METABOLISM

Teplizumab improves and stabilizes beta cell function in antibody-positive high-risk individuals

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We analyzed the effects of a single 14-day course of teplizumab treatment on metabolic function and immune cells among participants in a previously reported randomized controlled trial of nondiabetic relatives at high risk for type 1 diabetes (T1D). In an extended follow-up (923-day median) of a previous report of teplizumab treatment, we found that the median times to diagnosis were 59.6 and 27.1 months for teplizumab- and placebo-treated participants, respectively (HR = 0.457, $P = 0.01$). Fifty percent of teplizumab-treated but only 22% of the placebo-treated remained diabetes-free. Glucose tolerance, C-peptide area under the curve (AUC), and insulin secretory rates were calculated, and relationships to T cell subsets and function were analyzed. Teplizumab treatment improved beta cell function, reflected by average on-study C-peptide AUC (1.94 versus 1.72 pmol/ml; $P = 0.006$). Drug treatment reversed a decline in insulin secretion before enrollment, followed by stabilization of the declining C-peptide AUC seen with placebo treatment. Proinsulin:C-peptide ratios after drug treatment were similar between the treatment groups. The changes in C-peptide with teplizumab treatment were associated with increases in partially exhausted memory KLRG1$^+$TIGIT$^+$CD8$^+$ T cells ($r = 0.44$, $P = 0.014$) that showed reduced secretion of IFNγ and TNFα. A single course of teplizumab had lasting effects on delay of T1D diagnosis and improved beta cell function in high-risk individuals. Changes in CD8$^+$ T cell subsets indicated that partially exhausted effector cells were associated with clinical response. Thus, this trial showed improvement in metabolic responses and delay of diabetes with immune therapy.

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease characterized by T cell–mediated destruction of insulin-producing beta cells within the pancreatic islets of Langerhans. Longitudinal observational studies over more than 30 years have described the progression of this autoimmune disease from the first appearance of autoantibodies until critical impairment of beta cell function and the clinical diagnosis, often with ketoacidosis, occurs (*1*–*5*). T1D is associated with a need for lifelong exogenous insulin administration for survival; increased morbidity and mortality due to immediate hypoglycemia; and long-term complications such as vascular, renal, and ocular disease, along with reduced life span, life impairments, and considerable health care–related costs (*6*–*9*). Thus, approaches to prevent progression to clinical T1D before irremediable beta cell destruction and insulin deficiency are of paramount importance.

Changes in beta cell function precede the clinical diagnosis of T1D and have been studied in natural history cohorts of individuals who are identified as at risk for the disease on the basis of the presence of islet autoantibodies (*10*–*12*). Some studies suggest an ongoing and intermittently progressive decline in beta cell function that begins years before clinical diagnosis at a time when glucose tolerance is normal. During this period, there are signs of ongoing autoimmunity; on the basis of the findings of natural history studies, individuals with two or more islet autoantibodies have been classified using stages of T1D, with further specification according to the level of metabolic dysfunction: stage 1 before glucose abnormalities, stage 2 with dysglycemia during an oral glucose tolerance test (OGTT), and stage 3 at clinical presentation with hyperglycemia (*2*, *13*, *14*). However, the relationships between changes in beta cell function and clinical disease remain poorly defined. It is known, for example, that glucose tolerance, defined through responses to an OGTT, may fluctuate between abnormal and normal values within an individual who is at risk (*15*, *16*). In addition, OGTT glucose tolerance classifications used to designate a clinical diagnosis and beta cell function as measured by C-peptide responses to a metabolic challenge may not be closely related. Specifically, at the time of diagnosis based on an OGTT or mixed-meal tolerance test, many individuals have clinically meaningful C-peptide responses (*15*–*18*).

On the basis of successes from previous studies in patients with postdiagnosis stage 3 T1D taking teplizumab, an Fc receptor–nonbinding anti-CD3ε monoclonal antibody that showed reduced decline in stimulated C-peptide responses compared to placebo or control participants (*19*–*25*) in the TrialNet TN10 anti-CD3 prevention trial, we conducted a randomized phase 2 trial of teplizumab in individuals with stage 2 disease to test whether treatment would prevent or delay the clinical diagnosis of T1D (*26*). In this time-to-event study, we found a delay in the median time to diagnosis of 24 months with teplizumab versus placebo and a reduction in the rate of diabetes diagnoses from 35.9 to 14.9% per year (*26*). This study was the first trial to successfully show that immune therapy could delay or possibly prevent the diagnosis of T1D (*27*–*31*). Because the participants in the TrialNet TN10 anti-CD3 prevention trial were not diagnosed with clinical T1D at the time of study enrollment,

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the successful outcome of the trial has enabled us to evaluate the effects of the therapy on beta cell function and its relationship to immune modifications when the disease process was clinically silent.

To test the hypothesis that the immune therapy would improve beta cell function in the at-risk individuals from the TrialNet TN10 anti-CD3 prevention trial, we analyzed the results of metabolic studies performed before, during, and after conclusion of the original trial, as well as participant immune responses. Specifically, we collected data from OGTTs before randomization and follow-up OGTT data from nondiabetic participants available through monitoring in the TrialNet TN01 Pathway to Prevention Study. Our data indicate that even before clinical diagnosis, treatment with teplizumab can improve metabolic function associated with modulation of pathologic T cell signatures.

RESULTS

Teplizumab treatment results in a sustained delay in the diagnosis of T1D during extended follow-up studies

A total of 76 relatives at high risk but without a clinical diagnosis of T1D were enrolled into the TrialNet TN10 anti-CD3 prevention trial (*26*). The median age was 13 (range, 8 to 49), and all participants had two or more autoantibody tests within 6 months before enrollment. We previously reported that 42 of these individuals were diagnosed with T1D after a median follow-up of 742 days (range, 74 to 2683). We have since continued to follow the study participants for a median time of 923 days (range, 74 to 3119) (fig. S1). Over this extended period of follow-up, 25 of 32 (78%) of the placebo-treated and 22 of 44 (50%) of the teplizumab-treated participants were diagnosed with T1D (Cox model adjusting for stratification and age: hazard ratio = 0.457, $P = 0.01$) (Fig. 1). The median times to diagnosis of T1D were 59.6 and 27.1 months in the teplizumab and placebo treatment groups, respectively. Ten of 13 subjects followed beyond 60 months were not diagnosed with T1D. Of these individuals, eight were in the teplizumab group and two were in the placebo group.

