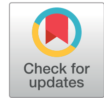

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Reversal of Diabetes in NOD Mice by Clinical-Grade Proinsulin and IL-10–Secreting *Lactococcus lactis* in Combination With Low-Dose Anti-CD3 Depends on the Induction of Foxp3-Positive T Cells

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The introduction of β-cell autoantigens via the gut through *Lactococcus lactis* (*L. lactis*) has been demonstrated to be a promising approach for diabetes reversal in NOD mice. Here we show that a combination therapy of low-dose anti-CD3 with a clinical-grade self-containing *L. lactis*, appropriate for human application, secreting human proinsulin and interleukin-10, cured 66% of mice with new-onset diabetes, which is comparable to therapy results with plasmid-driven *L. lactis*. Initial blood glucose concentrations (<350 mg/dL) and insulin autoantibody positivity were predictors of the stable reversal of hyperglycemia, and decline in insulin autoantibody positivity was an immune biomarker of therapeutic outcome. The assessment of the immune changes induced by the *L. lactis*–based therapy revealed elevated frequencies of CD4$^+$Foxp3$^+$ T cells in the pancreas-draining lymph nodes, pancreas, and peripheral blood of all treated mice, independent of metabolic outcome. Neutralization of cytotoxic T-lymphocyte antigen 4 and transforming growth factor-β partially abrogated the suppressive function of therapy-induced regulatory T cells (Tregs). Ablation or functional impairment of Foxp3$^+$ Tregs in vivo at the start or stop of therapy impaired immune tolerance, highlighting the dependence of the therapy-induced tolerance in mice with new-onset diabetes on the presence and functionality of CD4$^+$Foxp3$^+$ T cells. Biomarkers identified in this study can potentially be used in the future to tailor the *L. lactis*–based combination therapy for individual patients.

Clinical translation of antigen-based therapies has been disappointing so far, and, specifically in autoimmune type 1 diabetes (T1D), the administration of oral insulin or GAD has not been efficacious in preventing or halting the disease in high-risk individuals or in new-onset patients to date (1–3). Issues like the route and timing of vaccination and dosing strategy, but also the reliability of delivery of full protein or peptide due to gastric digestion may lie at the basis of these failures (4). The introduction of a delivery vehicle like the gram-positive food-grade lactic acid bacterium *Lactococcus lactis* (*L. lactis*), which is able to deliver intact antigen and immunomodulating cytokines directly into the gut, in proximity to the gut-associated lymphoid tissue, is an appealing tool (5,6). Another

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reason why antigen-based therapies did not succeed in stopping ongoing autoimmune processes may be that by the time the disease manifests, the strong pathogenic immune reactions overpower the regulatory mechanisms induced by the therapy. In light of this consideration, we and others (7,8) advocate that combinations of robust antigen-based interventions and systemic immune modulators may ultimately be needed to successfully reinstate long-term tolerance in ongoing autoimmunity without compromising immune function. Previously, we reported that a combination therapy consisting of a 5-day course of anti-CD3 antibodies at disease onset along with 6 weeks of oral administration of live, genetically modified *L. lactis* producing human proinsulin (PINS) and interleukin-10 (IL-10) safely restored durable normoglycemia in ~60% of nonobese diabetic (NOD) mice and elicited forkhead box p3–positive (Foxp3$^+$) T cells with a regulatory phenotype (9). The route to bring this successful antigen-based therapy to new-onset T1D patients will depend both on the generation of a clinical-grade self-containing *L. lactis* strain (10), but also on a profound understanding of the processes underlying this disease-modifying approach, and, consequently, on the implementation of certified biomarkers of both immune and therapeutic success.

Here, we demonstrated similar therapeutic efficacy in autoimmune diabetes remission using a clinical-grade self-containing *L. lactis* vaccine compared with the plasmid-driven *L. lactis* strain previously reported (9). In addition, we identified both functional β-cell reserve (initial blood glucose concentrations <350 mg/dL) and pretherapy insulin autoantibody (IAA) positivity as predictors of therapeutic efficacy, and proved that Foxp3$^+$ T cells are a prerequisite for the induction and maintenance of active tolerance induced by the *L. lactis*–based therapy.

RESEARCH DESIGN AND METHODS

Mice

NOD mice, originally obtained from Dr. C.Y. Wu (Department of Endocrinology, Peking Union Medical College Hospital, Beijing, People's Republic of China), were housed and inbred in the animal facility of Katholieke Universiteit Leuven (Leuven, Belgium) since 1989. NOD.Foxp3.DTR (diphtheria toxin receptor) mice and NOD.Foxp3.hCD2 mice were bred from stocks provided by Dr. C. Benoist (Harvard Medical School, Boston, MA) and Dr. S. Hori (RIKEN Research Center for Allergy and Immunology, Yokohama, Japan), respectively. The housing of all mice occurred under semibarrier conditions, and animals were fed sterile food and water ad libitum. Mice were screened for the onset of diabetes by evaluating glucose levels in urine (Diastix Reagent Strips; Bayer, Leverkusen, Germany) and venous blood (AccuCheck; Roche Diagnostics, Vilvoorde, Belgium). Mice were diagnosed as diabetic when they had glucosuria and two consecutive blood glucose measurements exceeding 200 mg/dL. NOD-scid and NOD-scid $\gamma c^{-/-}$ mice were bred from stocks purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were maintained in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, and all experimental procedures were approved and performed in accordance with the Ethics Committees of the Katholieke Universiteit Leuven under project no. 185-2009.

Bacteria and Media

Details on the construction, culture, and in vitro quantification of the lactococcal vectors used in the current study are available in the Supplementary Research Design and Methods (9). For intragastric inoculations, stock suspensions were diluted 1,000-fold in growth media and incubated for 16 h at 30°C, reaching a saturation density of 2×10^9 colony-forming units/mL. Bacteria were harvested by centrifugation and concentrated 10-fold in BM9 medium. Treatment doses consisted of 100 μL of this bacterial suspension.

New-Onset Diabetes Intervention

Upon diabetes determination, NOD or NOD transgenic mice were treated for 5 consecutive days intravenously (days 0–4; 2.5 μg/mouse) with hamster anti-mouse CD3 antibodies (clone 145–2C11; BioXCell, West Lebanon, NH). This therapy was given in combination with oral administration of either plasmid-driven or clinical-grade *L. lactis* strains (2×10^9 colony-forming units) 5 times/week over 6 weeks. Control mice were left untreated. Individual blood glucose concentrations at the start of treatment were recorded. Mice were tested three times weekly for their weight and blood glucose status. Remission was defined as the absence of glucosuria and a return to normal blood glucose concentrations. Experimental animals were sacrificed immediately or long after stopping therapy (6 or 14 weeks after treatment initiation). Peripheral blood, lymph organs, and pancreas were harvested, and single cells were assessed for phenotyping as described in the Supplementary Research Design and Methods. Detailed methodology and references on in vitro suppression assays are described in the Supplementary Research Design and Methods. Mice were removed from the study prior to the 14-week end point when blood glucose concentrations exceeded 600 mg/dL in two consecutive measurements.

Glucose Tolerance Test

One or two weeks prior to sacrifice, intraperitoneal glucose tolerance tests (IPGTTs) were performed. Mice were fasted for 16 h, injected with glucose (2 g/kg i.p.), and blood glucose concentrations were measured at 0, 15, 30, 60, 90, and 120 min.

IAA Measurement

Heparinized plasma was collected from NOD mice with new-onset diabetes before treatment randomization and at therapy discontinuation, and IAAs were analyzed at the University of Florida Department of Pathology, Immunology

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and Laboratory Medicine, College of Medicine (Gainesville, FL), as previously described (11).

