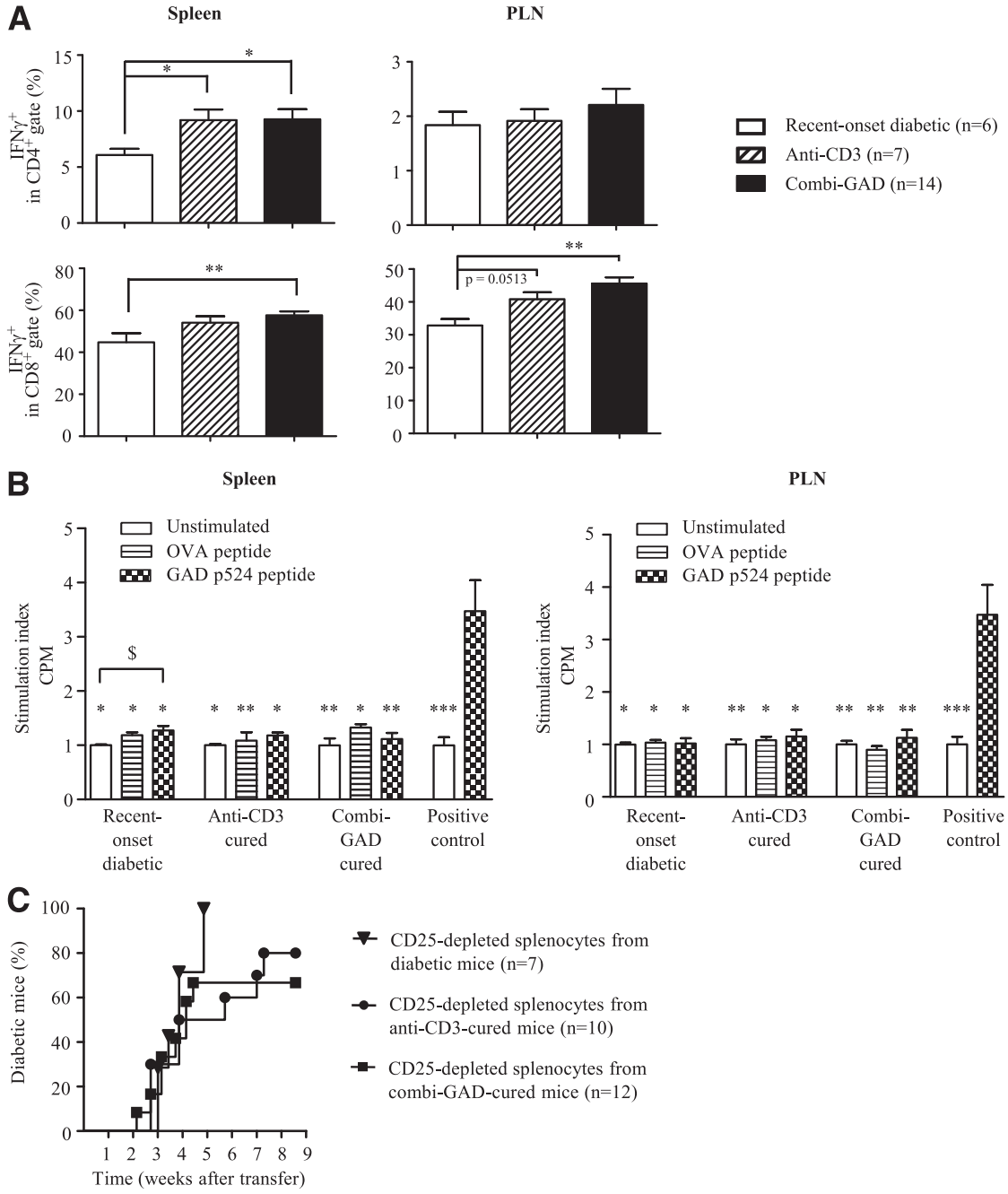

Figure 3—Combi-GAD therapy induces IFN-γ–producing T cells and does not eliminate effector T cells. *A*: IFN-γ^+ cells in the $CD4^+$ gate (*top*) and $CD8^+$ gate (*bottom*) of spleen (*left*) and PLNs (*right*) after in vitro restimulation. Statistical significance was calculated by Mann-Whitney *t* tests. $^*P < 0.05$, $^{**}P < 0.01$. *B*: Total splenocytes (5×10^5) (*left*) and PLNs ($1–2 \times 10^5$) (*right*) of indicated treatment groups were cultured in the presence of irrelevant ovalbumin peptide ($OVA_{323–339}$) or GAD p524 peptide for 3 days. PLNs were cocultured with mitomycin-treated splenocytes isolated from 10-week-old normoglycemic NOD mice. Proliferative responses were measured by ^3H-labeled thymidine incorporation. We used the proliferative response of popliteal lymph nodes isolated from GAD p524–immunized mice (footpath) as positive control. The proliferative response is expressed as a stimulation index calculated by dividing the mean cpm of the antigen-stimulated condition by the mean cpm of the unstimulated condition of the same treatment group. Results shown are the average of two pooled experiments. Data are mean ± SEM. Statistical significance between groups was calculated by Mann-Whitney *t* test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the positive control (GAD p524 peptide + immunized popliteal lymph nodes); $ < 0.05 between stimulation conditions in a treatment group. *C*: Adoptive transfers of CD25-depleted splenocytes (10×10^6) isolated from overtly diabetic, cured anti-CD3–treated, or cured combi-GAD–treated NOD mice, as indicated, into NOD/SCID mice. Shown is the diabetes incidence in the recipients. Statistical significance was calculated using Mantel-Cox log-rank test.

contains natural Tregs. We observed that this CD25-depleted population induced diabetes in 67% of the NOD/SCID recipients, which did not differ in incidence rate or speed from transfers using CD25-depleted splenocytes from cured anti-CD3–treated NOD mice or diabetic NOD mice (80% and 100%, respectively) (Fig. 3*C*), indicating that diabetogenic T cells were still present in cured combi-GAD–treated NOD mice. Taken together, these data indicate that combi-GAD therapy does not prime GAD65-specific responses and does not induce tolerance through the deletion of autoreactive T cells.