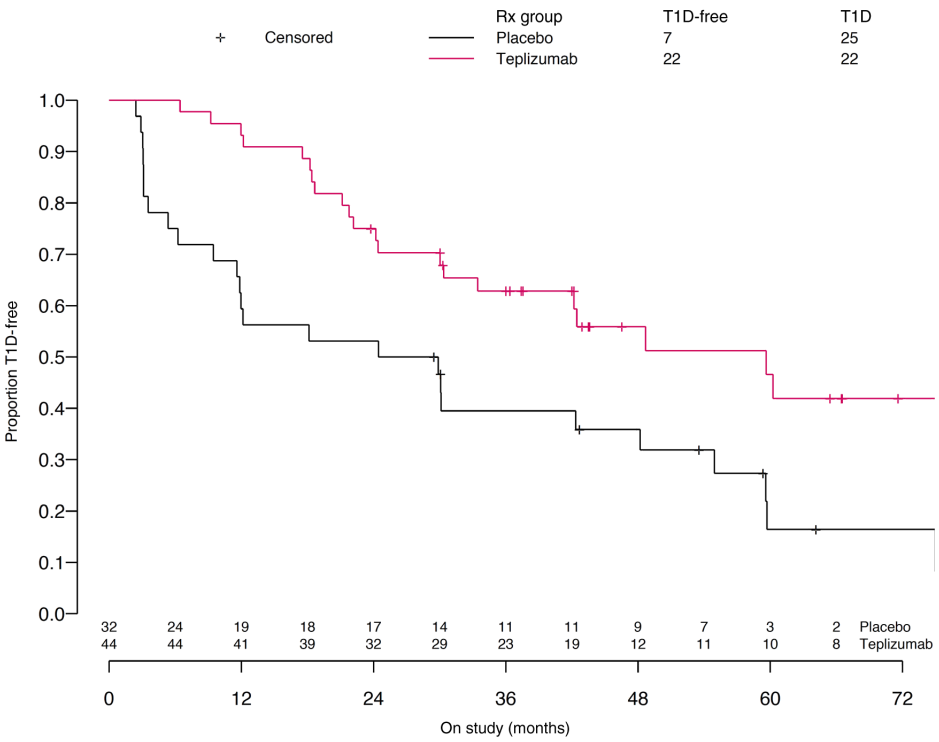

Fig. 1. Teplizumab treatment is associated with a sustained effect on T1D progression over 923 days of follow-up. Updated Kaplan-Meier curve based on 923 days of follow-up (range, 74 to 3119 days). The hazard ratio for development of T1D in teplizumab-treated participants versus placebo was 0.457; $P = 0.01$. The median time to diabetes was 27.1 and 59.6 months in the placebo and teplizumab treatment groups, respectively. At the conclusion of this period, 7 (22%) and 22 (50%) individuals, respectively, were not diagnosed with T1D.

Teplizumab treatment improves quantitative OGTT glucose area under the curve values

To determine how teplizumab treatment affected glucose tolerance, we classified the outcomes of the OGTTs as normal, dysglycemic, or diabetic at study entry and tallied the frequency of these outcomes at each study visit over the first 36 months of study and afterward (Fig. 2A and fig. S2). Study participants had been recruited on the basis of a dysglycemic OGTT test result. At randomization and consistent with the known variability of OGTT results, a small number of subjects had normal ($n = 3$) or diabetic ($n = 6$) glucose tolerance at that visit. The clinical diagnosis of T1D, the primary end point of the study, required two consecutive diabetic OGTTs; hence, participants could continue in the study with a single diabetic OGTT. At the 3-month visit after teplizumab or placebo administration, the frequency of dysglycemic OGTTs declined and the frequency of normal OGTTs increased in both groups (teplizumab-treated from 6.8 to 30.2%, McNemar test, $P = 0.009$; placebo from 15.6 to 36.7%, McNemar test, $P = 0.02$). Diabetic OGTTs also increased in both groups at this time point, particularly in the placebo group. Afterward, the frequency of normal and dysglycemic OGTTs remained relatively constant in the teplizumab group: The frequency of diabetic OGTTs increased in both groups but at a slower rate in the teplizumab-treated participants, who mostly maintained dysglycemic OGTT status.

Changes in OGTT classifications could overlook more subtle effects of treatment on the OGTT glucose responses. We therefore calculated and compared an average on-study glucose area under the curve (AUC) for each individual, which was corrected for the time in study. The average on-study glucose AUC was higher in those treated with placebo versus teplizumab {unadjusted mean [interquartile range (IQR)]: 175 (159, 195) mg/dl versus 165 (154, 180) mg/dl; when adjusted for baseline glucose and age using analysis of covariance (ANCOVA), $P = 0.02$} (Fig. 2B and table S1). The individual glucose AUC at the time of study entry was a predictor of the average on-study glucose AUC ($P = 0.0008$), but values at entry were similar between groups (unadjusted group geometric means: 155.5 mg/dl for placebo and 162.2 mg/dl for teplizumab, $P = 0.25$).

