Title

Immune therapy and β-cell death in type 1 diabetes.

Journal

Diabetes, 62(5)

Authors

Lebastchi, Jasmin
Deng, Songyan
Lebastchi, Amir
et al.

Publication Date

2013-05-01

DOI

10.2337/db12-1207

Immune Therapy and β-Cell Death in Type 1 Diabetes

Jasmin Lebastchi,[1] Songyan Deng,[1] Amir H. Lebastchi,[1] Isabel Beshar,[1] Stephen Gitelman,[2] Steven Willi,[3] Peter Gottlieb,[4] Eitan M. Akirav,[5] Jeffrey A. Bluestone,[6] and Kevan C. Herold[1]

Type 1 diabetes (T1D) results from immune-mediated destruction of insulin-producing β-cells. The killing of β-cells is not currently measurable; β-cell functional studies routinely used are affected by environmental factors such as glucose and cannot distinguish death from dysfunction. Moreover, it is not known whether immune therapies affect killing. We developed an assay to identify β-cell death by measuring relative levels of unmethylated *INS* DNA in serum and used it to measure β-cell death in a clinical trial of teplizumab. We studied 43 patients with recent-onset T1D, 13 nondiabetic subjects, and 37 patients with T1D treated with FcR nonbinding anti-CD3 monoclonal antibody (teplizumab) or placebo. Patients with recent-onset T1D had higher rates of β-cell death versus nondiabetic control subjects, but patients with long-standing T1D had lower levels. When patients with recent-onset T1D were treated with teplizumab, β-cell function was preserved ($P < 0.05$) and the rates of β-cell were reduced significantly ($P < 0.05$). We conclude that there are higher rates of β-cell death in patients with recent-onset T1D compared with nondiabetic subjects. Improvement in C-peptide responses with immune intervention is associated with decreased β-cell death. ***Diabetes*** **62:1676–1680, 2013**

Type 1 diabetes (T1D) is initiated years before clinical onset and continues until nearly all insulin-producing cells have been destroyed by autoimmune processes (1). It is estimated that at presentation 10–30% of β-cells remain and that β-cell death continues over the next 3–5 years until the majority of patients lose clinically significant levels of insulin production (2).

It has heretofore not been possible to directly measure β-cell destruction in patients. The measurements that are used actually measure β-cell function: they do not gauge the pathologic process and can be affected by metabolic factors such as ambient glucose levels. Moreover, newer immune therapies, such as FcR nonbinding anti-CD3 (teplizumab) and anti-CD20 monoclonal antibodies (rituximab) or CTLA4Ig (abatacept), have been shown to decrease the rate of decline in β-cell function, but owing to the lack of a more direct measure of β-cell destruction, their effects on the primary cause of the disease have not been assessed (3–8). As suggested by our previous studies, immune modulatory agents may cause functional recovery of degranulated β-cells even without eliminating β-cell killing (9).

We recently developed a method for detecting β-cell death in vivo by measuring the relative amount of β-cell–derived *INS* DNA in serum (10). This approach is based on the understanding that insulin is primarily transcribed by β-cells, in which epigenetic modifications of the *INS* gene such as unmethylation of CpG sites occur in the *INS* gene to achieve this task (11,12). When β-cells die and release their DNA into the circulation, the released DNA exhibits these modifications. Therefore, measurement of the amount of unmethylated *INS* DNA in the peripheral circulation may reflect the amount of β-cell death. We designed and validated a two-step nested PCR reaction to measure the levels of unmethylated *Ins1* DNA in mice with diabetes induced by streptozotocin and during development of diabetes in nonobese diabetic mice. We presented preliminary data in humans with T1D (10).

In this study, we modified the assay and used it to evaluate β-cell destruction in nondiabetic subjects, patients with new-onset T1D, and those treated with an immune modulator (teplizumab) that is known to preserve β-cell function (4,13,14). We show that individuals with new-onset T1D have increased rates of β-cell death compared with nondiabetic control subjects but individuals with long-standing disease have lower levels than healthy control subjects. We found a decreased rate of β-cell death in patients who were treated with teplizumab, suggesting that the drug may work by decreasing β-cell death.