Combi-GAD Therapy Reduces the Inflammatory Milieu in Insulitic Lesions

We know that combi-GAD therapy reduces the severity of insulitis (Fig. 2*D*), but it is also important to know whether this leads to a less inflammatory environment for the remaining β-cells. We thus wanted to analyze specifically the inflammatory nature of T cells that are in the insulitic lesions and used laser capture microdissection to isolate the lymphocytic infiltrate of the islets. Gene expression analysis showed that the amounts of proinflammatory molecules IFN-γ, IL-1β, and granzyme B were significantly decreased in the lymphocytic infiltrates of cured combi-GAD–treated mice compared with recent-onset diabetic and cured anti-CD3–treated mice (Fig. 4). Thus, combi-GAD therapy not only reduces the amount of lymphocytes infiltrating the pancreatic islets but also reduces the proinflammatory potential of local T cells in or around the pancreatic islets.

Combi-GAD Therapy Induces Foxp3$^+$ Tregs

To test whether combi-GAD therapy induces tolerance through Tregs, we first examined the frequencies of Tregs using flow cytometry. Foxp3$^+$ Tregs were present at higher frequencies in the spleens of NOD mice cured by combi-GAD therapy or by anti-CD3 alone compared with recent-onset diabetic mice (Fig. 5*A*). Of note, only combi-GAD therapy increased the frequency of Tregs in MLNs and PLNs, irrespective of identification as Foxp3$^+$ single-positive or Foxp3$^+$CD25$^+$ double-positive CD4$^+$ T cells (Fig. 5*A* and data not shown). Conversely, only anti-CD3 treatment significantly increased the numbers of Foxp3$^+$ Tregs in the pancreas (Fig. 5*B* and *C*). Frequencies of Foxp3$^+$ Tregs were not different in Peyer patches and lamina propria of small intestine and colon (data not shown). All CD4$^+$Foxp3$^+$ and CD4$^+$CD25$^+$Foxp3$^+$ Tregs expressed folate receptor 4 (FR4), and no treatment-induced differences in FR4 expression levels were observed (data not shown). Thus, Tregs accumulate in different locations after anti-CD3 therapy or combi-GAD therapy, namely combi-GAD therapy acts by inducing Tregs in the lymph nodes draining the target organ, whereas anti-CD3 treatment acts by increasing Tregs in the target organ itself.

We next evaluated whether Tregs in cured combi-GAD–treated NOD mice are functional. As a putative suppressor population, we used CD4$^+$CD25$^+$ T cells from cured NOD mice. As responder T cells, we used CD4$^+$CD25$^-$ T cells isolated from spleens of normoglycemic NOD mice. We found that CD4$^+$CD25$^+$ T cells from cured combi-GAD–treated animals efficiently suppressed proliferation and activation of responder T cells, as evidenced by reduced dilution of proliferation dye (Fig. 6*A*), lowered expression of the activation markers CD69 (Fig. 6*B*) and CD44 (Fig. 6*C*), and reduced secretion of the effector cytokine IFN-γ (Fig. 6*D*). Nevertheless, CD4$^+$CD25$^+$ T cells isolated from cured combi-GAD–

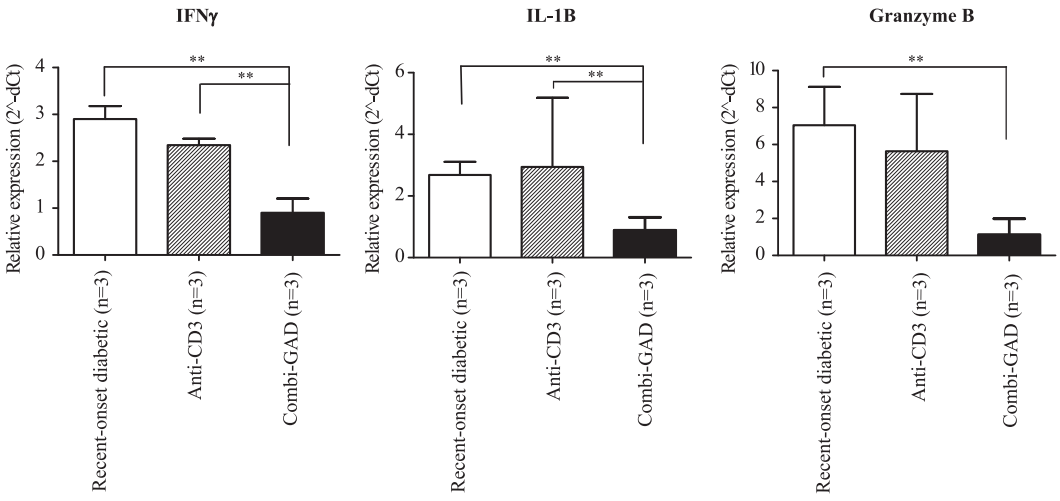

Figure 4—Combi-GAD therapy decreases the inflammatory profile of islet cell infiltrates locally in the pancreas. Shown is the gene expression of proinflammatory molecules in pancreatic lymphocytic infiltrates isolated by laser capture microdissection. Data are fold change in IFN-γ, IL-1B, or granzyme B expression after normalization to CD45 and are shown as mean ± SEM. Statistical analysis was calculated using Mann-Whitney *t* tests. $^{**}P < 0.01$.