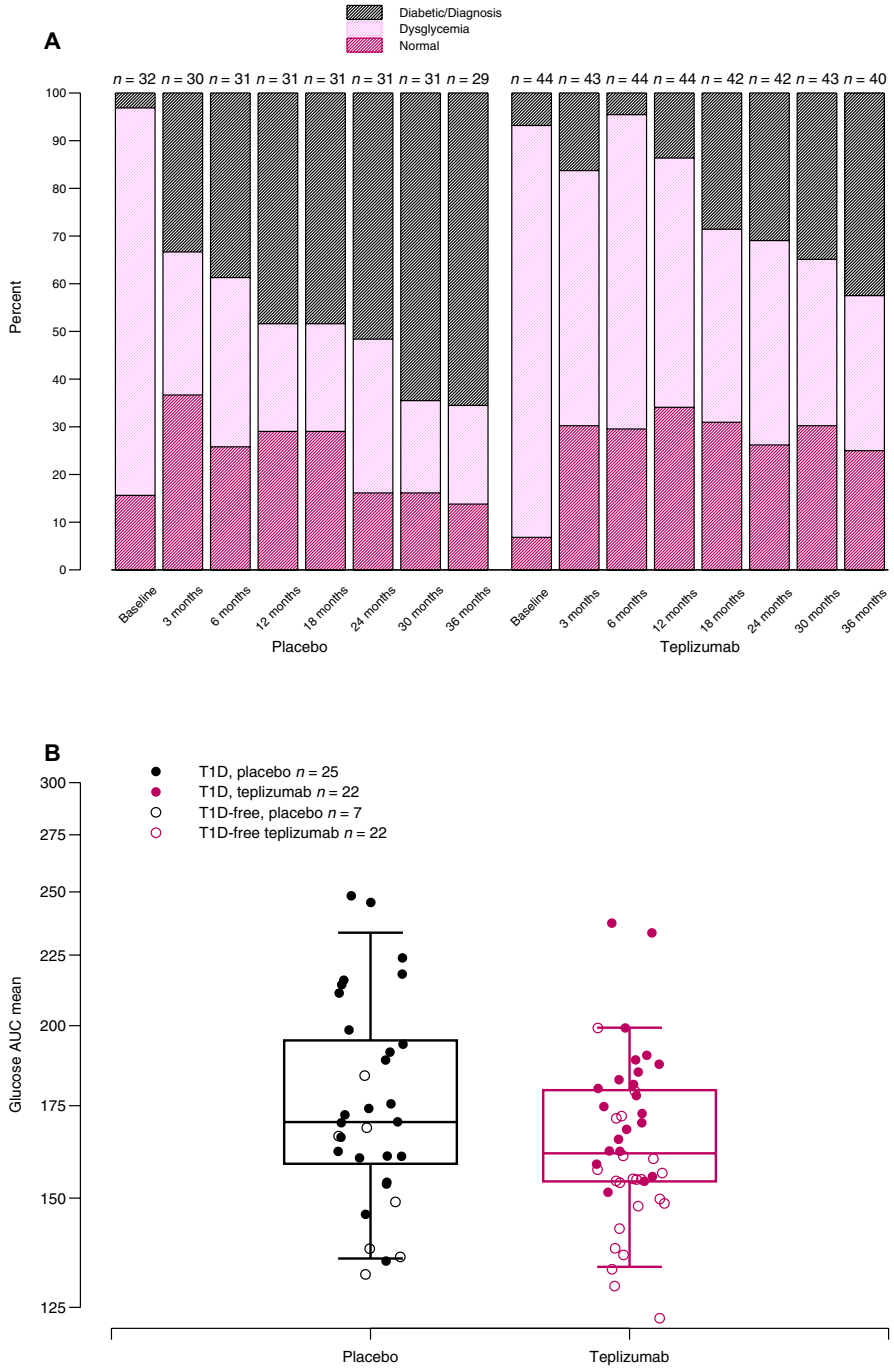

Fig. 2. Improved glycemia in teplizumab-treated participants is associated with maintenance of dysglycemic status. (**A**) OGTT classifications for participants in each group over 36 months of follow-up. The data are shown to 36 months because of loss of placebo-treated participants due to a clinical diagnosis of T1D (for individual participants see fig. S2). (**B**) Box plot displaying median and interquartile ranges for on-study OGTT glucose AUC mean for participants from placebo- and teplizumab-treated groups. An ANCOVA model incorporating baseline value, age, and treatment group showed that treatment significantly decreased average on-study glucose AUC (ANCOVA teplizumab effect: p = 0.02).

Average on-study hemoglobin A1c (HbA1c) AUC was also calculated and analyzed. In contrast to glucose, the average on-study HbA1c AUC was not statistically different in those treated with placebo versus teplizumab [mean (IQR): 5.42% (5.29, 5.57) versus 5.27% (4.99, 5.55); when adjusted for age and baseline HbA1c concentrations using ANCOVA treatment, $P = 0.13$] (fig. S3 and table S2). Because the frequency of diabetes was higher in the placebo group, given the similarity in the HbA1c, a measure of chronic glucose exposure, the higher average on-study glucose AUC in the placebo group was most likely due to acute rather than chronic changes in glucose concentrations.

Teplizumab treatment increases C-peptide responses

The average on-study C-peptide AUC was greater in the teplizumab treatment group versus placebo [unadjusted mean (IQR): 1.96 (1.48, 2.61) pmol/ml versus 1.68 (1.32, 2.11) pmol/ml; when adjusted for the age and baseline C-peptide AUC, the differences were highly significant ($P = 0.006$)] (Fig. 3 and table S3). To assess the relationship of this end point with diabetes development, we compared average on-study C-peptide AUC values between participants who did or did not develop T1D during the period of observation. For the entire study population, average on-study C-peptide AUC was greater in individuals who remained diabetes-free compared to those who progressed to T1D [unadjusted mean (IQR): 2.07 (1.55, 2.47) pmol/ml ($n = 29$) versus 1.71 (1.33, 2.13) pmol/ml ($n = 47$) ($P = 0.03$)]. However, there was no clear drug effect when the average C-peptide concentrations were compared with those who were diagnosed and remained diabetes-free {T1D-free [unadjusted mean (IQR)]: placebo, 1.95 (1.6, 2.33) pmol/ml ($n = 7$); teplizumab, 2.1 (1.55, 2.78) pmol/ml ($n = 22$); T1D: placebo, 1.61 (1.28, 2.04) pmol/ml ($n = 25$); teplizumab, 1.83 (1.41, 2.49) pmol/ml ($n = 22$); t test, $P = 0.26$} (Figure 3).

Baseline C-peptide AUC ($P < 0.0001$) was a significant determinant of the average on-study C-peptide AUC, but baseline values were similar between treatment groups [unadjusted group means (IQR) for placebo and teplizumab of 1.91 (1.56, 2.36) pmol/ml ($n = 32$) and 1.99 (1.47, 2.18) pmol/ml ($n = 44$) ($P = 0.661$)]. Compared to a control group of age- and sex-matched autoantibody-negative relatives, mean C-peptide AUC values in the teplizumab and placebo groups were reduced at baseline (Wilcoxon test, $P = 0.001$; table S4).

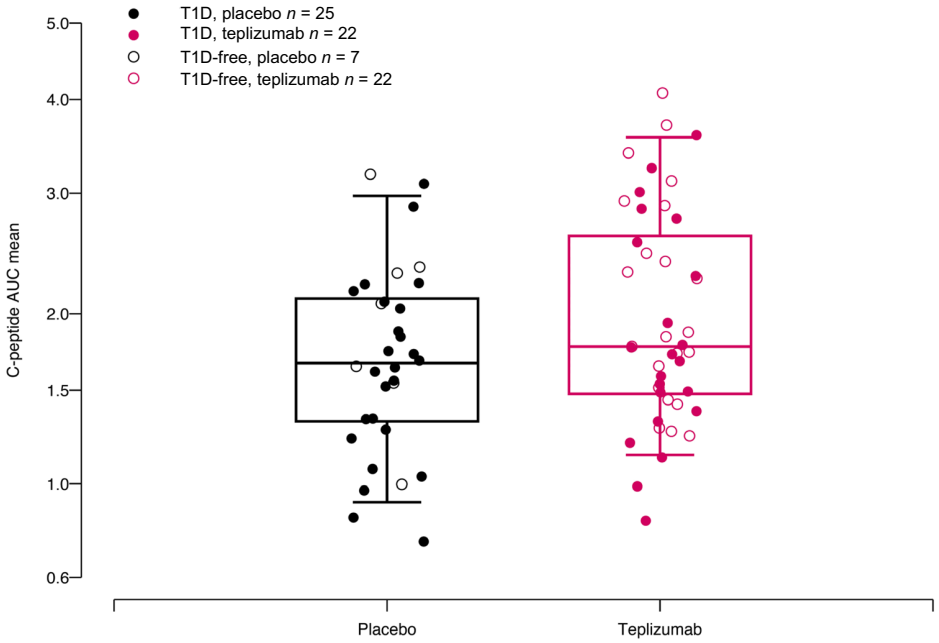

Fig. 3. Teplizumab treatment is associated with increased average on-study C-peptide AUC. Box plot displaying median and interquartile ranges for average on-study OGTT C-peptide AUC mean for participants from placebo- and teplizumab-treated groups. An ANCOVA model including baseline C-peptide AUC and age showed that treatment was associated with higher average on-study C-peptide AUC ($P = 0.006$).

