Oral delivery of Glutamic Acid Decarboxylase (GAD)-65 and IL10 by *Lactococcus lactis* reverses diabetes in recent-onset NOD mice.

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<td>Robert, Sofie; Katholieke Universiteit Leuven, Clinical and Experimental Medicine, Laboratory of Clinical and Experimental Endocrinology (CEE) Gysemans, Conny; Katholieke Universiteit Leuven, Clinical and Experimental Medicine, Laboratory of Clinical and Experimental Endocrinology (CEE) Takishii, Tatiana; Katholieke Universiteit Leuven, Clinical and Experimental Medicine, Laboratory of Clinical and Experimental Endocrinology (CEE) Korf, Hannelie; Katholieke Universiteit Leuven, Clinical and Experimental Medicine, Laboratory of Clinical and Experimental Endocrinology (CEE) Spagnuolo, Isabella; University of Siena and Fondazione Umberto Di Mario ONLUS, Department of Internal Medicine, Endocrine and Metabolic Sciences and Biochemistry Sebastiani, Guido; University of Siena and Fondazione Umberto Di Mario ONLUS, Department of Internal Medicine, Endocrine and Metabolic Sciences and Biochemistry Van Huynegem, Karolien; ActoGeniX nv, Technologiepark Steidler, Lothar; ActoGeniX nv, Technologiepark Caluwaerts, Silvia; ActoGeniX nv, Technologiepark Demetter, Pieter; Université Libre de Bruxelles (ULB), Department of Pathology Wasserfall, Clive; University of Florida, Department of Pathology, Immunology and Laboratory Medicine, College of Medicine Atkinson, Mark; University of Florida, Department of Pathology, Immunology and Laboratory Medicine, College of Medicine Dotta, Francesca; University of Siena and Fondazione Umberto Di Mario ONLUS, Department of Internal Medicine, Endocrine and Metabolic Sciences and Biochemistry Rottiers, Pieter; ActoGeniX nv, Technologiepark Van Belle, Tom; Katholieke Universiteit Leuven, Clinical and Experimental Medicine, Laboratory of Clinical and Experimental Endocrinology (CEE) Mathieu, Chantal; Katholieke Universiteit Leuven, Clinical and Experimental Medicine, Laboratory of Clinical and Experimental Endocrinology (CEE)</td>
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Oral delivery of Glutamic Acid Decarboxylase (GAD)-65 and IL10 by Lactococcus lactis reverses diabetes in recent-onset NOD mice

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

1Clinical and Experimental Endocrinology (CEE), KU Leuven, Leuven, Belgium
2Diabetes Unit, Department of Internal Medicine, Endocrine and Metabolic Sciences and Biochemistry, University of Siena and Fondazione Umberto Di Mario ONLUS, Siena, Italy
3ActoGeniX NV, Zwijnaarde, Belgium
4Department of Pathology, Université Libre de Bruxelles (ULB), Route de Lennik 808, 1070 Brussels, Belgium
5Department of Pathology, Immunology and Laboratory Medicine, College of Medicine, University of Florida, Gainesville, Florida, USA

* T.L.V.B and C.M. share senior authorship

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Corresponding author and to whom reprint requests should be addressed:

Chantal Mathieu, M.D., Ph.D.
Clinical and Experimental Endocrinology (CEE)
KU Leuven, Campus Gasthuisberg
O&N I Herestraat 49 – box 902
3000 Leuven, Belgium
E-mail: chantal.mathieu@med.kuleuven.be
Tel: +32 16 3 46023
Fax: +32 16 3 30718
Abstract

Growing insight into the pathogenesis of type 1 diabetes and numerous studies in preclinical models highlight the potential of antigen-specific approaches to restore tolerance in an efficient and safe manner. 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* (*L. lactis*) bacteria for controlled secretion of the type 1 diabetes autoantigen GAD65<sub>370-575</sub> and the anti-inflammatory cytokine IL10 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 nonobese diabetic (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<sup>+</sup>Foxp3<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Tregs). These preclinical data indicate a great therapeutic potential of orally-administered autoantigen-secreting *L. lactis* for tolerance induction in type 1 diabetes.
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, 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 the promise to target only β-cell-reactive T cells without impairing normal immune responses to diabetes-unrelated antigens.