In Vivo Blocking of Cytotoxic T-Lymphocyte Antigen 4 and Transforming Growth Factor-β

Mice tolerized by *L. lactis*–based therapy were injected intraperitoneally after therapy withdrawal with blocking antibodies against cytotoxic T-lymphocyte antigen 4 (CTLA4) (clone UC10–4F10; Bioceros) and transforming growth factor-β (TGF-β) (clone 1D11.16.8; BioXCell) in the following dose regimen: 250 μg at days 1 and 3, and then 100 μg at days 6, 8, 10, 13, and 18 for CTLA4; 200 μg three times per week over 3 weeks for TGF-β. Blood glucose concentrations were measured daily up to 25 days after the first injection.

Adoptive Transfer of Diabetes

To assess the diabetogenic potential of effector T cells (Teffs), all T cells from the spleens (1×10^7 cells) of control mice with new-onset diabetes, responders and nonresponders to *L. lactis*–based therapy, were transferred intravenously into the tail veins of 6- to 8-week-old immune-deficient NOD-scid mice. Recipient mice were monitored twice weekly for the development of diabetes up to 100 days after cell transfer.

Diphtheria Toxin–Mediated Depletion of Foxp3$^+$ T Cells in NOD.Foxp3.DTR Mice

NOD.Foxp3.DTR mice (expressing the human DTR under the control of Foxp3 transcriptional control elements) allow for the depletion of Foxp3$^+$ T cells upon diphtheria toxin (DT) administration (12). For regulatory T-cell (Treg) depletion, NOD.Foxp3.DTR mice (unmanipulated or tolerized after stopping the *L. lactis*–based therapy) were injected intravenously with 40 μg/kg body wt DT (Sigma-Aldrich) on days 1, 2, 4, and 7, and examined on day 8. After DT injections, weight, urine, and blood glucose status of mice were monitored. Foxp3$^+$ T cells were monitored in peripheral blood and pancreas by flow cytometry and histology, respectively, as previously described (13).

FOXP3-Inhibitory Peptide P60 in Combination With *L. lactis*–Based Therapy

P60 (a 15-mer synthetic peptide that can bind to and block FOXP3, 50 μg/dose i.p. daily, up to 14 doses) was administered at start of the *L. lactis*–based therapy, as previously described (14).

Histology of Pancreas and Insulitis Grading

Six-micrometer sections from formalin-fixed, paraffin-embedded pancreas tissues were cut and collected 100 μm apart, then stained with hematoxylin-eosin. Islets were observed under light microscopy at 20× or 40×, enumerated, and graded by an independent investigator in blinded fashion. At least 25 islets per pancreatic sample were scored for islet infiltration as follows: 0, no infiltration; 1, peri-insulitis; 2, islets with lymphocyte infiltration in <50% of the area; and 3, islets with lymphocyte infiltration in >50% of the area or completely destroyed.

Islet-Resident Foxp3$^+$ T-Cell Detection

Pancreas tissues were snap frozen in 2-methyl-butane 99% (ACROS Organics, Geel, Belgium), and cut into 12-μm tissue sections. Foxp3$^+$ T-cell detection was performed as previously described (9).

Statistics

All data were analyzed using GraphPad Prism 6 (GraphPad, La Jolla, CA). Survival curves were computed with the Kaplan-Meier test and were compared with the log-rank test. Groups were analyzed by ANOVA (nonparametric Kruskal-Wallis test) with the Dunn multiple comparison test or with the Mann-Whitney *U* test, as appropriate. Error bars represent the SEM. Unless otherwise indicated, differences are not significant. Symbols for *P* values are defined as follows: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

RESULTS

A Clinical-Grade Self-Containing *L. lactis* Vaccine Combined With Low-Dose Anti-CD3 Stably Reverts New-Onset Diabetes, Preserves Residual β-Cell Function, and Halts Insulitis Progression in NOD Mice

Using a clinical-grade self-containing *L. lactis* strain secreting human PINS along with IL-10 in combination with subtherapeutic doses of anti-CD3 antibodies, 66% of mice (23 of 35 mice) reverted to normoglycemia for at least 14 weeks after disease onset, which was significantly superior to the 43% of mice treated by anti-CD3 alone (Fig. 1*A*). This therapeutic efficacy obtained with the clinical-grade *L. lactis* strain was comparable to the combination therapy with plasmid-driven *L. lactis* strain (72% of mice [18 of 25 mice]; not significant). As expected, animals left untreated (n = 20) or treated with the empty vector bacterial strain *L. lactis*-pT1NX (n = 9) remained hyperglycemic and were sacrificed when 20% of their starting body weight was lost. Monotherapy with either the clinical-grade or plasmid-driven *L. lactis* strain secreting PINS and IL-10 was significantly less effective than the combination with anti-CD3 (0% [n = 8] and 17% [n = 8], respectively) (Fig. 1*A*).

During follow up, controls with new-onset diabetes and mice protected or not by *L. lactis*–based therapy were subjected to IPGTT and sacrificed 6 weeks after treatment initiation, at which time their pancreas tissues were assessed by histology. Only in the successfully treated animals, residual β-cell function (i.e., assessed as the area under the glucose tolerance curve) was preserved, and smaller proportions of islets had severe insulitis (Fig. 1*B*). Of interest, at the end of the combination therapy no difference in the severity of insulitis was observed between responders and nonresponders (Fig. 1*C*).

Starting Glycemia and IAA Positivity Predict Therapeutic Success of *L. lactis*–Based Therapy

No influence of the age or sex of mice was observed on the therapeutic success of the *L. lactis*–based therapy (data not shown). However, as previously shown (9), glycemic

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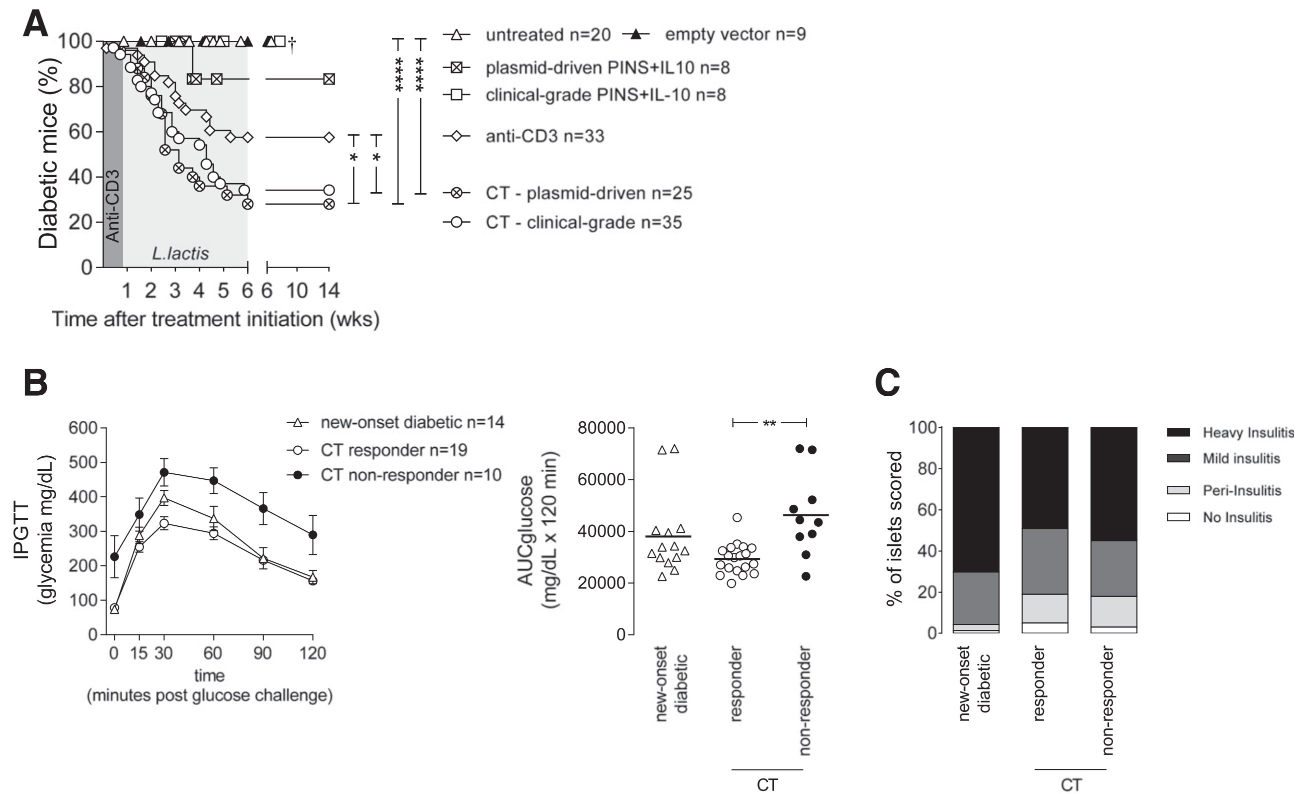