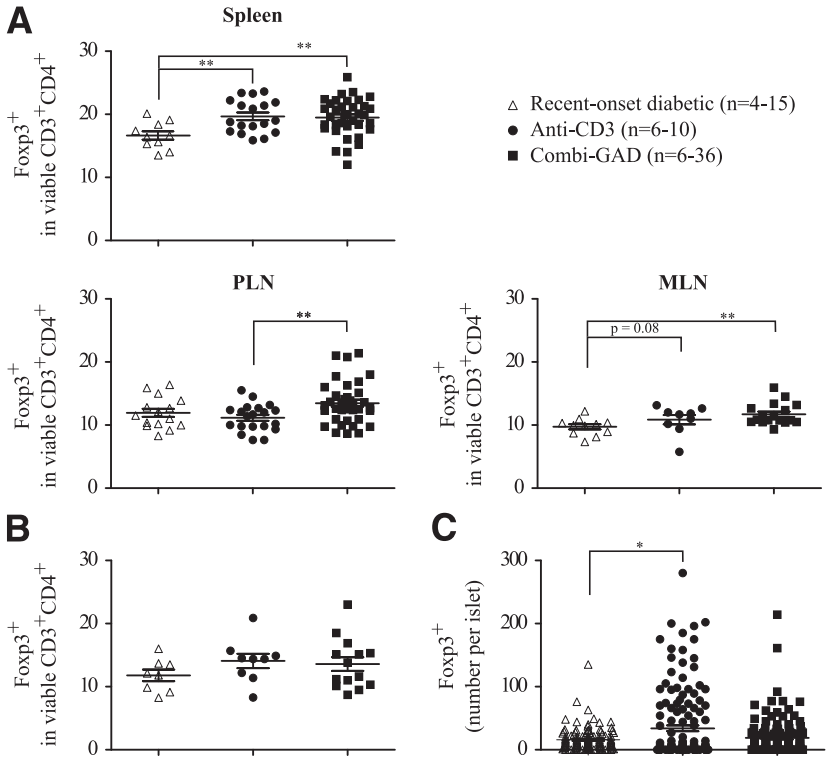

Figure 5—Combi-GAD therapy induces Foxp3$^+$ T cells in spleen, MLNs, and PLNs. *A* and *B*: Analysis of Foxp3$^+$ expression by flow cytometry in spleen, PLNs, and MLNs (*A*) and pancreas (*B*) of recent-onset diabetic and cured anti-CD3– and combi-GAD–treated NOD mice. Shown are scatterplots representing the frequencies of Foxp3$^+$ cells in CD4$^+$ population of individual mice (symbols) and as mean ± SEM (line and error bars) in viable CD3$^+$CD4$^+$ gate. *C*: Quantification of Foxp3$^+$ cells in or around the pancreatic islets by manual counting on immunostained paraffin-embedded sections (4–6 mice, 114–144 islets). Statistical analysis was calculated using Mann-Whitney *t* tests. $^*P < 0.05$, $^{**}P < 0.01$.

treated mice were similarly effective as those isolated from age-matched normoglycemic or cured anti-CD3–treated mice, indicating that combi-GAD therapy did not increase suppressive potency on a per-cell basis.

Finally, we evaluated whether CD4$^+$CD25$^+$ T cells from cured combi-GAD–treated NOD mice transferred disease-specific protection in vivo. For this purpose, NOD/SCID recipients were injected with CD25-depleted splenocytes isolated from overtly diabetic NOD mice, rendering them diabetic in all cases by 4 weeks (Fig. 6*E*). Cotransfer of CD4$^+$CD25$^+$ T cells from spleens and PLNs of cured anti-CD3– or combi-GAD–treated NOD mice similarly and significantly delayed the onset of diabetes in recipient mice (Fig. 6*E*). Taken together, these results indicate that combination therapy with LL-GAD65+IL10 plus low-dose anti-CD3 increases the frequency of functional Tregs that suppress polyclonally in vitro and disease specifically in vivo.

Tregs From Cured Combi-GAD–Treated Mice Are GAD Reactive

We next performed an antigen-specific suppressor assay in vitro to assess whether Tregs from cured combi-GAD–treated mice suppress GAD-specific responses. For this, we generated GAD-specific responders and control OVA-specific responders by immunizing mice. As expected, ex vivo incubation of these responder cells with their cognate antigen resulted in proliferation (Fig. 7*A*). The addition of CD4$^+$CD25$^+$ T cells from cured combi-GAD–treated mice suppressed GAD-specific proliferation of GAD-responder cells (Fig. 7*A*). In the absence of GAD peptide, CD4$^+$CD25$^+$ Tregs from cured combi-GAD–treated mice also suppressed responses of OVA-stimulated T cells (Fig. 7*A*), although with significantly less potency than responses of GAD-stimulated T cells (Fig. 7*B*), suggesting involvement of passive mechanisms such as IL-2 stealing through CD25. In the presence of both OVA and GAD peptide, CD4$^+$CD25$^+$ Tregs from cured combi-GAD–treated mice suppressed OVA-stimulated T cells more potently than in the absence of GAD peptide, suggesting involvement of antigen-induced active mechanisms, such as IL10 or transforming growth factor-β production. From this, we conclude that CD4$^+$CD25$^+$ T cells from cured combi-GAD–treated mice are GAD reactive.