There was also a direct relationship between the participant age and the average C-peptide AUC across both treatment arms and outcomes (Pearson's product-moment correlation, $\rho = 0.44$, $P = 0.0001$) (fig. S4), as has been noted previously in studies of individuals at risk and with new-onset T1D (*32*). In contrast to relationships with time to clinical diagnosis (*26*), Human Leukocyte Antigen-DR4 $(HLA\text{-}DR4)^+$, $HLA\text{-}DR3^-$, or anti-Zinc Transporter 8 (ZnT8) antibody status did not show significant interactions with treatment and the average on-study C-peptide AUC (Wald test: HLA-DR3, $P = 0.44$; HLA-DR4, $P = 0.84$; and ZnT8, $P = 0.84$; table S5).

Teplizumab treatment reverses declines in C-peptide AUC during the first 6 months of treatment

Because average on-study C-peptide AUC could obscure more pronounced between-group differences at individual study time points, we next analyzed the timing of the changes in C-peptide AUC relative to treatment and the insulin secretion patterns. Because the participants had been recruited from the TN01 Natural History Study, we were able to analyze the C-peptide response to OGTTs before enrollment and compare these to values after enrollment in this study. Geometric-like group means over a median of 2.4 months before randomization and over the 12 months after randomization are shown in Fig. 4. There was a decline in the C-peptide AUC before study enrollment in both those subsequently randomized to teplizumab and to placebo [before baseline and baseline: placebo, 1.94 (1.6, 2.37) and 1.83 pmol/ml (1.56 and 2.36); teplizumab, 2.01(1.47, 2.59) and 1.89 pmol/ml (1.47, 2.17) with a pretreatment slope of −0.0233 (−0.0557, 0.0175) for all participants ($n = 74$ total, 43 for teplizumab and 31 for placebo groups)]. In the participants treated with placebo, the decline in C-peptide persisted at the same rate for the first 6 months after enrollment [mean C-peptide AUC of 1.68 pmol/ml (1.2, 2.15) at 6 months], with no significant differences ($P > 0.05$) in pretreatment versus posttreatment slopes even after correction for age and the C-peptide at enrollment. In contrast, there was a significant increase in the C-peptide AUC in the teplizumab-treated participants at 6 months after enrollment [6-month mean C-peptide AUC of 2.06 pmol/ml (1.55, 2.58); Wilcoxon paired test, $P = 0.04$]. The posttreatment slopes differed significantly between the placebo- and teplizumab-treated participants by ANCOVA after correcting for age and pretreatment slope ($P = 0.0015$; table S6).

Insulin secretory dynamics are improved by teplizumab treatment

In addition to quantitative decreases in C-peptide AUC, studies by our group and others have identified qualitative abnormalities in beta cell secretory kinetics, with loss of early insulin secretion reflecting beta cell dysfunction before the onset of T1D (*10*, *32*–*35*). To determine whether the quantitative improvement in C-peptide AUC was associated with qualitative changes in the kinetics of insulin secretion, we determined the insulin secretory rates during the OGTTs using a two-compartment model and evaluated the kinetics and total insulin secretion (Fig. 5, A to G, and Table 1). We compared the OGTT insulin secretory responses and modeled the change in the secretory responses (slope) before and after drug treatment and between the two study arms. With this analysis of insulin secretion, we could distinguish the early and late secretory responses (first- and second-hour responses, respectively). The modeled slopes describing the change in the total, first-hour, and second-hour insulin secretion were similar in both groups before study enrollment ($P = 0.95$). After treatment with teplizumab, there was a significant increase in the total insulin secreted during the test in the teplizumab group ($P = 0.01$) that was significantly greater than that in the placebo group ($P = 0.0004$). The insulin secreted during the first hour continued to decline in the placebo group, whereas it increased in the teplizumab group ($P = 0.007$). The second hour of insulin secretion also improved in the teplizumab treatment group ($P = 0.03$) but not in the placebo group ($P = 0.38$) (Table 1). These results indicate that in the first 6 months after teplizumab treatment, there is improvement in insulin secretion, particularly within the first hour of the OGTT, suggesting improved beta cell function, whereas there is continuing deterioration in insulin secretion in the placebo-treated participants.

The relative abundance of proinsulin to C-peptide has been suggested as a measure of beta cell stress (*36*). Therefore, we compared the ratios of proinsulin:C-peptide at the times of the improved C-peptide and insulin secretion, beginning 6 months after study drug treatment. Coincident fasting proinsulin and C-peptide measures were available for 22 placebo-treated participants and 41 teplizumab-treated participants. Unlike the increased secretion of insulin in the first hour of the OGTT or the total C-peptide AUC,