Figure 1—A clinical-grade self-containing *L. lactis* vaccine combined with low-dose anti-CD3 stably reverts new-onset diabetes, preserves residual β-cell function, and halts insulitis progression in NOD mice. NOD mice with new-onset diabetes were treated as indicated, and blood glucose concentrations were followed up for 14 weeks post-treatment initiation. *A*: Shown is the percentage of mice that remained diabetic after treatment. †Dead or moribund mice. In all Kaplan-Meier survival curves, statistical significance between groups was determined by Mantel-Cox log-rank test: **P* < 0.05, *****P* < 0.0001. *B*: IPGTTs were performed on new-onset diabetic NOD mice in addition to *L. lactis*–based combination therapy (CT)–treated mice (both responders and nonresponders) 1–2 weeks prior to treatment termination. The corresponding area under the glucose tolerance curve (AUCglucose; mg/dL × 120 min) over 2 h is shown. *C*: Insulitis scoring was performed in a blinded manner on paraffin-embedded pancreatic sections of mice with new-onset diabetes and *L. lactis*–based CT-treated mice (both responders and nonresponders), as indicated, at the end of treatment. Statistical significance between groups was calculated using the Mann-Whitney *t* test; ***P* < 0.01.

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concentrations at the beginning of therapy predicted success, with 82% of mice starting with a glycemia <350 mg/dL cured (n = 22), compared with 38% of mice with a starting glycemia >350 mg/dL (n = 13) (Fig. 2*A*). In addition, positivity for IAAs at study entry seemed to correlate with therapeutic success (Fig. 2*B*). Interestingly, mice with blood glucose concentrations <350 mg/dL and IAA positivity at therapy start had a clearly superior diabetes remission rate (89%; n = 8) than mice with blood glucose levels >350 mg/dL and being IAA negative (33%; n = 5; P = 0.07) (Fig. 2*C*). Moreover, the *L. lactis*–based therapy significantly decreased IAA levels, particularly in mice responsive to the therapy (Fig. 2*D*).

L. lactis–Based Therapy Induces Higher Levels of Foxp3$^+$ T Cells With Regulatory Capacity but No Changes in Teffs

The mechanisms underlying disease remission induced by the *L. lactis*–based treatment were investigated by dissociating between the therapeutic immune effects in mice responsive or not to the intervention. We found that the percentages of CD4$^+$Foxp3$^+$ (both CD25$^+$ and CD25$^-$) T cells observed in the peripheral blood (Fig. 3*A*), the pancreas-draining lymph nodes (Fig. 3*B*), and the pancreas (Fig. 3*C*) were significantly higher in mice treated with the *L. lactis*–based therapy in comparison with untreated controls. Interestingly, in the pancreas-draining lymph nodes and pancreas, but not in peripheral blood, the increased frequency of CD4$^+$Foxp3$^+$ T cells was less pronounced in responders than in nonresponders. Using multicolor flow cytometry, we identified that most CD4$^+$Foxp3$^+$ Tregs were positive for CTLA4 and that the expression of this inhibitory marker was significantly higher in pancreas-draining lymph nodes (for both responders and nonresponders) and pancreata (only for responders) of treated mice compared with untreated controls (Supplementary Fig. 1*B* and *C*). Of interest, no differences in the percentages of CD4$^+$Foxp3$^+$CTLA4$^+$ T cells were observed in the peripheral blood of treated mice compared with untreated controls (Fig. 3*D*).

The percentages of naive (CD44$^-$CD62L$^+$CCR7$^+$), effector memory (CD44$^+$CD62L$^-$CCR7$^-$) and central