DISCUSSION

We report the preclinical success of an antigen-specific combination therapy for tolerance induction in T1D. Auto-antigen-specific strategies aim to restore the loss of tolerance that underlies T1D with fewer side-effects than antigen-

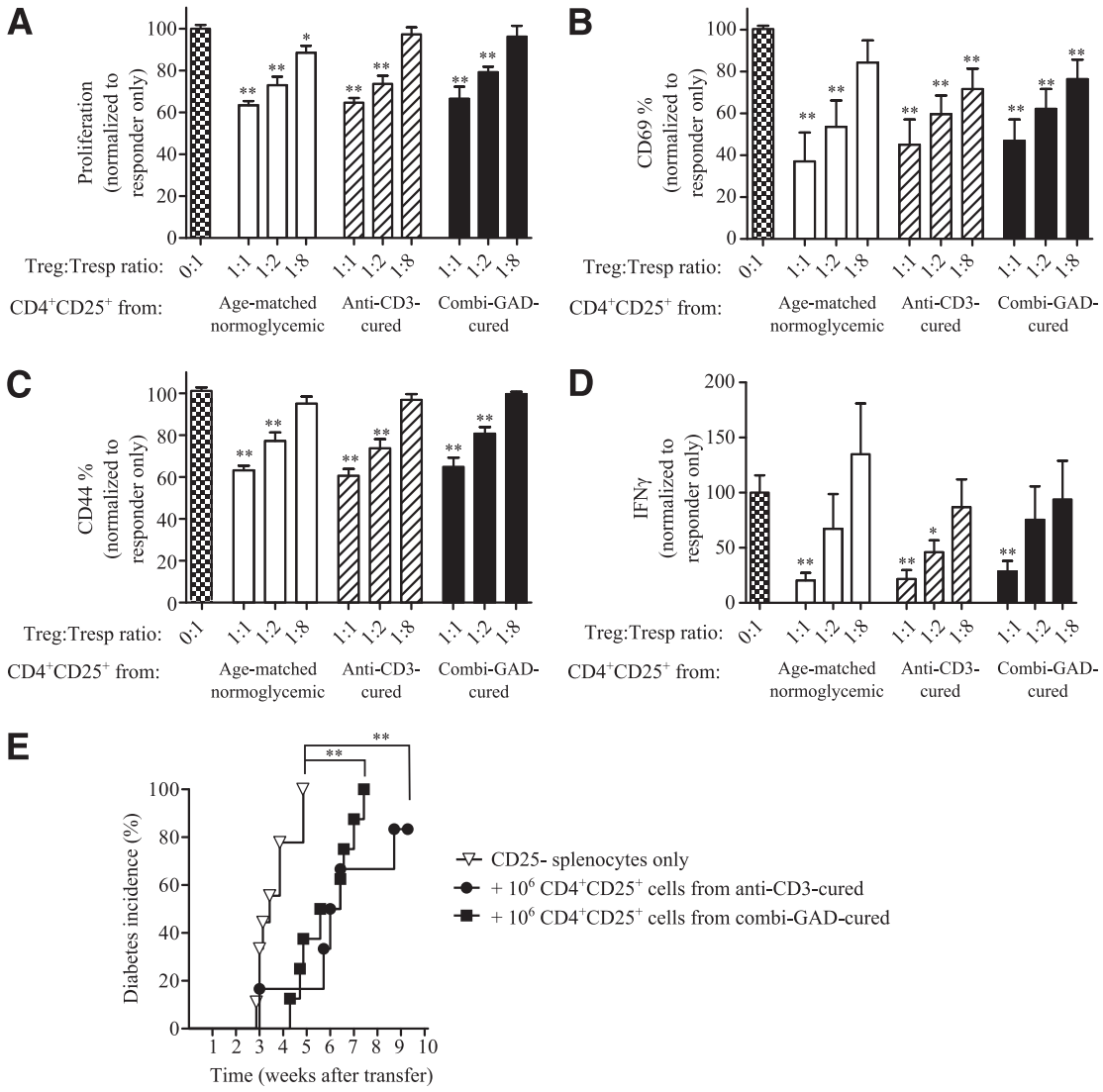

Figure 6—Combi-GAD therapy induces functional Tregs. *A–D*: In vitro polyclonal suppressor assay. $CD4^+CD25^-$ responder T cells (Tresp) isolated from normoglycemic NOD mice were dye labeled and stimulated using 0.5 μg/mL soluble anti-CD3 and accessory cells for 72 h in the presence of $CD4^+CD25^+$ Tregs isolated from cured mice at the end of the indicated treatment. Results of assays for in vitro suppressive capacity are shown. *A*: Proliferation of Tresp, shown as percentage of Tresp that had undergone one or more divisions, normalized to proliferation by Tresp-only culture. *B* and *C*: Activation of Tresp shown as a percentage of CD69 (*B*) or CD44 (*C*) expression, normalized to the percentage in Tresp-only culture. *D*: Measurement of IFN-γ in the coculture supernatant by cytometric bead assay, normalized to the concentration in Tresp-only culture. Results shown are the average of three pooled experiments. Statistical significance vs. Tresp alone and between corresponding dilutions of various treatment groups were calculated with Mann-Whitney *t* tests. $^*P < 0.05$, $^{**}P < 0.01$ vs. responders alone. *E*: In vivo assay for disease-specific suppressive capacity. CD25-depleted splenocytes from diabetic mice were transferred into NOD/SCID mice without (▽) or with $CD4^+CD25^+$ cells isolated from spleen and PLNs of cured anti-CD3–treated (●) or cured combi-GAD–treated (■) NOD mice. Shown is the diabetes incidence in recipients. Statistical significance was calculated using Mantel-Cox log-rank test. $^{**}P < 0.01$.