RESEARCH DESIGN AND METHODS

Sera were collected from nondiabetic control subjects, participants with T1D in a clinical trial of teplizumab (Delay), and patients with long-standing T1D. The Delay trial was a randomized placebo-controlled trial testing whether a course of treatment with teplizumab would reduce the decline in C-peptide after 12 months in patients with T1D for 4–12 months' duration (15). From the total of 58 subjects, baseline samples were available on 43 subjects and paired samples (at baseline and 1 year) on 37 subjects. Samples from the other subjects were either not available or did not meet quality-control standards. Serum was obtained from age-matched nondiabetic control subjects from the clinical laboratory at Yale New Haven Hospital. Institutional review board approval was obtained for the collection of serum for these studies.

DNA collection and bisulfite treatment. DNA was purified from 200 μL serum using QIAamp DNA Blood kits as suggested by the manufacturer (Qiagen, Valencia, CA), with a modified incubation period of 20 min at 45°C in the final step. DNA was bisulfite treated using an EZ DNA Methylation kit (Zymo Research, Irvine, CA).

PCR reactions. The assay design is shown in Fig. 1*A*. A methylation pattern–independent first-step reaction was performed with 8–20 ng bisulfite-treated DNA (10). The product of the first-step PCR reaction was used as template in a second reaction with methylation site-specific primers (Supplementary Fig. 1*A*). The threshold cycle (C_t) was determined for reactions with the unmethylated and methylated primer pairs. The relative abundance of

From the [1]Departments of Immunobiology and Internal Medicine and Surgery, Yale University School of Medicine, New Haven, Connecticut; the [2]Department of Pediatrics, University of California, San Francisco, San Francisco, California; the [3]Department of Endocrinology, Children's Hospital of Philadelphia, and Department of Pediatrics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania; the [4]Department of Internal Medicine, University of Colorado at Denver, Aurora, Colorado; [5]Diabetes and Obesity Center, Winthrop University Hospital, Mineola, New York; and the [6]Department of Internal Medicine, University of California, San Francisco, San Francisco, California.

Corresponding author: Kevan C. Herold, kevan.herold@yale.edu.

Received 2 September 2012 and accepted 23 November 2012.

DOI: 10.2337/db12-1207. Clinical trial reg. no. NCT00378508, clinicaltrials.gov.

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db12-1207/-/DC1.

The opinions expressed in this article are those of the authors and do not necessarily reflect the views of the Connecticut Department of Public Health or the State of Connecticut.