Studies in man and in NOD mice, the most widely used preclinical model of T1D, have identified GAD65 as one of the major autoantigens in this disease (5,6). GAD65 is not only expressed 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 NOD mice (5,8). The majority of pre-diabetic and newly-diagnosed T1D patients test positive for anti-GAD autoantibodies (6,9) or GAD-reactive T cells (10). In NOD mice, GAD65-specific T cell responses can be detected as early as four 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 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* (*L. lactis*) (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 *L. lactis* expressing GAD65_{370-575} and 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 (GM) *L. lactis* strains. *L. lactis* (LL) strains secreting human GAD65<sub>370-575</sub>, having 98% amino acid identity to mouse GAD370-575 (Supplemental Fig. 1A and B), with or without human IL10 were generated by transformation of the parental MG1363 strain or the IL10-secreting sAGX0037 strain with GAD65<sub>370-575</sub>-encoding plasmid (24,25). The amino acid sequence for GAD65 was retrieved from NCBI (NP_000809.1).

Bacteria and media. Bacterial strains were grown and harvested as previously described (24). Pharmacokinetics of LL-GAD65<sub>370-575</sub>+IL10 was evaluated in normoglycemic NOD mice receiving 7 serial inoculations of this strain (2×10<sup>9</sup> CFU, every 30 minutes). One hour after the final inoculation, blood, caecum, proximal and distal small intestine and colon were collected. Blood and tissue homogenates, including luminal content, were plated to quantify bacterial recovery.

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 (AccuCheck, Roche, Almere, The Netherlands).

Treatments and follow-up. Hamster anti-mouse CD3 monoclonal antibody (mAb) (145-2C11, BioXCell, New Hampshire, USA) was administered intravenously (2.5 µg/d) for 5 consecutive days. LL-pT1NX, LL-IL10, LL-OVA+IL10, LL-GAD65<sub>370-575</sub>, or LL-GAD65<sub>370-575</sub>+IL10, were given by intragastric inoculation (2×10<sup>9</sup> CFU/d) 5× per week for 6 weeks. Weight and glycemia were measured 3× per week. Diabetes remission was
defined as absence of glycosuria and glycemia values < 200 mg/dl on two consecutive days.

**Beta-cell parameters.** Pancreases were harvested for histological analyses and/or for insulin content determination as described (24). Intraperitoneal glucose tolerance tests (IPGTT) were performed one week prior to killing of the mice as described. Serum C-peptide levels were measured by ELISA (Millipore, Billerica, MA) (24). Insulin autoantibodies (IAA) were measured as published (26).

**Flow cytometry.** Single cell suspensions were prepared from lymphoid organs by mechanical disruption. Pancreases were digested in pre-warmed digestion medium (RPMI1640 medium plus 5% FCS, 2 mM L-glutamine, 0.05 mM β-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mg/ml collagenase NB8 and 0.02 mg/ml DNase I) for 30 minutes 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 IFNγ was performed after *in vitro* restimulation with 50 ng/ml PMA and 1 µg/ml ionomycin for 6 hours, in the presence of monensin and brefeldin A (eBioscience; 1:1000) for the last 4 hours. Samples were acquired on a Gallios™ flow cytometer and data were analyzed with Kaluza™ software (Beckman Coulter).