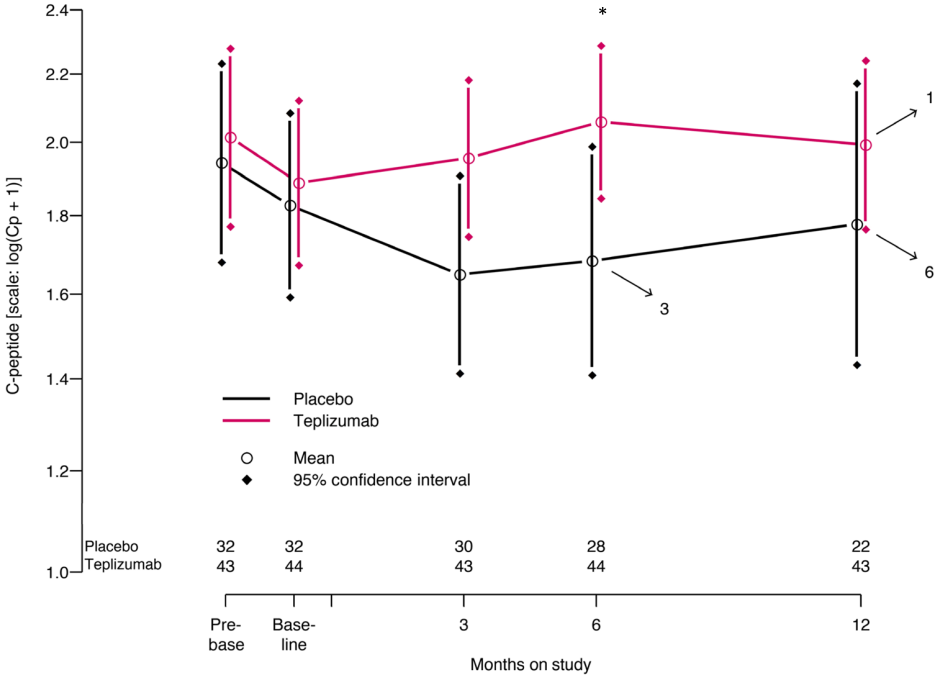

Fig. 4. C-peptide over time in the two treatment arms over the first year. The log-transformed mean C-peptide (Cp) AUC is shown. Arrows indicate the number of individuals who dropped out from OGTT monitoring because of diabetes development at each time point. Median C-peptide AUC value for "pre-baseline" time point was 24; median value for "baseline" time point was 0.85 months before randomization. *$P < 0.05$ for comparisons of 6-month on-treatment C-peptide AUC values to baseline in the teplizumab group and 6-month C-peptide AUC values in the teplizumab group to 6-month C-peptide AUC values in the placebo group.

we did not identify a significant difference in the ratios between the treatment arms at 6 months, after the C-peptide concentrations had improved in the teplizumab-treated patients [placebo median (range), 0.26 (0.05 to 2.14); teplizumab, 0.34 (0.1 to 2.44); $P > 0.05$] or when the average ratios after treatment up to 36 months were compared [placebo average on-study proinsulin:C-peptide ratio median (range), 0.38 (0.05 to 10.7) versus teplizumab, 0.42 (0.1 to 3.0); $P = 0.57$; fig. S3B].

Preservation of C-peptide is maintained until the last 6 months preceding clinical diagnosis

To determine the duration of these metabolic effects, we analyzed the C-peptide trajectories (least-square lines) over the entire study period or until the 6 months before the participant was diagnosed with T1D (Fig. 6, A and B). In this analysis, C-peptide AUC declined in the placebo group: The median slope was significantly less than 0 (median, IQR: −0.00382, −0.0107 to 0.000755; Wilcoxon one-sample, $P = 0.04$). The loss of C-peptide in the placebo group was even more pronounced in the 6 months between the penultimate and final OGTT [mean slope (IQR) of −0.0242 (−0.0469 to 0.0041); significantly nonzero (Wilcoxon one-sample, $P = 0.0001$] (Fig. 6, C and E).

In contrast, the median slope for the teplizumab group until the end of the study period or until the 6 months before the participant was diagnosed with T1D was not significantly different from 0 [mean (IQR): −0.000294 (−0.00372 to 0.00304); Wilcoxon one-sample, $P = 0.63$] (Fig. 6B), and thus, less C-peptide AUC was lost over time compared to the placebo-treated participants (Wilcoxon two-sample, $P = 0.04$). In the participants treated with teplizumab who were diagnosed with T1D, there was also a decline in the C-peptide AUC in the peridiagnostic period, but the median was not significantly less than 0 (Wilcoxon one-sample with a comparison to 0, $P = 0.09$) (Fig. 6D) and was modestly greater than those in the placebo treatment arm who were diagnosed with T1D (Wilcoxon two-sample comparing placebo and teplizumab slopes before diagnosis, $P = 0.06$) (Fig. 6E). A difference in insulin sensitivity between the two treatment arms was not a likely explanation for these findings because the ratios of the C-peptide AUC to glucose AUC were similar in the teplizumab and placebo groups at the time of T1D diagnosis ($P = 0.23$) (fig. S5, A and B).

C-peptide responses correlate with increases in partially exhausted CD8$^+$ T cells

We postulated that the rapid improvement in metabolic responses was related to the effects of teplizumab on T cells. There was a transient decline in the number of circulating lymphocytes during teplizumab treatment, but the number of cells was at the baseline level by day 42 (*37*). However, the improvement in C-peptide was not related to the changes in the number of circulating CD3$^+$ (Pearson $\rho = 0.19$, $P = 0.28$), CD4$^+$ ($\rho = 0.01$, $P = 0.97$), or CD8$^+$ ($\rho = 0.24$, $P = 0.17$) cells (fig. S6). We previously described an increase in the frequency of memory CD8$^+$ T cells with teplizumab treatment that we proposed were "partially exhausted" by expression of T cell immunoreceptor with Ig and ITIM domians (TIGIT) and killer cell lectin-like receptor subfamily G member 1 (KLRG1) (double-positive cells) and a transcriptional activation/exhaustion signature that could be further reduced by ligation of TIGIT (*21*, *23*, *25*, *26*, *38*). Thus, we tested whether the frequency of these or other cells was associated with C-peptide AUC during or shortly after the drug treatment period and whether they were functionally exhausted. We observed a significant correlation of the change in frequency of CD8$^+$KLRG1$^+$TIGIT$^+$ T cells at months 3, 6, and 18 but not between CD4$^+$ or other CD8$^+$ T cells and the fold change in C-peptide at month 6 (Table 2). The changes in T cell subsets most likely preceded the changes in C-peptide, and therefore, we also analyzed the fold changes in double-positive CD8$^+$ T cells at month 3 and the fold changes in C-peptide at month 6. There was a significant association between the frequency ($P = 0.014$) (Fig. 7A) as well as the absolute number of these cells ($P = 0.02$; fig. S6D) and the change in C-peptide.