nonspecific immunomodulatory and immunosuppressive compounds. Even though antigen-specific therapies hold the theoretical possibility of aggravating disease, numerous studies in murine models of diabetes and some clinical trials have proven that antigen-specific therapies can prevent diabetes, albeit in specific subgroups (29–32). Nevertheless, effective reversal of diabetes in preclinical models and in clinical trials by antigen-specific therapy alone is still lacking. It is known that efficacy critically depends on the proper choice of an adjuvant and an adequate route of delivery. Thus, the gut has been proposed as the optimal route for presenting potential autoantigens to an immune system prone to induce tolerance. A major issue with oral antigen administration is the availability of the antigen in the proper form at the site of the gut where tolerance is best induced (i.e., past the stomach) as well as correct timing of the presentation. This can explain why previous studies did not report positive findings with orally administered GAD65 (23). Antigen-specific therapies have the additional potential to

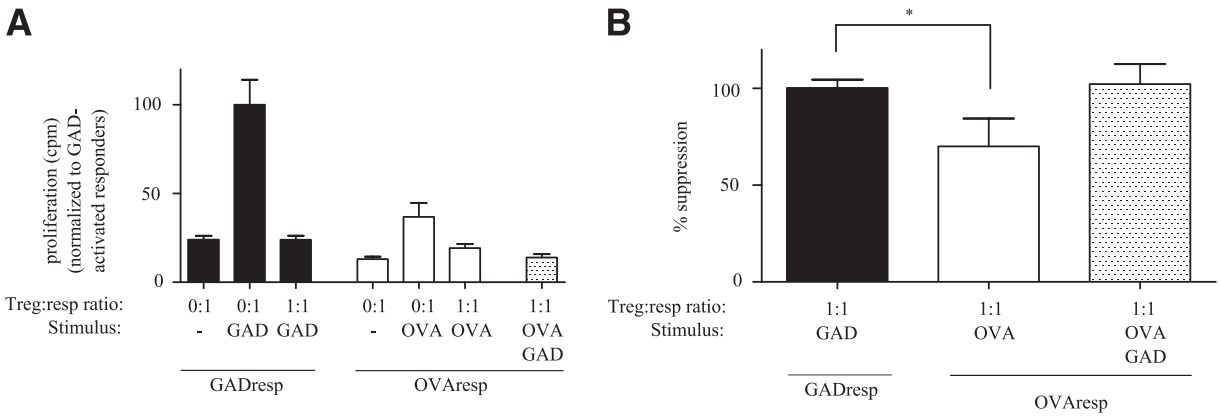

Figure 7—Cured combi-GAD–treated mice have GAD-reactive Tregs. *A* and *B*: In vitro antigen-specific suppressor assay. $CD4^+CD25^+$ Tregs isolated from cured mice at the end of combi-GAD therapy were cocultured with GAD-responder T cells (GADresp) or OVA-responder T cells (OVAresp) isolated from GAD- or OVA-immunized NOD mice, respectively, and GAD, OVA, or the combination of these two antigens, as indicated, for 4 days. *A*: Proliferative responses were measured by ^{3}H-labeled thymidine incorporation in the last 18 h. Data are expressed relative to GAD-activated GADresp. Results shown are the average of four pooled experiments. *B*: Percent suppression of GAD- and OVA-specific proliferative responses by $CD4^+CD25^+$ Tregs from combi-GAD–cured mice. The percentage of suppression was calculated by the equation 100 − [(cpm suppressed resp − cpm unstimulated resp) / (cpm activated resp − cpm unstimulated resp)]. Statistical significance was calculated with Mann-Whitney *t* test. $^*P < 0.05$.

induce bystander suppression toward other related antigens than the one administered and, thus, can circumvent the need for identification and targeting of the primary autoantigens that may initiate the autoreactive response in each individual. This is supported by the similar efficacies of the present combi-GAD therapy and our previously reported combi-PINS therapy (oral administration of LL-secreting proinsulin and IL10 combined with systemic delivery of anti-CD3) (24). Both therapies could reverse diabetes in overtly diabetic NOD mice, but small differences in immune effects could be observed. Although in combi-PINS–treated mice, Tregs preferentially accumulated in pancreatic islets, in the present combi-GAD–treated mice, $Foxp3^+$ Tregs were typically found in PLNs. Of note, in contrast with anti-CD3 alone or with combi-PINS therapy (24), combi-GAD therapy also reversed diabetes in severely hyperglycemic NOD mice, suggesting that combi-GAD therapy is more widely applicable and advocating the targeting of GAD65 also in more advanced stages of T1D. This is in line with other studies pointing to the primary position of proinsulin in the antigen-spreading cascade, hypothesizing that more GAD65-specific T cells originate at later stages, which can then be targeted by combi-GAD therapy (33).