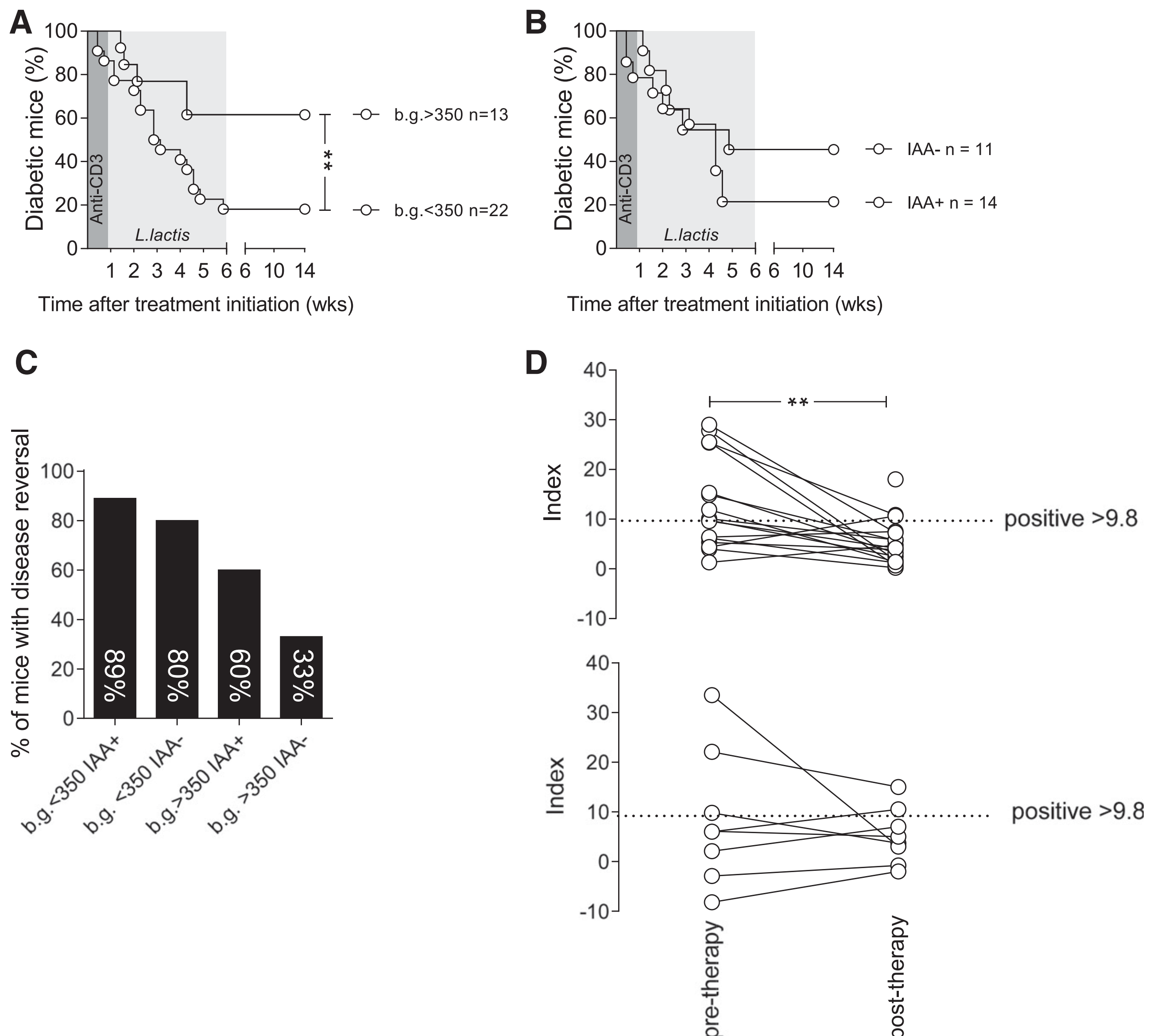

Figure 2—Starting glycemia and IAA positivity at study entry predict the therapeutic success of *L. lactis*–based combination therapy. *A*: NOD mice with new-onset diabetes were stratified based on starting blood glucose concentrations (b.g.) under or above 350 mg/dL at study entry. Shown is the percentage of mice that remained diabetic after combination treatment with the clinical-grade *L. lactis* strain. In the Kaplan-Meier survival curve, statistical significance between groups was determined by the Mantel-Cox log-rank test; $^{**}P < 0.01$. *B*: Before treatment randomization, the serum of each individual NOD mouse was collected to measure IAA levels in a blinded fashion. The percentage of mice responsive or not to *L. lactis*–based combination therapy for IAA positivity. *C*: NOD mice new-onset diabetic were stratified based on starting b.g. under or above 350 mg/dL and IAA status (positive or negative) at study entry. Shown is the percentage of mice that were tolerized after therapy. *D*: IAA levels at diabetes diagnosis and after *L. lactis*–based combination treatment follow-up in therapy responders (top panel) and nonresponders (bottom panel). Statistical significance between groups was calculated using the Mann-Whitney *t* test; $^{**}P < 0.01$. Starting glycemia and positivity for IAAs at study entry correlated with therapeutic success. Change in IAA positivity between pretherapy and post-therapy was significantly different in therapy responders.

memory ($CD44^+CD62L^+CCR7^+$) $CD4^+$ T cells were not altered in any recipient group with respect to therapeutic success or failure (data not shown). The transfer of splenocytes from responders and nonresponders of *L. lactis*–based treatment caused diabetes in NOD-scid recipients with similar disease kinetics as seen with the transfer of splenocytes isolated from untreated controls with new-onset diabetes, suggesting that circulating diabetogenic cells were not depleted from treated mice (Supplementary Fig. 2).

Diabetes Reversal Induced by *L. lactis*–Based Therapy Is Accompanied by and Depends on the Generation of Functional $Foxp3^+$ Tregs

Using NOD.Foxp3.hCD2 mice treated by *L. lactis*–based therapy, we could isolate $CD4^+CD25^+Foxp3^+$ T cells for

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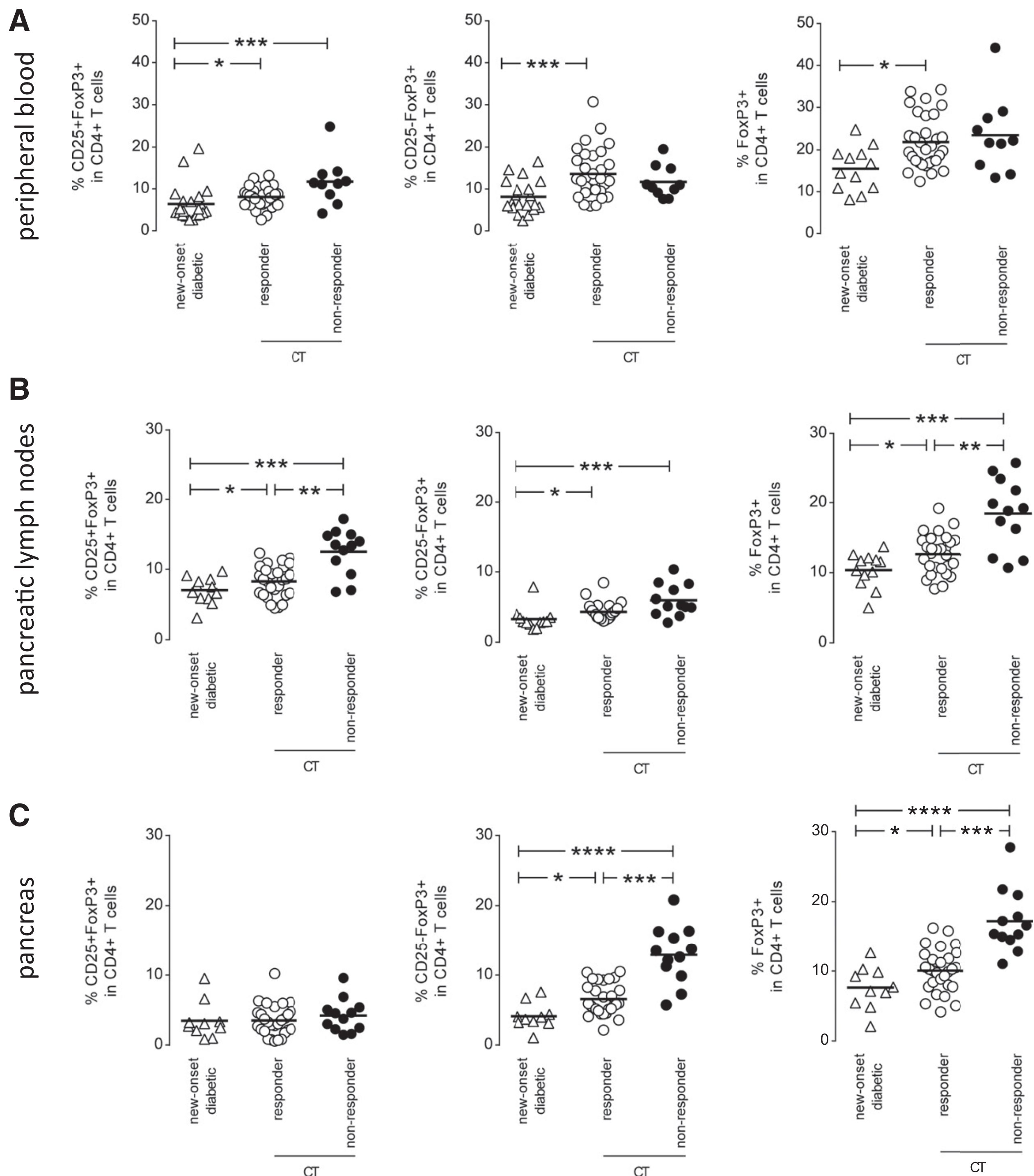