**Antigen-specific proliferation assay.** Total splenocytes (5×10⁵) and pancreatic lymph node (PLN) cells (1-2×10⁵) were cultured with irrelevant ovalbumin peptide (OVA323-339 peptide; Innovagen) or GAD p524 (524-543 peptide; Eurogentec, Belgium) for 72 hours. PLN were cultured with mitomycin-treated splenocytes (1-2×10⁵) isolated from
normoglycemic NOD mice (10 weeks). $^3$H-thymidine (Amersham, Buckinghamshire, UK) was added for the last 18 hours (1 µCi). Cells were harvested on glass filter paper and counts per minute (cpm) determined in a liquid scintillation counter. Cells isolated from mice primed with GAD p524 were used as positive control.

**Laser Capture Microdissection (LCM) and Real-Time PCR.** Tissue slides were processed as previously described (28). Lymphocytic infiltrates were captured using HS Caps support. Real-Time PCR analyses were performed according to manufacturer’s instructions using Verity Thermalcycler and VIIA7 system for Real-Time PCR. Data analysis was performed using VIIA7 RUO 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.** CD$_4^+$CD$_{25}^-$ T cells were isolated by negative selection using antibodies to CD25, CD8, B220, CD11c, CD11b, MHC Class II, and sheep anti-rat IgG Dynabeads (Invitrogen, Merelbeke, Belgium). CD$_4^+$CD$_{25}^+$ T cells were isolated by positive selection from CD$_4^+$ T cells using anti-mouse CD25 biotin (eBioscience) and anti-biotin MicroBeads (Miltenyi Biotec).

**In vitro polyclonal suppression assay and IFNγ detection.** Suppressive function of CD$_4^+$CD$_{25}^+$ Tregs was assessed in an in vitro polyclonal suppression assay conducted as previously described (24). IFNγ was measured in cell-free supernatants as previously described (24).
**In vivo suppression assay.** CD4^+^CD25^+^ T cells from cured anti-CD3-treated or combi-GAD-treated mice were co-injected 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’s adjuvant (CFA; Difco Laboratories, Detroit, MI). Control mice were injected with PBS:CFA emulsion. After 11 days, popliteal LNs 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 LNs of cured combi-GAD-treated mice. ^3^H-thymidine addition as in 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-values < 0.05 were considered statistically significant. Graphs were plotted and statistics calculated with GraphPad Prism.
RESULTS

Diabetes reversal by LL-GAD65\textsubscript{370-575}+IL10 plus low-dose anti-CD3 (combi-GAD therapy). We constructed various bacterial strains secreting full human GAD65 protein or fragments thereof. For in vivo validation, we selected \textit{L. lactis} secreting human GAD65\textsubscript{370-575} (LL-GAD65\textsubscript{370-575}), or secreting human GAD65\textsubscript{370-575} in combination with human IL10 (LL-GAD65\textsubscript{370-575}+IL10) based on secretion capacity, verified by Western blotting and ELISA, bacterial growth profile and the presence of relevant CD4\textsuperscript{+} T cell epitopes (8) (data not shown). We examined whether oral administration of \textit{L. lactis} secreting GAD65\textsubscript{370-575} could reverse autoimmune diabetes by treating recent-onset diabetic NOD mice with LL-GAD65\textsubscript{370-575}+IL10, LL-GAD65\textsubscript{370-575}, LL-IL10 or LL-OVA+IL10 (2×10\textsuperscript{9} CFU) via gavage for 5 days per week during 6 weeks with or without an initial course (days 0-4) of systemic low-dose anti-CD3 mAb (2.5 µg per day) (24). As expected, in the absence of treatment none of the recent-onset diabetic NOD mice returned to normoglycemia (Fig. 1A). Administration of LL-GAD65\textsubscript{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 respectively 32% and 34% of treated mice, indicating that the anti-CD3 dose used is indeed suboptimal. Combination treatments of anti-CD3 with LL-GAD65\textsubscript{370-575} alone or anti-CD3 with LL-IL10 alone resulted in respectively 50% and 39% diabetes remission. Importantly, we found synergistic effects of combining LL-GAD65\textsubscript{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. 1A), 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. 1A). Also, 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. 1B).