Preclinical studies had demonstrated that GAD65 vaccination can prevent but not reverse autoimmune diabetes in murine models, such as the NOD mouse (11–13,17,34). In addition, the promising results of C-peptide preservation in a small study testing GAD/alum formulation could not be confirmed in subsequent phase II/III clinical trials in T1D patients (4,18,19). In our opinion, the synergistic efficacy of the combi-GAD therapy comes from the multipronged attack on the autoimmune response. First, we delivered viable LL capable of secreting antigen to the intestine, which is a safe way to introduce autoantigens because the immune system of the gut is a tolerogenic microenvironment (22). Second, we codelivered IL10, an anti-inflammatory cytokine that can induce anergy in T cells (35) and stabilize Treg function (36). IL10 also affects cytokine production and antigen presentation by antigen-presenting cells (37), favoring a tolerant status (38) and thus reducing any risk of autoimmune (re)activation. Third, we optimized this antigen-specific mucosal vaccination by coadministration of a short-course systemic immunomodulating anti-CD3 mAb because anti-CD3 provides an immediate freezing effect on the immune response (39), thus buying time for the antigen-specific immune response to peak by days 10–14. In addition, anti-CD3 treatment can recruit Tregs to the gastrointestinal mucosa, thus aiding induction of antigen-specific oral tolerance (40,41).

Oral tolerance can be achieved in multiple nonexclusive ways: administration of antigen at a high dose favors deletion of effector T cells or clonal anergy, whereas a low dose favors induction and activation of Tregs (22). We conclude that our combi-GAD therapy favors induction of Tregs because cured combi-GAD–treated mice contained more $CD4^+$ T cells expressing Foxp3 and CD25 and diabetogenic effector T cells were not deleted from cured animals. Tregs from cured combi-GAD–treated mice delayed diabetes transfer in vivo. Moreover, these Tregs have increased suppressive activity after in vitro GAD stimulation compared with OVA stimulation. This finding is important because Tregs are functionally compromised in T1D patients (42,43).

It remains to be determined whether the observed increase in IFN-γ production is involved in the mechanism of action of combi-GAD treatment, for instance, by

suppressing IL17-secreting pathogenic T cells (44,45). It is also not clear why combi-GAD and combi-PINS treatments both increased Treg frequencies in the PLNs but only combi-GAD therapy increased the Treg frequencies in the MLNs and only combi-PINS therapy allocated $Foxp3^+$ cells to the pancreas. It is known that regulation by Tregs can occur in the PLNs (17) and that MLNs are essential in oral tolerance induction by supporting the generation of $Foxp3^+$ Tregs that then undergo antigen-specific expansion in the small intestine (46–48).

In summary, mucosal delivery of $GAD65_{370–575}$+IL10 by genetically modified LL and a systemic low-dose of anti-CD3 mAb stably reversed diabetes in recent-onset diabetic NOD mice. The results further underscore the potential of using genetically modified LL as part of a therapy for autoimmune diabetes, as we had previously demonstrated with combi-PINS therapy (24). Because of its effectiveness even in mice with severe hyperglycemia, combi-GAD therapy shows additional promise to be deployed in late-stage disease and/or as part of a patient-tailored therapeutic approach based on autoantibody risk scores to have a sustained effect on the course of T1D. Nevertheless, as exemplified by the success of other antigen-specific therapies in preclinical models but failure in clinical trials, the promising potential of combi-GAD therapy needs clinical validation.

Acknowledgments. The authors thank Jos Depovere and Jos Laureys (CEE, KU Leuven, Belgium) for technical assistance; Tine Paessens, Annemie Ducatteeuw, Flore Thielemans, Anuschka Haegeman, and Peter Vanhoenacker (ActoGeniX NV, Zwijnaarde, Belgium) for help with the LL engineering and processing and ELISA analysis; and Gianluca Matteoli and Guy Boeckxstaens (Translational Research Center for Gastrointestinal Disorders, KU Leuven, Belgium) for help with isolating the lamina propria.

Funding. This work was supported by grants from the Fund for Scientific Research Flanders (FWO-Vlaanderen G.0554.13N), KU Leuven (GOA 2009/10), the Health Seventh Framework Programme of the European Community (FP7/2009-2014 under grant agreement 241447 with acronym NAIMIT), and the JDRF (17-2011-524). S.R. holds a Baekeland Fellowship from the Agency for Innovation by Science and Technology in Flanders (IWT-90702). C.G. is supported by KU Leuven. T.T. is supported through a Selective Bilateral Agreement fellowship from KU Leuven. T.L.V.B. is supported by the Health Seventh Framework Programme of the European Community (FP7/2009-2014 under grant agreement 241447 with acronym NAIMIT). H.K. is a postdoctoral fellow of the FWO-Vlaanderen. F.D. received support from the Italian Ministry of Research, the Italian Ministry of Health, and the Italian Foundation for Diabetes Research. C.M. is a clinical researcher of the FWO-Vlaanderen.

Duality of Interest. K.V.H., L.S., S.C., and P.R. have financial interests in ActoGeniX NV, including employment and stock options. No other potential conflicts of interest relevant to this article were reported.