Figure 3—*L. lactis*–based combination therapy induces higher levels of Foxp3$^+$ T cells in responders than in nonresponders. *A*–*C*: The percentages of CD25$^+$Foxp3$^+$ cells (left panel), CD25$^-$Foxp3$^+$ cells (middle panel), and total Foxp3$^+$ cells (right panel) within the CD4$^+$ T-cell population in peripheral blood (*A*), pancreas-draining lymph nodes (*B*), and pancreata (*C*) of mice with new-onset diabetes and *L. lactis*–based combination therapy (CT)–treated mice (both responders and nonresponders). *D*–*F*: The percentages of CD25$^+$Foxp3$^+$CTLA4$^+$ cells (left panel), CD25$^-$Foxp3$^+$CTLA4$^+$ cells (middle panel), and Foxp3$^+$CTLA4$^+$ cells (right panel) within the CD4$^+$ T-cell population in peripheral blood (*D*), pancreas-draining lymph nodes (*E*), and pancreata (*F*) of mice with new-onset diabetes and *L. lactis*–based CT-treated mice (both responders and nonresponders). Each symbol represents one mouse, and horizontal bars indicate the median value. Statistical significance was calculated using Mann-Whitney *t* test; *P < 0.05, **P < 0.01, ***P < 0.001; ****P < 0.0001.

functional in vitro studies, in which they suppressed proliferation, CD69 activation, and interferon-γ production of pathogenic CD4$^+$CD25$^-$ Teffs. These Tregs produced IL-10 (and TGF-β) when they were cocultured and stimulated with anti-CD3 antibody in the presence of splenic antigen-presenting cells (APCs) isolated from NOD-scid γc$^{-/-}$ mice (Fig. 4

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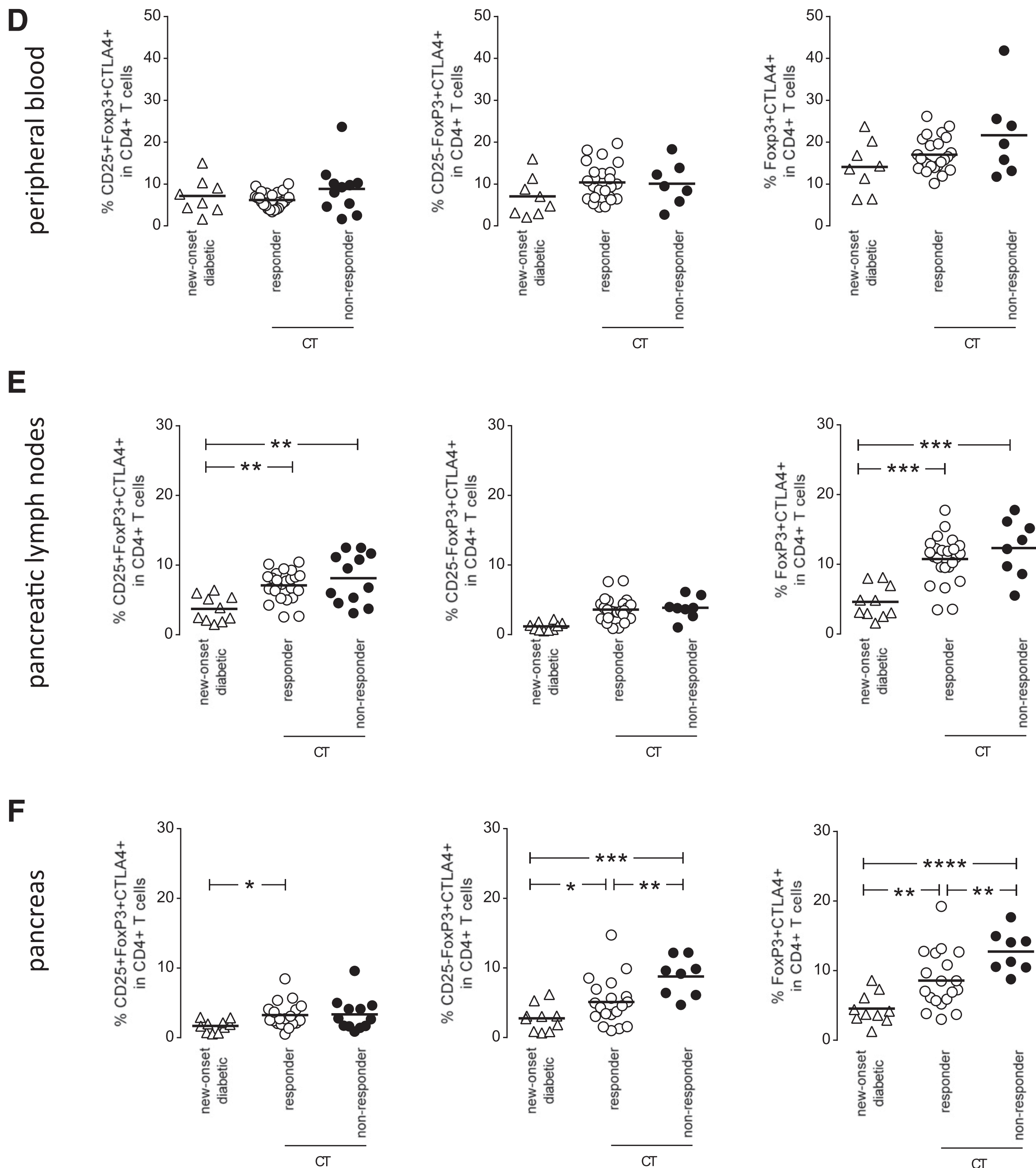

Figure 3—*Continued.*

and data not shown). No difference in the regulatory capacity of $CD4^+CD25^-Foxp3^+$ T cells was seen between therapy responders and nonresponders.

The addition of anti-CTLA4 Ig (clone UC10–4F10) or a TGF-β neutralizing antibody (clone 1D11.16.8) significantly reduced the suppression by the $CD4^+CD25^+Foxp3^+$ T cells (Fig. 5*A*), suggesting that $CD4^+CD25^+Foxp3^+$ Tregs of cured mice inhibit Teff proliferation via a CTLA4-dependent and TGF-β–dependent fashion in vitro. Adding anti–IL-10 (clone JES5–2A5) did not alter the direct suppressive effect of the Tregs. On the other hand, these regulatory mechanisms were not demonstrated with the $CD4^+CD25^-Foxp3^+$ T-cell fraction from therapy responders and nonresponders (Fig. 5*B*). Treating