Important towards potential clinical application, the combi-GAD therapy cured NOD mice with severe hyperglycemia at diagnosis (> 350 mg/ml) with similarly 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. 1C). 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. 1D). Finally, combi-GAD therapy reversed diabetes in NOD mice independent of sex or age (Fig. 1E and data not shown).

With regards 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-treated and cured anti-CD3-treated mice (data not shown), and stable weight evolution (data not shown).

**Combi-GAD therapy preserves β-cell function and prevents progression of insulitis.**

Pancreases of cured combi-GAD-treated animals had a similar insulin content as those
from age-matched normoglycemic NOD mice (Fig. 2A), and more than pancreases of untreated recent-onset, longstanding diabetic mice or cured anti-CD3-treated mice. In addition, concentrations of random C-peptide in serum of cured combi-GAD-treated mice and cured anti-CD3-treated mice were similar to those in recent-onset diabetic mice, but significantly higher than those in NOD mice with longstanding diabetes, indicating preservation of functional β-cells in cured mice (Fig. 2B). This was supported by intraperitoneal glucose tolerance tests (IPGTT) 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. 2C). Histological analysis furthermore showed that combi-GAD therapy not only prevented the worsening of insulitis that is normally observed from recent-onset to longstanding diabetes, but even ameliorated insulitis to similar degrees of age-matched normoglycemic mice (Fig. 2D).

**Combi-GAD therapy does not eliminate autoreactive T cells.** Tolerance can be installed via anergy or deletion of pathogenic effector T cells and/or via 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 pancreatic lymph nodes (PLN) and mesenteric lymph nodes (MLN) in comparison with untreated recent-onset diabetic animals (Supplemental Table 1). As a result, the CD4:CD8 ratio dropped more in combi-GAD-treated mice than in anti-CD3-treated mice as compared with recent-onset diabetic NOD mice (Supplemental Table 1).
We then examined if combi-GAD therapy changed effector T cell responses and analyzed the fraction of T cells producing the proinflammatory Th1 cytokine IFNγ. We found increased frequencies of IFNγ⁺ CD8⁺ and CD4⁺ T cells upon *in vitro* restimulation of spleen and PLN cells isolated from cured combi-GAD-treated mice, as compared with recent-onset diabetic NOD mice (Fig. 3A). This suggests that combi-GAD therapy does not eliminate pathogenic effector T cells.

To verify that mucosal GAD65₃₇₀₋₅₇₅ 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 PLN of recent-onset diabetic, cured anti-CD3 or combi-GAD-treated animals in the presence of irrelevant ovalbumin peptide (OVA₃₂₃₋₃₃₉) or GAD65 p524 peptide for 3 days (Fig. 3B). This showed that combi-GAD therapy did not boost GAD65-specific T cell responses.

To test if 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 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% or 100% respectively) (Fig. 3C), indicating 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 also does not induce tolerance via 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. 2D), 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 micro-dissection (LCM) to isolate the lymphocytic infiltrate of the islets. Gene expression analysis showed that the amounts of the pro-inflammatory molecules IFNγ, IL-1β and granzyme B were significantly decreased in the lymphocytic infiltrates of cured combi-GAD-treated mice in comparison 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 pro-inflammatory potential of local T cells in or around the pancreatic islets.