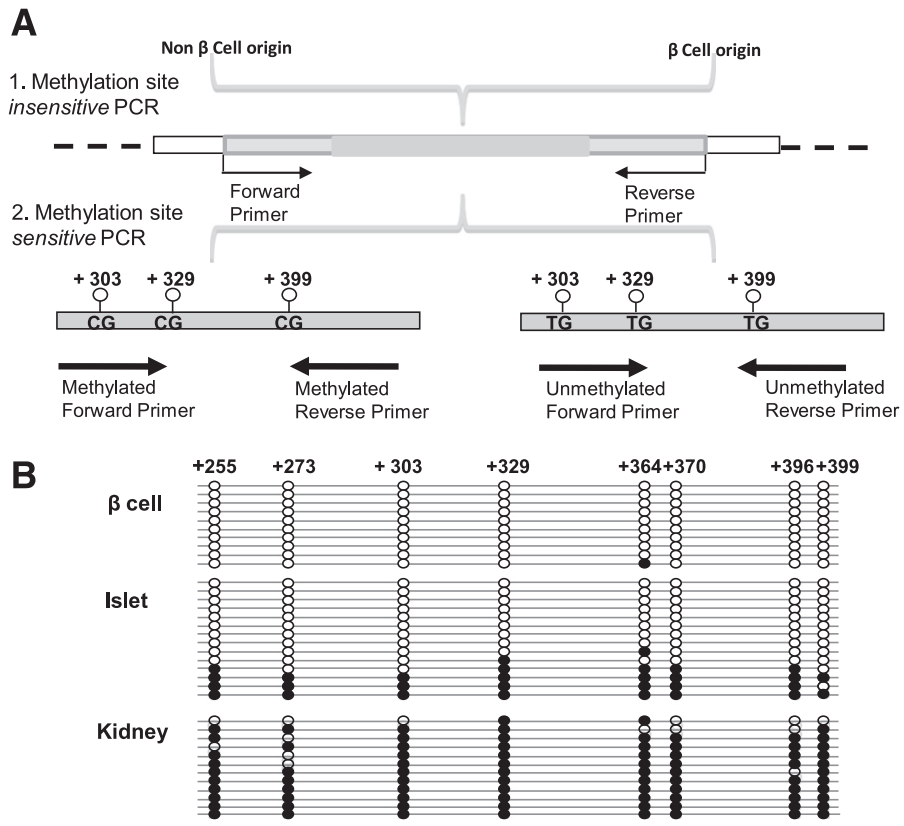

FIG. 1. Measurement of unmethylated *INS* DNA by real-time PCR. DNA extraction of sera, tissue, and cells and bisulfite treatment. *A*: DNA was isolated from serum and treated with bisulfite. The treated DNA was amplified using a first-step, methylation-insensitive reaction between bp 329 and 399 from the transcription start site. The products of this reaction were used in a second-step RT-PCR reaction with primers specific for methylated or unmethylated DNA sequences. The difference in the C_t values (methylated − unmethylated) was determined and is represented by the Δ. *B*: The methylated and unmethylated sequences from 10 clones from human β-cells, 14 clones from human islet cells, and 12 clones from human kidney cells are indicated at eight CpG sites (○, unmethylated cytosines; ●, methylated cytosines). The methylation pattern of the CpG sites in β-cells and kidney has previously been published (10). The bp are indicated downstream from the transcription start site. The majority of the CpG sites in purified β-cells are unmethylated, whereas there is a mixture of methylated and unmethylated sites in the products of the first-step PCR reaction from islet DNA reflecting the mixture of endocrine cells. The products of the PCR reaction with kidney DNA are predominantly methylated. C, cytosine; G, guanine; T, thymine. CG (cytosine-guanine) refers to methylated CpG sites, and TG (thymine-guanine) refers to unmethylated CpG sites.

unmethylated DNA was expressed as the difference (Δ) in the C_t value for methylated *INS* DNA − C_t value for unmethylated *INS* DNA. The assay performance was evaluated with templates of synthetic methylated and unmethylated DNA and with repeated draws of serum samples from healthy control subjects (Supplementary Fig. 1).

Statistical analyses. Data are expressed as means ± SEM. The differences between groups and time were compared by an unpaired and paired *t* test, respectively. Multiple groups were compared by one-way ANOVA with Tukey post hoc analysis, and linear regression was performed using Prism 5 (GraphPad, Carey, NC). A *P* value of <0.05 was considered statistically significant. Samples with undetectable levels of unmethylated *INS* DNA were assigned a Δ of −21.

RESULTS

Detection of unmethylated *INS* DNA: assay performance. We modified our previous reported assay to improve the specificity of detection, performed recovery studies to determine the limit of detection of islet derived DNA, and evaluated the interassay reproducibility (10) (Fig. 1 and Supplementary Fig. 1). Sequencing studies of fluorescence-activated cell sorter–sorted β-cells, islets, or kidney confirmed the identification of unmethylated *INS* DNA within mixtures of human cells. When islet-derived DNA was added at a 1:2 ratio to serum-derived DNA, the difference in the C_t values of the methylated and unmethylated products was 5.5 (or ~45-fold) compared with methylated DNA. However, below a concentration of islet DNA of 16 pg/μL, the Δ was indistinguishable from the sample without islet DNA added. We determined the reproducibility of the measurements with repeated blood draws in healthy control subjects (1.27%) and the interassay coefficient of variation (with samples from patients with new-onset T1D, 6.43%) (Supplementary Fig. 1*C*).

Circulating levels of unmethylated insulin DNA in patients with T1D. The Delay trial was a randomized placebo-controlled study of teplizumab in subjects with recent-onset (i.e., 4–12 months' duration) T1D, which tested whether a single course of the drug would attenuate the decline in C-peptide after the new-onset period. We first compared the levels of unmethylated *INS* DNA in serum from patients at study entry with age-matched nondiabetic individuals (Table 1). The levels of unmethylated *INS* DNA were significantly higher in the patients (Δ = −11.9 ± 0.63) compared with control subjects (−15.9 ± 0.54 [Fig. 2*A*]; $P = 0.001$). In addition, there was an inverse relationship between the relative level of unmethylated insulin and are under the curve (AUC) of the C-peptide responses to a mixed meal (Fig. 2*B*) ($r = -0.34$, $P = 0.03$).