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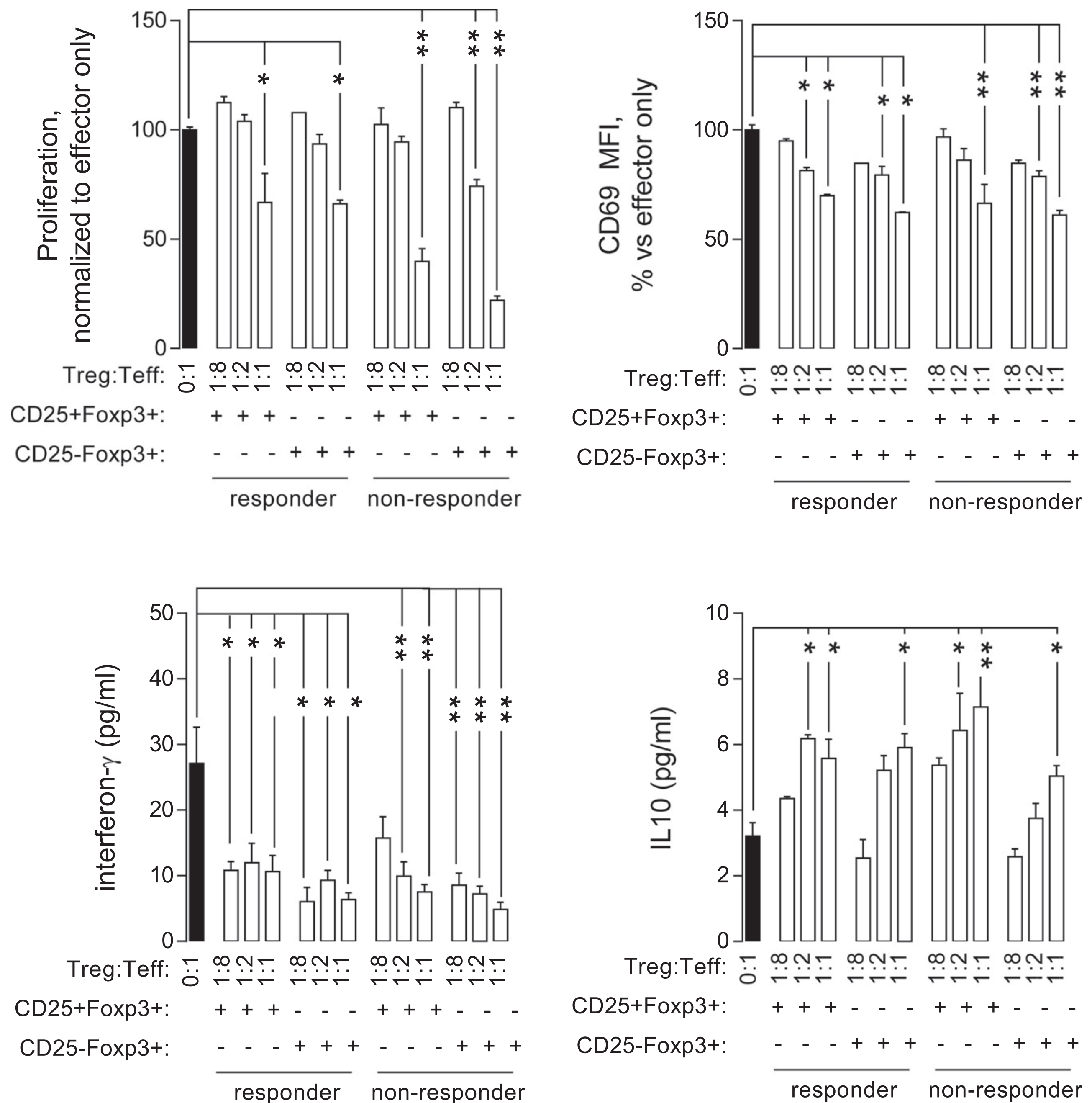

Figure 4—*L. lactis*–based combination therapy induces suppressive IL-10–secreting Foxp3$^+$ T cells in responders and nonresponders. In vitro polyclonal suppressor assay. CD4$^+$CD25$^-$ Teffs were isolated from normoglycemic NOD mice, dye-labeled, and stimulated for 72 h using soluble anti-CD3 in the presence of accessory cells and increasing ratios of CD4$^+$CD25$^+$Foxp3$^+$ or CD4$^+$CD25$^-$Foxp3$^+$ T cells (Tregs), isolated from *L. lactis*–based combination therapy (CT)–treated NOD.Foxp3.hCD2 mice (both responders and nonresponders) at the end of the indicated 6-week treatment. The proliferation of Teffs was measured by flow cytometric analysis of dye dilution and shown as the percentage of Teffs that had undergone two or more divisions, normalized to effector-only culture. The activation of Teffs was measured by flow cytometric analysis of CD69 and shown as the mean fluorescence intensity (MFI), normalized to effector-only culture. Meso Scale Diagnostics high-sensitivity multiplex assay of interferon-γ and IL-10 concentrations in the Treg/Teff cultures. Statistical significance between groups was calculated using the Kruskal-Wallis test followed by Dunnett multiple testing: *$P < 0.05$, **$P < 0.01$.

stably cured mice in vivo with a combination of anti-CTLA4 Ig (clone UC10–4F10) and anti–TGF-β (clone 1D11.16.8) led to diabetes recurrence in two of five mice (Fig. 5*C*).

Finally, we investigated whether the therapeutic success of *L. lactis*–based therapy was dependent on the presence and functionality of Foxp3$^+$ T cells. For this, NOD mice with new-onset diabetes were simultaneously treated with the *L. lactis*–based therapy and the FOXP3-inhibitory peptide P60 for a period of 14 days (Fig. 6*A*). Interestingly, none of the mice ($n = 6$) developed normoglycemia, while mice treated with the *L. lactis*–based therapy and vehicle ($n = 11$)

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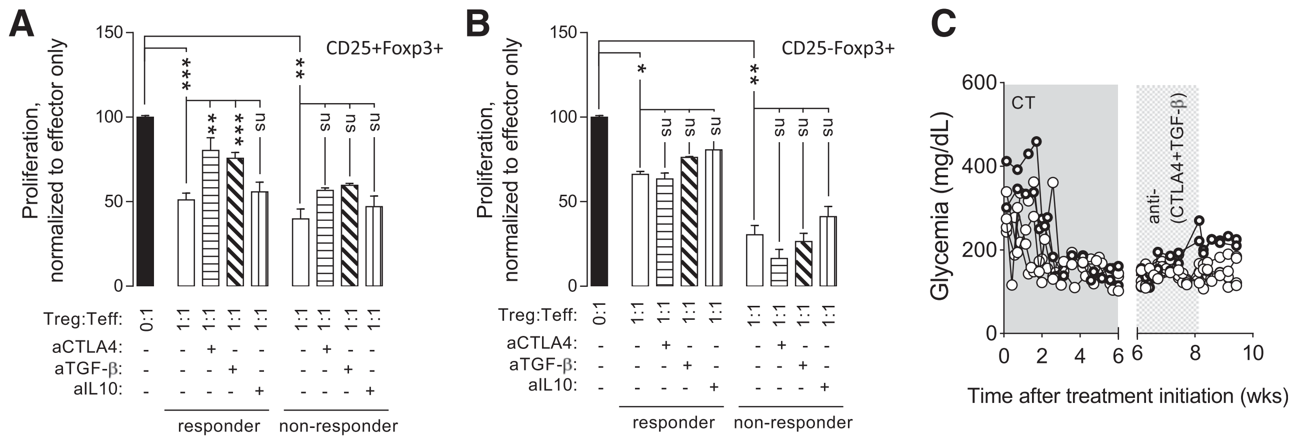

Figure 5—*L. lactis*–based combination therapy–induced Tregs depend on CTLA4 and TGF-β to control Teff responses. Teff proliferation is shown as the percentage of Teffs that had undergone two or more divisions, normalized to proliferation by effector-only culture. Dye-labeled $CD4^+CD25^-$ T cells (Teff) were stimulated with anti-CD3 (0.5 μg/mL) in the presence of accessory cells and $CD4^+CD25^+Foxp3^+$ (*A*) or $CD4^+CD25^-Foxp3^+$ (*B*) cells (Tregs), isolated from *L. lactis*–based combination therapy (CT)–treated NOD.Foxp3.hCD2 mice (both responders and nonresponders), and indicated neutralizing antibodies (10 μg/mL). Statistical significance between groups was calculated using the Kruskal-Wallis test followed by Dunnett multiple testing; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. *C*: *L. lactis*–based CT-cured mice were injected with anti-CTLA4 and anti–TGF-β antibodies (n = 5), and were followed up for diabetes recurrence (mice with glucosuria and blood glucose measurements >200 mg/dL are represented by the dark circles).

already had a 60% diabetes remission rate, indicating that Tregs are crucial for the induction of therapy-induced tolerance (Fig. 6*B*).