**Combi-GAD therapy induces Foxp3⁺ Tregs.** To test whether combi-GAD therapy induced tolerance via Tregs, we first examined the frequencies of Tregs using flow cytometry. Foxp3⁺ Tregs were present at higher frequencies in spleens of NOD mice cured by combi-GAD therapy or by anti-CD3 alone, as compared with recent-onset diabetic mice (Fig. 5A). Interestingly, only combi-GAD therapy increased the frequency of Tregs in MLN and PLN, irrespective of identification as Foxp3⁺ single-positive or Foxp3⁺CD25⁺ double-positive CD4⁺ T cells (Fig. 5A and data not shown). Conversely, only anti-CD3 treatment significantly increased the numbers of Foxp3⁺ Tregs in the pancreas (Fig. 5B and C). Frequencies of Foxp3⁺ Tregs were not different in Peyer’s 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 LN 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 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. 6A), lowered expression of the activation markers CD69 (Fig. 6B) and CD44 (Fig. 6C), and reduced secretion of the effector cytokine IFN\(\gamma\) (Fig. 6D). Nevertheless, CD4\(^{+}\)CD25\(^{+}\) T cells isolated from cured combi-GAD-treated mice were similarly effective as those isolated from age-matched normoglycemic or cured anti-CD3-treated mice, indicating 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. 6E). Co-transfer of CD4\(^{+}\)CD25\(^{+}\) T cells from spleens and PLN of cured anti-CD3-treated or combi-GAD-treated NOD mice similarly and significantly delayed the onset of diabetes in recipient mice (Fig. 6E). 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. 7A). Addition of CD4⁺CD25⁺ T cells from cured combi-GAD-treated mice suppressed GAD-specific proliferation of GAD-responder cells (Fig. 7A). In the absence of GAD peptide, CD4⁺CD25⁺ Tregs from cured combi-GAD-treated mice also suppressed responses of OVA-stimulated T cells (Fig. 7A), although with significantly less potency than responses of GAD-stimulated T cells (Fig. 7B), suggesting involvement of passive mechanisms such as IL-2 stealing via 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 TGF-β production. From this, we conclude that CD4⁺CD25⁺ T cells from cured combi-GAD-treated mice are GAD-reactive.
DISCUSSION

We report here the preclinical success of an antigen-specific combination therapy for tolerance induction in T1D. Autoantigen-specific strategies aim to restore the loss of tolerance that underlies T1D with less side-effects than antigen-non-specific 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 proper choice of adjuvant and adequate route of delivery. Thus, the gut has been proposed as the optimal route, 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 presentation. This can explain why previous studies did not report positive findings with orally administered GAD65 (23). Antigen-specific therapies do have the additional potential to induce bystander suppression towards 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 our combi-GAD therapy and our previously reported combi-PINS therapy (24). Both therapies could reverse diabetes in overtly diabetic NOD mice, but small differences in immune effects could be observed. Whereas in combi-PINS-treated mice,
Tregs preferentially accumulated in pancreatic islets, in the present combi-GAD-treated mice, Foxp3+ Tregs were typically found in PLN. Interestingly, in contrast to anti-CD3 alone or to 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 our 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 our combi-GAD therapy comes from the multi-pronged attack on the autoimmune response. First, we deliver viable *L. lactis* 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 co-deliver 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 co-administration 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 day 10 to 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 non-exclusive ways: administration of antigen at 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 is important because Tregs are functionally compromised in T1D patients (42,43).

It remains to be determined whether the observed increase in IFNγ 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 treatment both increased Treg frequencies in the PLN, but only combi-GAD therapy increased the Treg frequencies in the MLN and only combi-PINS therapy allocated Foxp3+ cells to the pancreas. It is known that regulation by Tregs can occur in the PLN (17) and that MLN 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 GAD65370-575 with IL10 by GM L. lactis and a systemic low-dose of anti-CD3 mAb stably reversed diabetes in recent-onset diabetic
NOD mice. Our results further underscore the potential of using genetically-modified \textit{L. lactis} as part of a therapy for autoimmune diabetes, as we had previously demonstrated with orally-administered \textit{L. lactis} secreting PINS and IL10 combined with systemic delivery of anti-CD3 (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 other antigen-specific therapies’ success in preclinical models but failure in clinical trials, the promising potential of combi-GAD therapy needs clinical validation.
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K.V.H., L.S., S.C., and P.R. have financial interests in ActoGeniX NV, including employment and stock options. Otherwise we declare no conflict of interest.