Author Contributions. S.R. contributed to the study design, research, data analysis, and writing of the manuscript. C.G. and T.L.V.B. contributed to the study design, research, and writing and editing of the manuscript. T.T. contributed to the research. H.K., K.V.H., L.S., S.C., and F.D. contributed to the study design and research. I.S. and G.S. contributed to the study of pancreatic islet histology and immunohistochemistry. P.D. contributed to the study of gut histology. C.H.W. contributed to the insulin autoantibody measurements. M.A.A. contributed to editing the manuscript. P.R. contributed to the study design and editing of the manuscript. C.M. contributed to the study design and writing and editing of the manuscript. C.G., T.L.V.B., and C.M. are guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation. Parts of this study were presented in abstract form at the Belgian Immunological Society Annual Meeting, Liège, Belgium, 8 November 2013; the 12th International Conference of the Immunology of Diabetes Society, Victoria, BC, Canada, 15–19 June 2012; and the Keystone Symposium Immunopathology of Type 1 Diabetes, Whistler, BC, Canada, 4–9 April 2013.

References

1. van Belle TL, Coppieters KT, von Herrath MG. Type 1 diabetes: etiology, immunology, and therapeutic strategies. Physiol Rev 2011;91:79–118

2. Keymeulen B, Vandemeulebroucke E, Ziegler AG, et al. Insulin needs after CD3-antibody therapy in new-onset type 1 diabetes. N Engl J Med 2005;352:2598–2608

3. Herold KC, Gitelman S, Greenbaum C, et al.; Immune Tolerance Network ITN007AI Study Group. Treatment of patients with new onset type 1 diabetes with a single course of anti-CD3 mAb teplizumab preserves insulin production for up to 5 years. Clin Immunol 2009;132:166–173

4. Ludvigsson J, Faresjö M, Hjorth M, et al. GAD treatment and insulin secretion in recent-onset type 1 diabetes. N Engl J Med 2008;359:1909–1920

5. Tisch R, Yang XD, Singer SM, Liblau RS, Fugger L, McDevitt HO. Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice. Nature 1993;366:72–75

6. Atkinson MA, Maclaren NK, Scharp DW, Lacy PE, Riley WJ. 64,000 Mr autoantibodies as predictors of insulin-dependent diabetes. Lancet 1990;335:1357–1360

7. Jun HS, Khil LY, Yoon JW. Role of glutamic acid decarboxylase in the pathogenesis of type 1 diabetes. Cell Mol Life Sci 2002;59:1892–1901

8. Di Lorenzo TP, Peakman M, Roep BO. Translational mini-review series on type 1 diabetes: systematic analysis of T cell epitopes in autoimmune diabetes. Clin Exp Immunol 2007;148:1–16

9. Baekkeskov S, Nielsen JH, Marner B, Bilde T, Ludvigsson J, Lernmark A. Autoantibodies in newly diagnosed diabetic children immunoprecipitate human pancreatic islet cell proteins. Nature 1982;298:167–169

10. Endl J, Otto H, Jung G, et al. Identification of naturally processed T cell epitopes from glutamic acid decarboxylase presented in the context of HLA-DR alleles by T lymphocytes of recent onset IDDM patients. J Clin Invest 1997;99:2405–2415

11. Tian J, Atkinson MA, Clare-Salzler M, et al. Nasal administration of glutamate decarboxylase (GAD65) peptides induces Th2 responses and prevents murine insulin-dependent diabetes. J Exp Med 1996;183:1561–1567

12. Tisch R, Liblau RS, Yang XD, Liblau P, McDevitt HO. Induction of GAD65-specific regulatory T-cells inhibits ongoing autoimmune diabetes in nonobese diabetic mice. Diabetes 1998;47:894–899

13. Pleau JM, Fernandez-Saravia F, Esling A, Homo-Delarche F, Dardenne M. Prevention of autoimmune diabetes in nonobese diabetic female mice by treatment with recombinant glutamic acid decarboxylase (GAD 65). Clin Immunol Immunopathol 1995;76:90–95

14. Yoon JW, Yoon CS, Lim HW, et al. Control of autoimmune diabetes in NOD mice by GAD expression or suppression in beta cells. Science 1999;284:1183–1187

15. Tisch R, Wang B, Serreze DV. Induction of glutamic acid decarboxylase 65-specific Th2 cells and suppression of autoimmune diabetes at late stages of disease is epitope dependent. J Immunol 1999;163:1178–1187

16. Olcott AP, Tian J, Walker V, et al. Antigen-based therapies using ignored determinants of beta cell antigens can more effectively inhibit late-stage autoimmune disease in diabetes-prone mice. J Immunol 2005;175:1991–1999

17. Bresson D, Fradkin M, Manenkova Y, Rottembourg D, von Herrath M. Genetic-induced variations in the GAD65 T-cell repertoire governs efficacy of anti-CD3/GAD65 combination therapy in new-onset type 1 diabetes. Mol Ther 2010;18:307–316