Next, NOD.Foxp3.DTR mice with new-onset (spontaneous) diabetes were treated with the *L. lactis*–based therapy, and, after stable diabetes reversal was observed, $Foxp3^+$ T cells were eliminated using DT as described in the scheme depicted in Fig. 7*A*. First, we established in unmanipulated NOD.Foxp3.DTR mice that the selected DT regimen eliminated >90% of $CD4^+Foxp3^+$ T cells, with the remaining Tregs expressing low or no CD25, in the peripheral blood within 3 days after the first DT injection (Supplementary Fig. 3*A*–*C*). A progressive repopulation of these cells started from day 5 after the first DT injection, as has been reported for several Foxp3.DTR strains (15–17). This DT regimen also dramatically decreased the number of $Foxp3^+$ T cells residing in the pancreas, consequently leading to the development of autoimmune diabetes (Supplementary Figs. 2*D* and 3*B*). Next, comparable to wild-type NOD mice, the *L. lactis*–based treatment induced autoimmune diabetes remission in 57% of NOD.Foxp3.DTR mice (four of seven mice) (Fig. 7*B*). Transient $Foxp3^+$ T-cell depletion resulted in a complete reversal to the diabetic state in all mice (n = 4) that were initially cured by the therapy, as evidenced by the reappearance of glucosuria along with severe hyperglycemia starting from day 2 after the first DT injection (Fig. 7*B*). This breach of immune tolerance to insulin-producing β-cells was also accompanied by the induction of severe insulitis (Fig. 7*C*) and the ablation of the islet-resident $Foxp3^+$ Treg pool (Fig. 7*D*). Collectively, these data demonstrated that the therapeutic effect from the *L. lactis*–based intervention depended on the presence and functionality of $Foxp3^+$ Tregs.

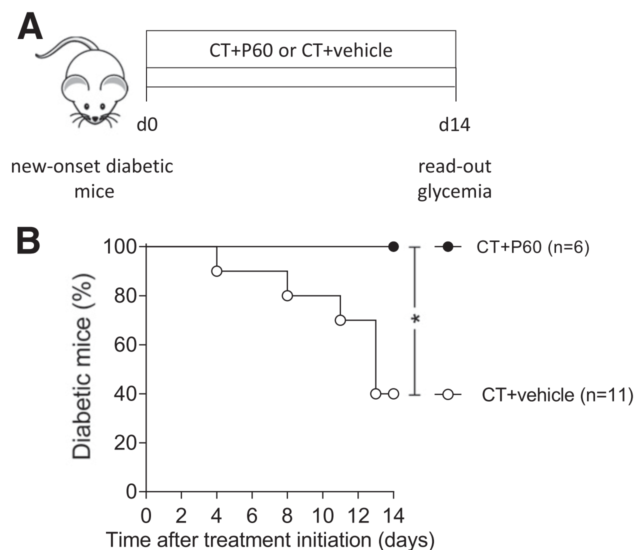

Figure 6—Specific inhibition of Treg function impairs therapy-induced tolerance. *A*: Treatment scheme for the simultaneous administration of *L. lactis*–based combination therapy (CT) and the specific FOXP3 inhibitor P60 (50 μg/daily i.p.) in NOD mice with new-onset diabetes. *B*: Shown is the percentage of mice that remained diabetic after treatment. In the Kaplan-Meier survival curve, statistical significance between groups was determined by Mantel-Cox log-rank test; *$P < 0.05$.

DISCUSSION

Oral tolerance as a means of intervention to arrest disease has been extensively explored in various animal models of autoimmune disease, including T1D (18). We previously

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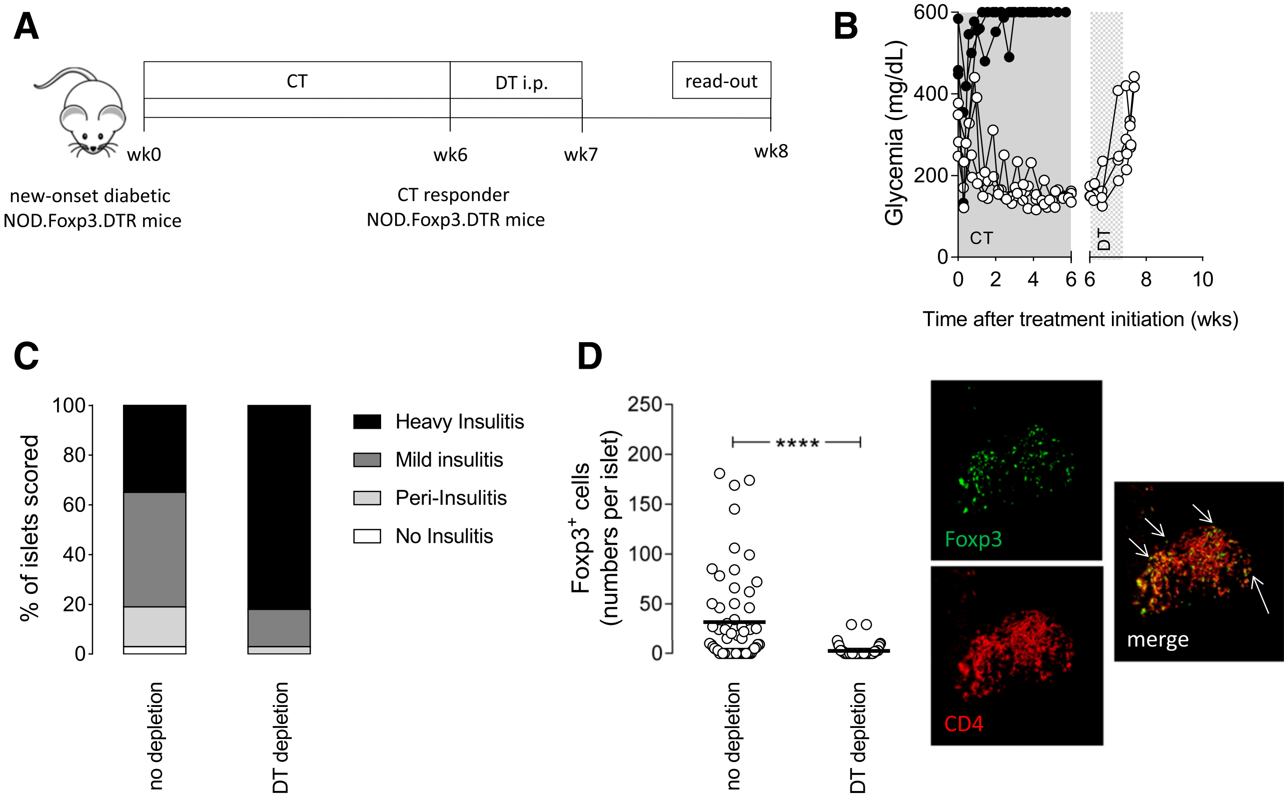

Figure 7—Foxp3$^+$ T-cell depletion breaches *L. lactis*–based combination therapy–induced tolerance in NOD.Foxp3.DTR mice. *A*: Treatment scheme for Foxp3$^+$ T-cell depletion by DT in *L. lactis*–based combination therapy (CT)–cured NOD.Foxp3.DTR mice. *B*: Blood glucose measurements in NOD.Foxp3.DTR mice with new-onset diabetes during *L. lactis*–based CT ($n = 7$) and after DT treatment ($n = 4$). Mice were considered cured (white symbols) when random blood glucose concentrations recovered to <200 mg/dL or were considered noncured (black symbols) when mice sustained blood glucose concentrations >200 mg/dL. Insulitis scoring (*C*) and quantification (*D*) of islet-resident Foxp3$^+$ T cells in the pancreata of therapy-cured NOD.Foxp3.DTR mice before and after DT treatement. Staining of a representative pancreas section from a CT-tolerized mice for CD4 (red) and Foxp3 (green) in which the white arrows indicate the presence of Foxp3$^+$ T cells within an islet of Langerhans. Statistical significance was calculated using the Mann-Whitney *t* test; ****$P < 0.0001$.

described the reversal of new-onset autoimmune diabetes in mice by the oral administration of plasmid-driven *L. lactis* strains secreting diabetes-relevant antigens (i.e., whole PINS or GAD65 peptide) and IL-10 in combination with systemic low-dose anti-CD3 (9,11). In both antigen-based therapies, induction of CD4$^+$CD25$^+$Foxp3$^+$ T cells accompanied the therapeutic success.