To determine whether β-cell mass affected the level of *INS* DNA, we compared the levels of unmethylated *INS* DNA in patients with long-standing T1D, with and without residual C-peptide responses to a mixed meal (Table 1),

TABLE 1
Clinical characteristics of patients with recent-onset T1D and long-standing T1D

	Delay subjects (T1D)	Nondiabetic control subjects	Long-standing T1D		Nondiabetic control subjects
			C-peptide ≥0.2 pmol/mL	C-peptide <0.2 pmol/mL	
Sex (male/female)	24/19	7/6	2/3	5/1	3/3
Age (years)	12.51 ± 0.618	11.68 ± 0.538	21.6 ± 2.25	22.6 ± 1.49	20.8 ± 2.2
Duration (years)	0.61 ± 0.031		8.8 ± 2.31	7.2 ± 1.06	
HbA_{1c} (%)	6.64 ± 0.157		6.44 ± 0.23	6.95 ± 0.07*	
Average insulin use (units/kg/day)	0.379 ± 0.026		0.754 ± 0.252	0.897 ± 0.083	
C-peptide AUC (pmol/mL)	0.829 ± 0.069		0.824 ± 0.29	0.039 ± 0.023**	

Data are means ± SEM. AUC, area under the curve. *P = 0.04, **P = 0.015 vs. subjects with C-peptide ≥0.2 pmol/mL.

with levels in age-matched nondiabetic control subjects. Subjects with long-standing T1D with or without significant levels of insulin production (i.e., <0.2 pmol/mL) had undetectable levels of unmethylated *INS* DNA (not shown).

Effects of treatment with teplizumab on the levels of unmethylated insulin DNA. Paired samples for analysis of the level of unmethylated *INS* DNA were available from 39 subjects in the Delay trial. Drug-treated subjects showed a significantly reduced decline in C-peptide 1 year after treatment (17.7 ± 6.39 vs. 40.2 ± 7.69%, P = 0.03 [Fig. 3*A*]) and reduced change in insulin requirements (change in insulin dose from baseline of 0.06 ± 0.05 vs. 0.25 ± 0.04 units/kg/day, P = 0.009 [Fig. 3*B*]), but the HbA_{1c} levels were not significantly changed (not shown). Teplizumab- and placebo-treated subjects had a similar level of unmethylated *INS* DNA at baseline (Fig. 3*C*). In the teplizumab-treated subjects but not in the placebo-treated subjects, the relative level of unmethylated *INS* DNA declined significantly at the 1-year end point (P = 0.005 and P = ns, respectively), suggesting a reduced level of β-cell death. The decline in the level of unmethylated *INS* DNA was significantly greater in the teplizumab-treated subjects (P = 0.04) (Fig. 3*D*).

DISCUSSION

These studies show that the levels of unmethylated *INS* DNA are elevated in subjects with new-onset T1D compared with nondiabetic control subjects and suggest active β-cell destruction at the time of onset of disease. The levels of *INS* DNA do not simply reflect β-cell mass because they were elevated in the subjects with new-onset disease versus age-matched nondiabetic control subjects even though their β-cell mass is clearly reduced. Nonetheless, the quantitative measure of β-cell death is affected by the total β-cell mass because in long-standing patients, with reduced mass, the levels were lower than those even in nondiabetic control subjects.

The mechanism of improvement in β-cell function after immune therapy is not known: our preclinical studies in NOD mice showed that there was functional recovery of degranulated β-cells (9). This is the first evidence indicating that immune therapy that reduces the decline in β-cell functional responses in T1D does so by decreasing β-cell death. We cannot, however, be certain that teplizumab treatment alone accounts for all of the improvement in C-peptide responses in the drug-treated group. The average HbA_{1c} in the drug-treated group was lower than in the placebo group at baseline (6.28 ± 0.15 vs. 7.02 ± 0.42%, P = 0.04), which may have affected the C-peptide responses. However, the HbA_{1c} levels were not affected by drug treatment in either group, and the levels of unmethylated *INS* DNA in the two groups were similar at baseline. Therefore, the effects of the imbalance of the HbA_{1c} levels did not account for differences in the rates of β-cell death.