T cell exhaustion has been associated with reduced cytokine production after activation (*39*). We therefore measured intracellular cytokines after stimulation of peripheral blood mononuclear cells (PBMCs) with anti-CD3 and anti-CD28. Among the double-positive CD8$^+$ T cells, the frequency of interferon-γ

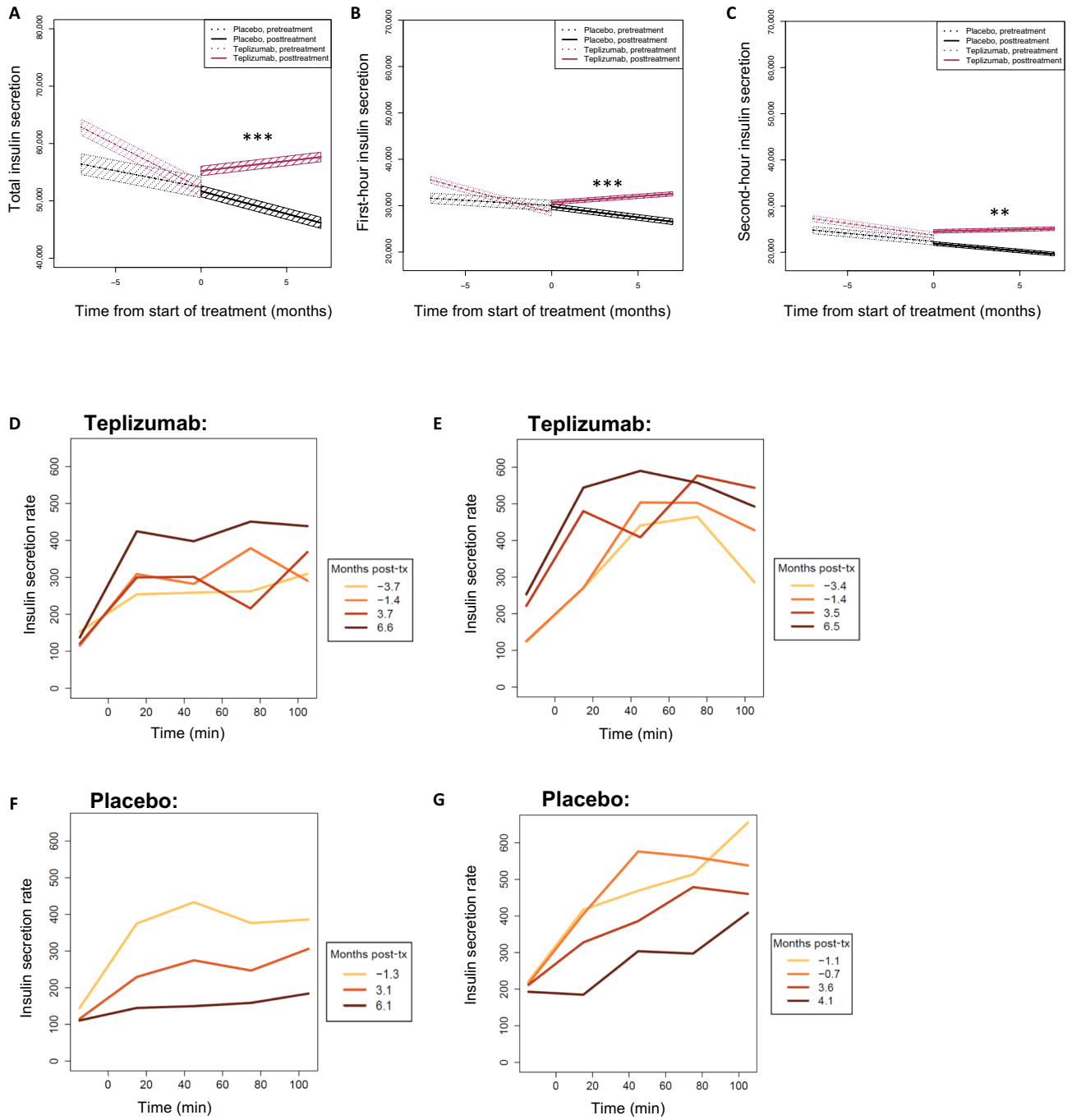

Fig. 5. Insulin secretion after treatment with teplizumab or placebo. Estimated slopes for the insulin secreted (pmol) during the total (**A**), first hour (**B**), and second hour (**C**) of the OGTT at the visits before enrollment and over the first 6 months after study drug treatment. Median values (and 95% confidence intervals in shaded colors) are shown. Significance for Wilcoxon signed-rank test for comparison of posttreatment slopes between treatment groups are shown in each panel. Please refer to Table 1 for full statistical analyses. (**D** and **E**) Representative insulin secretion rates during serial OGTTs for two teplizumab-treated participants who were not diagnosed with T1D (aged 11 and 12 years) and (**F** and **G**) two placebo-treated individuals (both aged 13 years) who were diagnosed with T1D. The colored lines indicate the time of the visits in relationship to study drug administration. tx, treatment. $^{**}P < 0.01$ and $^{***}P < 0.001$.

(IFNγ)–producing and tumor necrosis factor–α (TNFα)–producing cells ($P < 0.0001$ for both) were decreased at 3 months (Fig. 7, B and C, and Table 2) in the teplizumab-treated but not placebo-treated participants, and the decline was associated with the improvement in C-peptide. In contrast, relative proportions of IFNγ- and TNFα-producing cells among the double-positive memory

Table 1. Analysis of insulin secretion to oral glucose in the first 6 months after treatment. diff = (post-rx slope) – (pre-rx slope)

Measure to be compared between arms	Median		*P* value
	Placebo (*n* = 31)	Teplizumab (*n* = 44)	
First-hour insulin interval secretion			
Pretreatment slope	−259.5	−422.7	0.79
Posttreatment slope	−476.2	371.0	0.0003
Paired pre-rx versus post-rx *P* values* within arms	*P* = 0.86	*P* = 0.007	–
Second-hour insulin interval secretion			
Pretreatment slope	−728.2	−383.6	0.78
Posttreatment slope	−186.8	442.5	0.003
Paired pre-rx versus post-rx *P* values* within arms	*P* = 0.38	*P* = 0.03	–
Insulin interval secretion (2 hours)			
Pretreatment slope	−1245.0	−1024.0	0.95
Posttreatment slope	−1037.4	1085.8	0.0004
Paired pre-rx versus post-rx *P* values* within arms	*P* = 0.80	*P* = 0.01	–

**P* values based on Wilcoxon signed-rank (paired) test comparing the pre-rx versus post-rx slopes by subject and across those subjects in each treatment arm. *n* = 31 for placebo-treated group and *n* = 44 for teplizumab-treated group.

$CD8^+$ T cells remained stable in the placebo group at 3 months of follow-up.

DISCUSSION

Studies of natural history cohorts have described changes in metabolic function during the progression to T1D in relatives at risk. Our successful intervention trial, with teplizumab, in the at-risk population has given us a unique opportunity to directly assess how changing immune cells can affect metabolic function and progression to the clinical diagnosis of T1D in relatives at high risk. The trial design was a time-to-event analysis (*40*). The current work used an extended period of follow-up, as well as OGTT data obtained before enrollment in the TN10 anti-CD3 prevention trial to provide metabolic characterization of study participants. In this extended follow-up, we showed that the effects of the single 14-day course of teplizumab treatment persisted: The median time to diabetes in the teplizumab group was about 5 years compared to slightly over 2 years in the placebo-treated participants, with 50% of the teplizumab-treated participants versus 22% of the placebo-treated participants not diagnosed with T1D. Eighteen percent of the teplizumab-treated participants versus 6% of the placebo-treated participants were followed for more than 5 years and were not diagnosed. The current study shows successful modulation of the progression of beta cell failure before the diagnosis of T1D with immunologic intervention.