S.R. designed and performed research, analyzed data and wrote the paper. T.T. performed research. K.V.H., L.S., and S.C. designed and performed research. I.S. and G.S.
performed the pancreatic islet histology and immunohistochemistry. F.D. designed research. P.D. performed the gut histology. C.H.W. performed the insulin autoantibody measurements. M.A.A. edited the paper. H.K. designed and performed research. P.R. designed research and edited the paper. T.V.B. and C.G. designed and performed research and wrote and edited the paper. C.M. designed research and wrote and edited the paper. T.V.B., C.G., 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.

These data have already been published in abstract form on the annual meeting of Belgian Immunological Society (BIS), 12th International Conference on the Immunology of Diabetes (IDS) and the Keystone symposium Immunopathology of Type 1 Diabetes.
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TABLE LEGENDS

Supplemental table 1. Percentage of CD4$^+$ cells, CD8$^+$ cells and the CD4/CD8 ratio in the pancreas, PLN, MLN and spleen as analyzed by flow cytometry. Data are shown as mean ± SEM in viable CD3$^+$ gate (8-33 mice per group). PLN, pancreatic-draining lymph nodes; MLN, mesenteric lymph nodes. Statistical significance was calculated by Mann-Whitney or unpaired t-test. * $p < 0.05$ and ** $p < 0.01$ versus recent-onset diabetic.
FIGURE LEGENDS

FIG. 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 post-treatment initiation. A: Percentage of diabetic mice per treatment group. † indicates dead or moribund mice. B: Individual glycemia values of combi-GAD-treated (left) and anti-CD3-treated (right) recent-onset diabetic NOD mice. Open and closed symbols represent animals that were non-cured and cured at the end of treatment, respectively. C-E: Therapeutic efficacy of combi-GAD-treated (left) and anti-CD3-treated (right) mice was stratified (C) according to blood glucose concentrations at diabetes diagnosis, or (D) according to the presence of insulin autoantibodies in the serum at the start of treatment, or (E) according to gender. LL: *L. lactis*; autoAb: autoantibody. Statistical significance between groups was calculated using Mantel-Cox Log-rank test. *p < 0.05, **p < 0.01, ***p < 0.001.

FIG. 2. Combi-GAD treatment preserves β-cell function and prevents insulitis progression. A: Pancreatic insulin content (ng insulin/mg pancreas) and B: C-peptide concentration in serum (pM) for control groups and cured anti-CD3- and combi-GAD-treated mice at treatment stop as measured by insulin and C-peptide ELISA, respectively. ND: Not detectable. C: Intraperitoneal glucose tolerance tests (IPGTT) were performed on control groups and cured mice 1 to 2 weeks prior to treatment stop. Corresponding area under the curve (AUC) over 2 hours is shown. Statistical significance between groups was calculated by Mann-Whitney t-test. Bar graphs represent mean ± SEM. *p < 0.05, **p < 0.01 and ***p < 0.001 versus longstanding diabetic, and $p < 0.05 versus
recent-onset diabetic. D: Insulitis was blindly scored as indicated in pancreatic sections of control mice and cured anti-CD3-treated and combi-GAD-treated mice at treatment stop.

FIG. 3. Combi-GAD therapy induces IFNγ-producing T cells and does not eliminate T effector cells. A: Bar graphs showing IFNγ+ cells in the CD4+ gate (top) and CD8+ gate (bottom) of spleen (left) and pancreatic lymph node (PLN; 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 PLN (1-2×10^5; right) of indicated treatment groups were cultured in the presence of irrelevant ovalbumin peptide (OVA 323-339) or GAD p524 peptide for 3 days. PLN were cocultured with mitomycin-treated splenocytes isolated from 10 weeks old normoglycemic NOD mice. Proliferative responses were measured by ³H-labeled thymidin incorporation. We used the proliferative response of popliteal lymph nodes (popliteal LN) isolated from GAD p524-immunized mice (footpath) as positive control. The proliferative response is expressed as a stimulation index calculated by dividing the mean counts per minute (cpm) of the Ag-stimulated condition by the mean cpm of the unstimulated condition of the same treatment group. Results shown are the average of 2 pooled experiments. Statistical significance between groups was calculated by Mann-Whitney t-test. Bar graphs represent mean ± SEM. *p < 0.05, **p < 0.01 and ***p < 0.001 versus the positive control (GAD p524 peptide + immunized popliteal LN), and $p < 0.05$ 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.