18. Ludvigsson J, Krisky D, Casas R, et al. GAD65 antigen therapy in recently diagnosed type 1 diabetes mellitus. N Engl J Med 2012;366:433–442
19. Wherrett DK, Bundy B, Becker DJ, et al.; Type 1 Diabetes TrialNet GAD Study Group. Antigen-based therapy with glutamic acid decarboxylase (GAD) vaccine in patients with recent-onset type 1 diabetes: a randomised double-blind trial. Lancet 2011;378:319–327
20. Li AF, Escher A. Intradermal or oral delivery of GAD-encoding genetic vaccines suppresses type 1 diabetes. DNA Cell Biol 2003;22:227–232
21. Miller A, Lider O, Weiner HL. Antigen-driven bystander suppression after oral administration of antigens. J Exp Med 1991;174:791–798
22. Mayer L, Shao L. Therapeutic potential of oral tolerance. Nat Rev Immunol 2004;4:407–419
23. Ma S, Huang Y, Yin Z, Menassa R, Brandle JE, Jevnikar AM. Induction of oral tolerance to prevent diabetes with transgenic plants requires glutamic acid decarboxylase (GAD) and IL-4. Proc Natl Acad Sci U S A 2004;101:5680–5685
24. Takiishi T, Korf H, Van Belle TL, et al. Reversal of autoimmune diabetes by restoration of antigen-specific tolerance using genetically modified *Lactococcus lactis* in mice. J Clin Invest 2012;122:1717–1725
25. Rottiers P, De Smedt T, Steidler L. Modulation of gut-associated lymphoid tissue functions with genetically modified *Lactococcus lactis*. Int Rev Immunol 2009;28:465–486
26. Yu L, Eisenbarth G, Bonifacio E, Thomas J, Atkinson M, Wasserfall C. The second murine autoantibody workshop: remarkable interlaboratory concordance for radiobinding assays to identify insulin autoantibodies in nonobese diabetic mice. Ann N Y Acad Sci 2003;1005:1–12
27. Matteoli G, Mazzini E, Iliev ID, et al. Gut CD103+ dendritic cells express indoleamine 2,3-dioxygenase which influences T regulatory/T effector cell balance and oral tolerance induction. Gut 2010;59:595–604
28. Grieco FA, Moretti M, Sebastiani G, et al. Delta-cell-specific expression of hedgehog pathway Ptch1 receptor in murine and human endocrine pancreas. Diabetes Metab Res Rev 2011;27:755–760
29. Fife BT, Guleria I, Gubbels Bupp M, et al. Insulin-induced remission in new-onset NOD mice is maintained by the PD-1-PD-L1 pathway. J Exp Med 2006; 203:2737–2747
30. Daniel D, Wegmann DR. Protection of nonobese diabetic mice from diabetes by intranasal or subcutaneous administration of insulin peptide B-(9-23). Proc Natl Acad Sci U S A 1996;93:956–960
31. Vehik K, Cuthbertson D, Ruhlig H, Schatz DA, Peakman M, Krischer JP; DPT-1 and TrialNet Study Groups. Long-term outcome of individuals treated with oral insulin: diabetes prevention trial-type 1 (DPT-1) oral insulin trial. Diabetes Care 2011;34:1585–1590
32. Skyler JS, Krischer JP, Wolfsdorf J, et al. Effects of oral insulin in relatives of patients with type 1 diabetes: The Diabetes Prevention Trial—Type 1. Diabetes Care 2005;28:1068–1076
33. Krishnamurthy B, Dudek NL, McKenzie MD, et al. Responses against islet antigens in NOD mice are prevented by tolerance to proinsulin but not IGRP. J Clin Invest 2006;116:3258–3265
34. Boettler T, Pagni PP, Jaffe R, Cheng Y, Zerhouni P, von Herrath M. The clinical and immunological significance of GAD-specific autoantibody and T-cell responses in type 1 diabetes. J Autoimmun 2013;44:40–48
35. Groux H, Bigler M, de Vries JE, Roncarolo MG. Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells. J Exp Med 1996;184:19–29
36. Murai M, Turovskaya O, Kim G, et al. Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. Nat Immunol 2009;10:1178–1184
37. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 2001;19:683–765
38. Slavin AJ, Maron R, Weiner HL. Mucosal administration of IL-10 enhances oral tolerance in autoimmune encephalomyelitis and diabetes. Int Immunol 2001; 13:825–833
39. Chatenoud L, Bluestone JA. CD3-specific antibodies: a portal to the treatment of autoimmunity. Nat Rev Immunol 2007;7:622–632
40. Waldron-Lynch F, Henegariu O, Deng S, et al. Teplizumab induces human gut-tropic regulatory cells in humanized mice and patients. *Sci Transl Med* 2012; 4:118ra12
41. Esplugues E, Huber S, Gagliani N, et al. Control of TH17 cells occurs in the small intestine. Nature 2011;475:514–518
42. Lindley S, Dayan CM, Bishop A, Roep BO, Peakman M, Tree TI. Defective suppressor function in CD4(+)CD25(+) T-cells from patients with type 1 diabetes. Diabetes 2005;54:92–99
43. Kukreja A, Cost G, Marker J, et al. Multiple immuno-regulatory defects in type-1 diabetes. J Clin Invest 2002;109:131–140
44. Park H, Li Z, Yang XO, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol 2005;6:1133–1141
45. Jain R, Tartar DM, Gregg RK, et al. Innocuous IFNgamma induced by adjuvant-free antigen restores normoglycemia in NOD mice through inhibition of IL-17 production. J Exp Med 2008;205:207–218
46. Spahn TW, Weiner HL, Rennert PD, et al. Mesenteric lymph nodes are critical for the induction of high-dose oral tolerance in the absence of Peyer's patches. Eur J Immunol 2002;32:1109–1113
47. Kraus TA, Brimnes J, Muong C, et al. Induction of mucosal tolerance in Peyer's patch-deficient, ligated small bowel loops. J Clin Invest 2005;115:2234–2243
48. Hadis U, Wahl B, Schulz O, et al. Intestinal tolerance requires gut homing and expansion of FoxP3+ regulatory T cells in the lamina propria. Immunity 2011; 34:237–246