Teplizumab treatment improved beta cell function, even in those who were not diagnosed with T1D. The average OGTT glucose concentrations were lower, and C-peptide responses were higher with teplizumab treatment. There was improvement in total and early insulin secretion rates, identifying a functional and quantitative improvement in insulin release. The early secretion of insulin, a feature of normal beta cell function, was the most changed, indicating that the impaired "beta cell glucose sensitivity" that has been described in patients who progress to clinical diabetes was improved and that effects on C-peptide AUC were not solely a reflection of improved beta cell mass (*33*). Metabolic changes were associated with an increased frequency of $TIGIT^+KLRG1^+$ memory $CD8^+$ T cells and a reduced secretion of cytokines (TNFα and IFNγ) that have been associated with pathology in T1D, indicating that the T cells had functional exhaustion (*21*, *23*, *25*, *26*, *38*). In contrast, early increases in C-peptide were not correlated with individual changes in total number of $CD3^+$ T cells or other T cell subsets. The lack of correlation between increases in C-peptide AUC and reductions in total $CD3^+$ cells within the teplizumab-treated group, combined with the stabilization of C-peptide despite recovery of circulating $CD3^+$ T cells, points away from a direct effect of the treatment-induced $CD3^+$ nadir on beta cell function. Previous successful interventions that have shown preservation of C-peptide responses with immune or other interventions have all been done in patients with stage 3 disease (*20*–*22*, *41*–*44*). This study shows metabolic improvement even in the absence of hyperglycemia.

Because the clinical trial was designed as a time-to-event protocol, the variable time in the study for each participant created a challenge in analyzing the metabolic responses during the study OGTTs. Hence, we used the average on-study C-peptide, glucose, and HbA1c AUCs, which included all of the available data for each participant. Moving forward, this approach could prove useful in metabolic analyses of other diabetes prevention studies designed as time-to-event trials.

Although the time in the trial was not a determinant of the average C-peptide AUC, there were time-dependent metabolic effects of the drug treatment. Beta cell function was declining in the participants as they were enrolling in the TN10 anti-CD3 prevention trial.

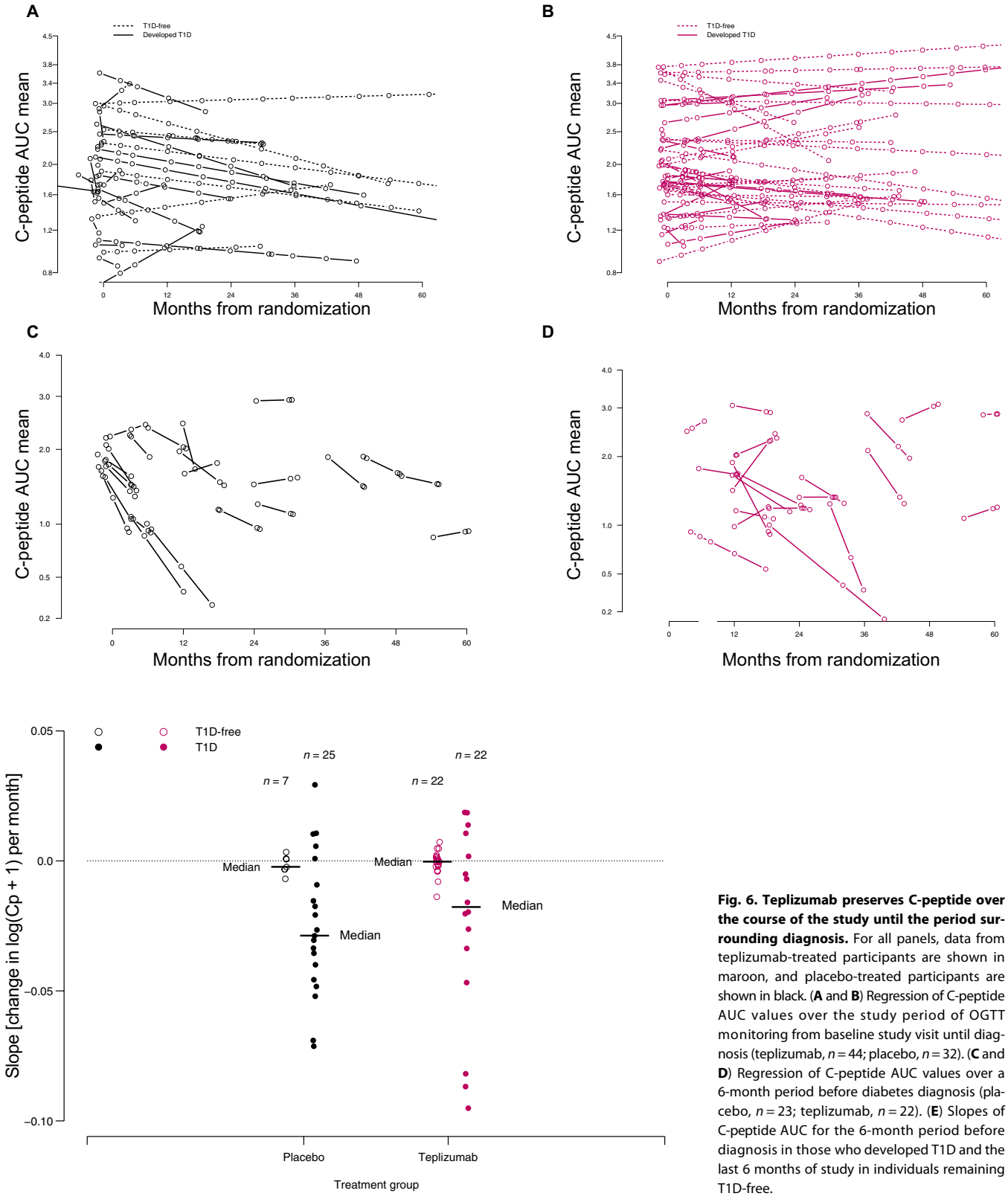

Fig. 6. Teplizumab preserves C-peptide over the course of the study until the period surrounding diagnosis. For all panels, data from teplizumab-treated participants are shown in maroon, and placebo-treated participants are shown in black. (**A** and **B**) Regression of C-peptide AUC values over the study period of OGTT monitoring from baseline study visit until diagnosis (teplizumab, $n = 44$; placebo, $n = 32$). (**C** and **D**) Regression of C-peptide AUC values over a 6-month period before diabetes diagnosis (placebo, $n = 23$; teplizumab, $n = 22$). (**E**) Slopes of C-peptide AUC for the 6-month period before diagnosis in those who developed T1D and the last 6 months of study in individuals remaining T1D-free.