In the current study, we designed an oral clinical-grade self-containing *L. lactis* strain secreting chromosomal-integrated human PINS and IL-10. When combined with a short course of subtherapeutic doses of anti-CD3, the intervention was safe and highly effective in inducing long-term normoglycemia in mice with new-onset diabetes. Initial blood glucose concentrations (<350 mg/dL) in addition to IAA positivity at disease onset were predictors of therapeutic outcome, whereas the preservation of residual β-cell function and decline in IAA positivity were markers of therapeutic success. It is encouraging that studies with anti-CD3 monotherapy in patients with new-onset T1D already revealed that subjects who enrolled within 6 weeks of diagnosis and with higher levels of C-peptide at entry responded better to therapy (19–21). These observations suggest that some degree of residual β-cell mass will be necessary for therapeutic success when intervening at the moment of diabetes diagnosis, namely when dysglycemia is present. Likewise, a post hoc analysis of participants with new-onset T1D of the otelixizumab trial found a good correlation between pre-existing IAA levels and clinical outcome (22). IAA positivity at study entry was also found to distinguish responders from nonresponders among recipients of oral insulin (1).

Our previous studies suggested that the mechanism of *L. lactis*–based therapy involved the induction of CD4$^+$CD25$^+$Foxp3$^+$ T cells (9). By dissociating between the immune effects of the *L. lactis*–based intervention in mice responsive or not to the therapy, we were able to further characterize the nature and role of the immune processes accompanying the treatment. The *L. lactis*–based therapy induced suppressive IL-10–secreting CD4$^+$Foxp3$^+$ (both CD25$^+$ and CD25$^-$) T cells in the pancreas-draining lymph nodes and pancreata of responders, and even more so of nonresponders, suggesting enhanced recruitment of Tregs to the inflamed target tissues. In the periphery, the frequency of CD4$^+$Foxp3$^+$ T cells was also increased in treated

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mice compared with untreated controls, pointing toward a possible value for this cell population as an immune marker. Interestingly, the frequency of CTLA4$^+$ T cells among various Treg subsets was significantly higher in the pancreata of combination therapy-treated responder mice compared with mice with new-onset diabetes and in contrast to combination therapy–treated nonresponder mice. CTLA4 by Tregs has a nonredundant role to limit lymphopenia-induced uncontrolled proliferation of autoreactive Teffs in vivo (23). Alternatively, CTLA4 expression by Tregs may prolong the contact time between Tregs and dendritic cells via lymphocyte function–associated antigen 1 activation (24), increasing the efficiency of Treg suppression in a (transient) lymphopenic environment, similar to the one induced by low-dose anti-CD3. Because various immune- or tissue-specific mediators are important for Treg-suppressive functions at these inflammatory sites, more in-depth studies looking at the expression of chemokines, adhesion molecules, and extracellular matrix components of site-specific Tregs can provide more insights into follow-up studies. Of note, no difference was seen in the degree of insulitis between responder and nonresponder mice, suggesting also alterations in other lymphocyte subsets besides Tregs.

It is still not fully understood how Tregs control immune effector responses in autoimmune diseases and inflammation. Several studies (25) demonstrated that peripheral Tregs can use different regulatory mechanisms according to their environmental milieu and stimulatory conditions. In our case, CTLA4 and TGF-β were important for the regulatory activity of therapy-expanded CD4$^+$CD25$^+$Foxp3$^+$ T cells in vitro, and partially in vivo, whereas IL-10 was not. There is no consensus on the role of CTLA4 for Treg function, and several effects have been reported: the induction of cell-intrinsic negative signals to activated Teffs, the modulation of the development of APCs, and the stable function of Foxp3$^+$ Tregs (26,27). With regard to the involvement of TGF-β in therapy-mediated suppression, this cytokine can regulate several immunological processes, such as inflammation, lineage commitment, antibody generation, as well as tolerance induction (28). Moreover, it can preserve Foxp3 expression and support the differentiation of other T cells into Treg-like cells (29). In fact, TGF-β can promote the development of IL-10–secreting Tregs, because treatment of mice with anti–TGF-β prevented the conversion of CD4$^+$Foxp3$^-$ cells into CD4$^+$Foxp3$^+$IL-10$^+$ cells in intestine-associated lymphoid tissues (30). Although IL-10 seemed to be a good marker for the identification of our *L. lactis*–based therapy–induced Tregs, the role of IL-10 in their regulator function remains controversial. Others also demonstrated (31) that anti–IL-10 antibodies did not abrogate established tolerance in vivo. Here, it has been discussed whether IL-10 modulated the maturation phenotype of the APCs inducing anergy in both antigen-specific CD4$^+$ and CD8$^+$ T cells, and preferentially converting truly naive CD4$^+$ T cells into suppressor cells expressing Foxp3, rather than through direct activity on T cells (32). Based on our observations, it is intriguing to speculate that Treg production of IL-10 is a major mechanism by which Tregs regulate inflammation at environmental interfaces, whereas TGF-β- and CTLA4-dependent regulation of the function of APCs may be a regulatory mechanism that predominates in secondary lymphoid tissues, where it controls the initial activation and expansion of naive autoreactive T-cells.

As in mice, human Tregs are defined by having a suppressive phenotype endowed by high and sustained expression of the transcription factor Foxp3 (33), and the loss of function/mutation in the Foxp3 gene leads to severe fatal autoimmune disorders (15,34). In the current study, we discovered that the specific inhibition of Treg functionality by the P60 peptide (14) at the start of *L. lactis*–based therapy completely impaired the induction of therapy-induced tolerance. Moreover, transient depletion of Foxp3$^+$ Tregs from therapy-tolerized NOD.Foxp3. DTR mice was sufficient to induce complete disease relapse in all animals, demonstrating that the presence of Foxp3$^+$ T cells was crucial to maintain therapeutic tolerance and control pathogenic Teffs, which were still present in mice responsive to *L. lactis*–based therapy. A recent study (35) suggested that antigen-specific Foxp3$^+$ Tregs can also mediate tolerance both by diminishing recruitment of antigen-carrying inflammatory APCs to lymph nodes and by impairing their function.

In conclusion, our data demonstrated that combining a clinical-grade self-containing *L. lactis*–secreting human PINS and IL-10 with low-dose anti-CD3 increased the frequency of diabetes reversal compared with anti-CD3 monotherapy. Both therapy responders and nonresponders had increased frequencies of CD4$^+$Foxp3$^+$ T cells, suggesting that immune effects induced by the *L. lactis*–based therapy occurred in each individual recipient, but that therapeutic success (defined as a return to stable normoglycemia) depended on other parameters, such as functional β-cell mass still present at disease onset. This idea was further strengthened by the observation that therapeutic success was correlated with starting glycemia. Next to initial blood glucose concentrations at study entry, IAA levels also predicted the outcome of this *L. lactis*–based therapy using PINS as antigen. Finally, we showed that Foxp3$^+$ Tregs were essential to induce and maintain active tolerance and control diabetogenic immune responses in tolerized mice. These findings provide all the ingredients for testing this intervention in humans: a clinical-grade self-containing *L. lactis*–secreting islet antigens, biomarkers for predicting therapeutic success, and the demonstration that the induction of Foxp3$^+$ T cells is the basis of the *L. lactis*–based therapy–induced cure.

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