While the levels of unmethylated *INS* DNA decreased in teplizumab-treated subjects together with reduced decline in C-peptide AUC and reduced need for exogenous insulin, we did not find a direct relationship between the changes in *INS* DNA and these clinical parameters. Several factors may affect this relationship. At diagnosis, there may be

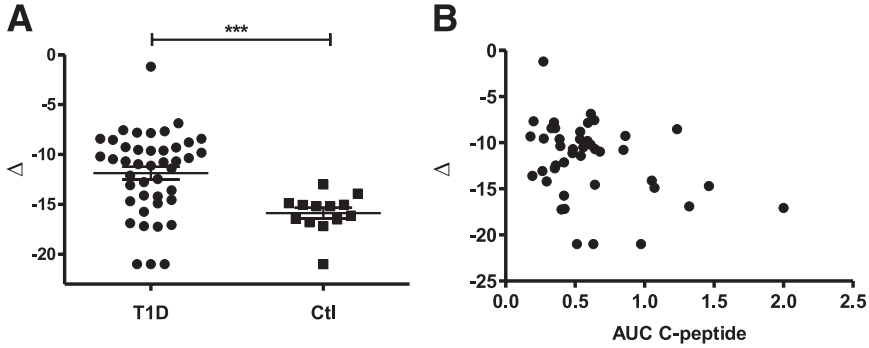

FIG. 2. Unmethylated *INS* DNA in patients with T1D. *A*: The levels of unmethylated *INS* DNA were measured in 43 subjects with recent-onset T1D and 13 nondiabetic control (Ctl) subjects of similar age (unpaired t test, ***P = 0.001). In the patients and control subjects, unmethylated C_t values ranged from 29.8 to 37.2 and from 22.3 to 37.3, respectively, and the methylated C_t values ranged from 15.4 to 30.7 and from 15.1 to 24.2. *B*: The C-peptide responses to a mixed meal (area under the curve [AUC]) and the corresponding Δ are shown for the subjects with recent-onset T1D (r = 0.34, P = 0.03).