FIG. 4. Combi-GAD therapy decreases inflammatory profile of islet cell infiltrates locally in the pancreas. Shown is the gene expression of pro-inflammatory molecules in pancreatic lymphocytic infiltrates isolated by laser capture microdissection. Data are expressed as fold change in IFNγ, 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.

FIG. 5. Combi-GAD therapy induces Foxp3+ T cells in spleen, mesenteric and pancreatic lymph nodes. A and B: Analysis of Foxp3 expression by flow cytometry in (A) spleen, pancreatic lymph nodes (PLN), mesenteric lymph nodes (MLN) and (B) pancreas of recent-onset diabetics (triangle), cured anti-CD3-treated (circle) and cured combi-GAD-treated (square) NOD mice. Shown are scatter plots 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.

FIG. 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 hours in the presence of CD4+CD25+ Tregs (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 1 or more divisions, normalized to proliferation by Tresp-only culture. B and C: Activation of Tresp, shown as percentage of CD69 (B) or CD44 (C) expression, normalized to the percentage in Tresp-only culture. D: Measurement of IFNγ in the co-culture supernatant by cytometric bead assay, normalized to the concentration in Tresp-only culture. Results shown are the average of 3 pooled experiments. Statistical significance versus Tresp alone and between corresponding dilutions of different treatment groups was calculated with Mann-Whitney t-tests. * p < 0.05 and ** p < 0.01 versus responders alone. E: In vivo assay for disease-specific suppressive capacity. CD25-depleted splenocytes from diabetic mice were transferred into NOD/SCID mice without (triangles) or with CD4⁺CD25⁺ cells isolated from spleen and PLN of cured anti-CD3-treated (circles) or cured combi-GAD-treated (squares) NOD mice. Shown is the diabetes incidence in recipients. Statistical significance was calculated using Mantel-Cox Log-rank test. ** p < 0.01.

FIG. 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 co-cultured 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 ³H-labeled thymidine incorporation in the last 18 hours. Data are expressed relative to GAD-activated GADresp. Results shown are the average of 4 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 formula 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.
SUPPLEMENTAL FIGURE LEGENDS

SUPPLEMENTAL FIG. 1. Representation linear amino acid sequence of human and mouse GAD65_{370-575}. A: *L. lactis* was genetically modified to secrete a C-terminal fragment of GAD65, namely GAD65_{370-575}, in combination with (LL-GAD65_{370-575}+IL10) or without IL10 (LL-GAD65_{370-575}). B: Human and mouse GAD65_{370-575} amino acid sequence alignment.
Figure 1.

A

![Graph showing changes in glycemia over time for different treatments.](image)

B

**Combi-GAD**

- Non-cured (n=12)
- Cured (n=24)

C

- < 350 mg/dl (n=21)
- > 350 mg/dl (n=15)

D

- AutoAb - (n=17)
- AutoAb + (n=7)

E

- Male (n=12)
- Female (n=24)

Time (weeks after treatment initiation)

- Non-treated diabetics (n=15)
- LL-GAD65 370-575 + IL10 (n=10)
- Anti-CD3 (n=44)
- Anti-CD3 + LL-pTINX (n=35)
- Anti-CD3 + LL-IL10 (n=33)
- Anti-CD3 + LL-OVA + IL10 (n=10)
- Anti-CD3 + LL-GAD65 370-575 (n=12)
- Anti-CD3 + LL-GAD65 370-575 + IL10 (n=36)
Figure 2.