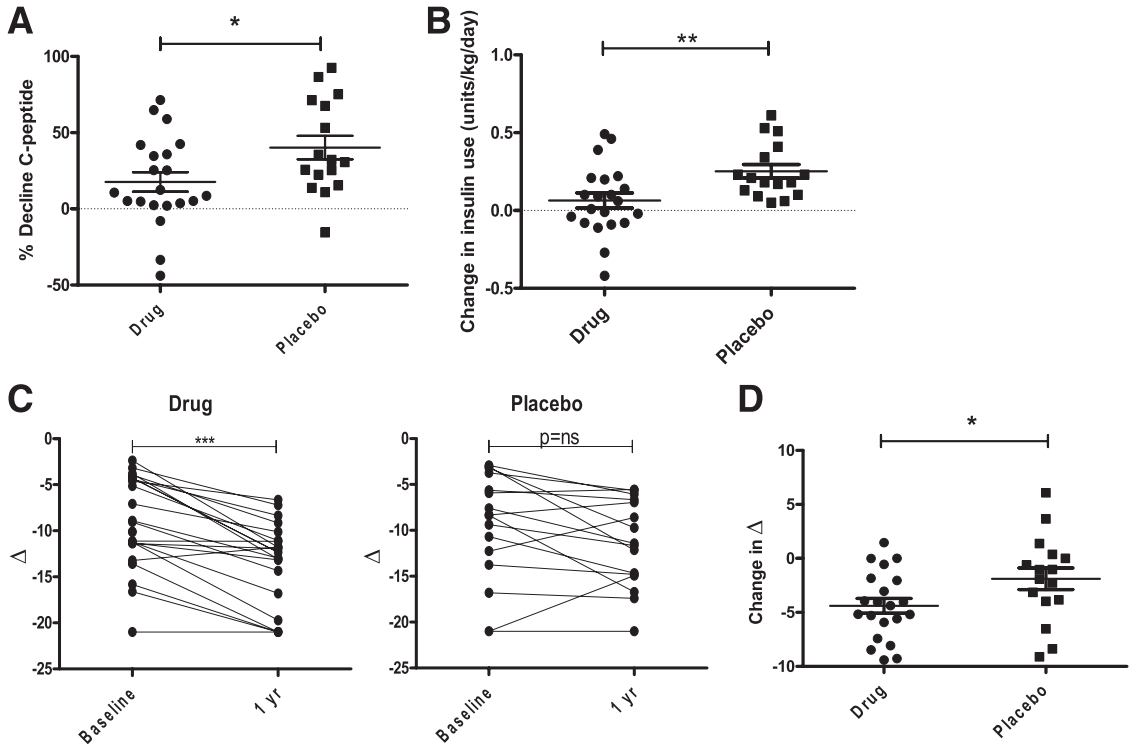

FIG. 3. Effects of teplizumab therapy on clinical responses and levels of unmethylated *INS* DNA. *A* and *B*: Percent decline in C-peptide (P* = 0.03) and increase in insulin use (***P* < 0.009) from baseline are shown for drug- and placebo-treated subjects. The baseline C-peptide and insulin in the drug group was 0.53 ± 0.07 pmol/mL/min and 0.38 ± 0.03 units/kg/day, respectively, and in the placebo group 0.53 ± 0.08 pmol/mL/min and 0.38 ± 0.04 units/kg/day (*P* = 0.18 and 0.94). *C*: Relative β-cell–derived DNA levels before and after treatment with teplizumab or placebo. There was a significant reduction in the Δ in subjects treated with teplizumab (****P* < 0.0001) but not placebo (*P* = 0.08). At baseline, the Δs in the drug- and placebo-treated groups were similar (*P* = 0.8). *D*: Subjects treated with teplizumab had a greater decline in the Δ (**P* = 0.04).**

a functional component to the impaired metabolic response that may or may not have reversed after study entry. Moreover, the kinetics of β-cell death is not known. Cell death may be greater before diagnosis, and the levels that we found may represent a decline from those peak levels (10). Our findings also imply that β-cell death may continue for an extended period of time after the diagnosis of T1D. The study subjects had been diagnosed with diabetes for an average of 7 months prior to entry, and the placebo-treated subjects still had an increased level of unmethylated *INS* DNA 1 year later compared with the nondiabetic control subjects (*P* = 0.013) (Fig. 3*C*). We showed that reduced β-cell mass may affect the level of β-cell INS DNA, suggested by our analysis of subjects with long-standing T1D. The levels we measured may also vary considerably between individuals based on age as well as the absolute β-cell mass as suggested by our studies in patients with long-standing disease.

One limitation of this approach is that β-cell death can only be detected if β-cells release their DNA into the serum. This is likely to occur during necrotic cell death associated with immune destruction by cytolytic T cells or by cytokines, but we do not know how β-cells die in human T1D (16–18). Other mechanisms of cell death, such as autophagy, or clearing of dying β-cells by phagocytic cells may not be identified with this approach (19). Therefore, it will be important to confirm our finding in other clinical studies that may affect β-cell destruction.

In summary, we identified higher rates of β-cell death in patients with recent-onset T1D versus nondiabetic control subjects and have shown that teplizumab treatment is associated with reduced level of β-cell death. The method that we have developed may help investigators understand the pathogenesis and treatment of human T1D including the relationships between functional and pathologic changes in β-cells. Moreover, this tool may be useful for decisions regarding for whom and when immune intervention would be most appropriate.

ACKNOWLEDGMENTS

This study was supported by grants 2008-1012, 2007-502, 2007-1059, and 2006-351 from the JDRF and R01 DK057846, P30 DK20495, UL1 RR024139, UL1 RR025780, UL1 RR024131, and UL1 RR024134 from the National Institutes of Health and was supported by the Howalt family, the Tobacco Trust Fund, and the Department of Public Health, State of Connecticut contract number 2012-0222.

J.A.B. has a patent application for teplizumab. E.M.A. and K.C.H. have a patent application for the assay of unmethylated insulin DNA and are members of the scientific advisory board of Islet Sciences, Inc. No other potential conflicts of interest relevant to this article were reported.

J.L. designed, modified, and performed the assay; analyzed data; and wrote the manuscript. S.D., A.H.L., and I.B. performed studies and analyzed data. S.G. designed the clinical trial, carried out the clinical trial, collected samples, and wrote and reviewed the manuscript. S.W. and P.G. carried out the clinical trial, collected samples, and wrote and reviewed the manuscript. E.M.A. wrote and reviewed the manuscript. J.A.B. wrote and reviewed the manuscript and designed the clinical trial. K.C.H. analyzed data, wrote the manuscript, and designed the clinical trial. K.C.H. is the guarantor of this work and, as

such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

REFERENCES

1. Bluestone JA, Herold K, Eisenbarth G. Genetics, pathogenesis and clinical interventions in type 1 diabetes. Nature 2010;464:1293–1300
2. Schölin A, Nyström L, Arnqvist H, et al.; Diabetes Incidence Study Group in Sweden (DISS). Proinsulin/C-peptide ratio, glucagon and remission in new-onset Type 1 diabetes mellitus in young adults. Diabet Med 2011;28:156–161
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. Herold KC, Gitelman SE, Masharani U, et al. A single course of anti-CD3 monoclonal antibody hOKT3gamma1(Ala-Ala) results in improvement in C-peptide responses and clinical parameters for at least 2 years after onset of type 1 diabetes. Diabetes 2005;54:1763–1769
5. 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
6. Orban T, Bundy B, Becker DJ, et al.; Type 1 Diabetes TrialNet Abatacept Study Group. Co-stimulation modulation with abatacept in patients with recent-onset type 1 diabetes: a randomised, double-blind, placebo-controlled trial. Lancet 2011;378:412–419
7. Pescovitz MD, Greenbaum CJ, Krause-Steinrauf H, et al.; Type 1 Diabetes TrialNet Anti-CD20 Study Group. Rituximab, B-lymphocyte depletion, and preservation of beta-cell function. N Engl J Med 2009;361:2143–2152
8. Waldron-Lynch F, Herold KC. Immunomodulatory therapy to preserve pancreatic β-cell function in type 1 diabetes. Nat Rev Drug Discov 2011;10: 439–452
9. Sherry NA, Kushner JA, Glandt M, Kitamura T, Brillantes AM, Herold KC. Effects of autoimmunity and immune therapy on beta-cell turnover in type 1 diabetes. Diabetes 2006;55:3238–3245
10. Akirav EM, Lebastchi J, Galvan EM, et al. Detection of β cell death in diabetes using differentially methylated circulating DNA. Proc Natl Acad Sci USA 2011;108:19018–19023
11. Kim MS, Kondo T, Takada I, et al. DNA demethylation in hormone-induced transcriptional derepression. Nature 2009;461:1007–1012
12. Kuroda A, Rauch TA, Todorov I, et al. Insulin gene expression is regulated by DNA methylation. PLoS ONE 2009;4:e6953
13. Herold KC, Hagopian W, Auger JA, et al. Anti-CD3 monoclonal antibody in new-onset type 1 diabetes mellitus. N Engl J Med 2002;346:1692–1698
14. Sherry N, Hagopian W, Ludvigsson J, et al.; Protégé Trial Investigators. Teplizumab for treatment of type 1 diabetes (Protégé study): 1-year results from a randomised, placebo-controlled trial. Lancet 2011;378:487–497
15. Herold KC, Gitelman SE, Willi SM, et al. Teplizumab treatment may improve C-peptide responses in participants with type 1 diabetes after the new-onset period: a randomised controlled trial. Diabetologia 2013;56:391–400
16. Campbell IL, Iscaro A, Harrison LC. IFN-gamma and tumor necrosis factor-alpha. Cytotoxicity to murine islets of Langerhans. J Immunol 1988;141: 2325–2329
17. Rabinovitch A, Sumoski W, Rajotte RV, Warnock GL. Cytotoxic effects of cytokines on human pancreatic islet cells in monolayer culture. J Clin Endocrinol Metab 1990;71:152–156
18. Skowera A, Ellis RJ, Varela-Calviño R, et al. CTLs are targeted to kill beta cells in patients with type 1 diabetes through recognition of a glucose-regulated preproinsulin epitope. J Clin Invest 2008;118:3390–3402
19. Fujimoto K, Hanson PT, Tran H, et al. Autophagy regulates pancreatic beta cell death in response to Pdx1 deficiency and nutrient deprivation. J Biol Chem 2009;284:27664–27673