**A**

![Bar graph](image1)

**B**

![Bar graph](image2)

**C**

![Bar graph](image3)

**D**

![Bar graph](image4)

Legend:
- Age-matched normoglycemic (n=4-13)
- Recent-onset diabetic (n=4-8)
- Longstanding diabetic (n=5-10)
- Anti-CD3 (n=7-10)
- Combi-GAD (n=10-14)

*No insulitis*
- Peri-insulitis
- Mild insulitis
- Heavy insulitis

For Peer Review Only
Figure 3

A

Spleen

PLN

B

Spleen

PLN

C

CD25-depleted splenocytes from diabetic mice (n=7)

CD25-depleted splenocytes from anti-CD3-cured mice (n=10)

CD25-depleted splenocytes from combi-GAD-cured mice (n=12)
Figure 4

Relative expression ($2^{-\Delta\Delta C_T}$)

IFNγ
- Recent-onset diabetic (n=3)
- Anti-CD3 (n=3)
- Combi-GAD (n=3)

IL-1β
- Recent-onset diabetic (n=3)
- Anti-CD3 (n=3)
- Combi-GAD (n=3)

Granzyme B
- Recent-onset diabetic (n=3)
- Anti-CD3 (n=3)
- Combi-GAD (n=3)

**
Figure 5

A

Spleen

B

PLN

C

MLN

**  

*p = 0.08  

^ Recent-onset diabetic (n=4-15)  

• Anti-CD3 (n=6-10)  

■ Combi-GAD (n=6-36)  

Foxp3+ in viable CD3+CD4+
Figure 6

A

Proliferation (normalized to responder only)

Treg:Tresp ratio: CD4+CD25+ from: Age-matched normoglycemic Anti-CD3-cured Combi-GAD-cured

B

CD69 % (normalized to responder only)

Treg:Tresp ratio: CD4+CD25+ from: Age-matched normoglycemic Anti-CD3-cured Combi-GAD-cured

C

CD44 % (normalized to responder only)

Treg:Tresp ratio: CD4+CD25+ from: Age-matched normoglycemic Anti-CD3-cured Combi-GAD-cured

D

IFN-γ (normalized to responder only)

Treg:Tresp ratio: CD4+CD25+ from: Age-matched normoglycemic Anti-CD3-cured Combi-GAD-cured

E

Diabetes incidence (%)

Time (weeks after transfer)

- CD25- splenocytes only
- $10^6$ CD4+CD25+ cells from anti-CD3-cured
- $10^6$ CD4+CD25+ cells from combi-GAD-cured
Figure 7

A

B

For Peer Review Only
A

hGAD65

NH₂ - 370 575 - COOH

B

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<tr>
<th>Species</th>
<th>Sequence</th>
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<td>GLLMSRKHKW KLSGVERANS VTWNPHKMMG VPLQCSALLV REEGLMQNCN QMHASYLFQQ DKHYDLSYDT</td>
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<td>Mouse 370 - 439</td>
<td>GLLMSRKHKW KLSGVERANS VTWNPHKMMG VPLQCSALLV REEGLMQNCN QMHASYLFQQ DKHYDLSYDT</td>
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<tr>
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<td>PPSLRTLEDN EERMSRLSKV APVIKARMME YGTTMVSYQP LGDKVNFFRM VISNPAATHQ DIDFLI</td>
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<tr>
<td>Mouse 510 - 575</td>
<td>PPSLRTLEDN EERMSRLSKV APVIKARMME YGTTMVSYQP LGDKVNFFRM VISNPAATHQ DIDFLI</td>
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<tr>
<td></td>
<td>Recent-onset diabetic</td>
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<tr>
<td><strong>Pancreas</strong></td>
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<tr>
<td>CD4$^+$</td>
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<td>CD4/CD8</td>
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<td>CD4$^+$</td>
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<td>CD8$^+$</td>
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