Table 2. Pearson correlations between percent change in C-peptide from baseline to month 6 and CD8$^+$ T cell subpopulations.

	At month 3		At month 6		At month 18	
	ρ	*P*	ρ	*P*	ρ	*P*
CD8$^+$ T cell subsets*						
KLRG1$^+$TIGIT$^+$ of CD8$^+$ central memory T cells	0.429	0.016	0.433	0.01	0.463	0.011
KLRG1$^+$TIGIT$^+$ of CD8$^+$ effector memory T cells	0.421	0.018	0.460	0.006	0.461	0.012
KLRG1$^-$TIGIT$^-$ naïve CD8$^+$	0.049	0.79	0.17	0.34	−0.126	0.52
CD4$^+$ T cell subsets*						
KLRG1$^+$TIGIT$^+$CD4$^+$	0.016	0.93	0.026	0.883	0.217	0.26
PD-1$^+$TIGIT$^+$ memory CD4$^+$ regulatory T cells (FoxP3$^+$ and CD127^{lo})	−0.195	0.27	−0.04	0.82	0.3	0.1

*For CD8$^+$ T cell subsets, $n = 21$ for placebo-treated group and $n = 32$ for teplizumab-treated group. For CD4$^+$ T cell subsets, $n = 23$ for placebo-treated group and $n = 35$ for teplizumab-treated group.

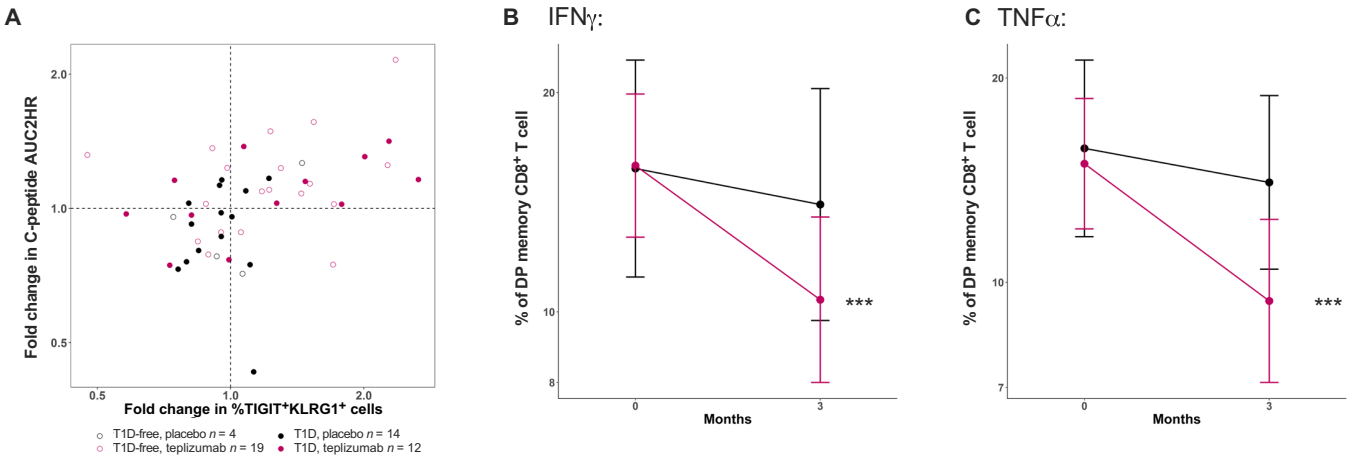

Fig. 7. Functional changes in T cells are associated with improvements in metabolic function. (**A**) The changes in TIGIT$^+$KLRG1$^+$CD45RO$^+$CD8$^+$ T cells between baseline and 3 months and the change in the C-peptide AUC between the baseline and 6 months are shown. There was a significant correlation between the changes in this cell subset and C-peptide in the teplizumab-treated (Pearson ρ = 0.44, $P = 0.014$, $n = 31$) but not the placebo-treated (ρ = 0.28, $P = 0.25$, $n = 18$) participants. (**B** and **C**) The frequency of double-positive (DP) CD8$^+$ memory cells that produce IFNγ or TNFα are shown for the placebo-treated (black, $n = 16$) and drug-treated (maroon, $n = 24$) participants at baseline and month 3. The frequency of the IFNγ- and TNFα-producing cells were reduced in the teplizumab-treated participants (paired *t* test, ***$P < 0.0001$).

In previous studies, we found that the degree of beta cell death was high among similarly high-risk individuals, and other studies have documented beta cell dysfunction in the peridiagnosis period (*34*, *45*, *46*). These metabolic data together with the relatively short median time to diagnosis of T1D in the placebo group indicate that the screening methods used identified an active time of disease and individuals at very high risk for progression. Consistent with preclinical studies, the effects during this period of active disease support the concept that this intervention may be most effective when there is immune cell activation (*47*). The greatest increase in C-peptide occurred shortly after teplizumab treatment, followed by stabilization of beta cell function, whereas in the placebo group, beta cell function declined gradually over time. Consistent with prior reports, in those who developed clinical diabetes in both treatment arms, there was a precipitous decline in the stimulated C-peptide levels seen about 6 months before T1D onset (*45*, *46*).

One measure of beta cell dysfunction, the impaired early secretion of insulin, and total C-peptide AUC both improved with teplizumab treatment. However, another measure of beta cell dysfunction, the ratio of proinsulin:C-peptide, was not different between groups

at 6 months or over the 36 months after treatment. Moreover, the OGTTs did not uniformly normalize in those who were not diagnosed with T1D because the outcomes of the OGTTs fluctuated even within individuals that did and did not develop T1D. Most likely, this variability reflects the tenuous degree of residual insulin production or even the ongoing presence of metabolic stressors. Consistent with this, we did not find a relationship between average on-study glucose AUC and C-peptide AUC. These clinical outcomes are similar to the effects of anti-CD3 monoclonal antibody in the nonobese diabetic model of T1D before the onset of diabetes, in which insulin granularity was improved but beta cell mass did not recover to normal amounts (*48*, *49*). Thus, qualitative and quantitative measures of beta cell function may be affected differently by immune therapy. Further studies with metabolic clamps might refine our analysis of the metabolic function, but such studies were impractical in this clinical trial setting.

The factors that precipitated disease in the 6 months before clinical T1D diagnosis in both the treatment and placebo arms are not clear at this point. The similar relation between C-peptide and glucose in the two treatment arms among those who were diagnosed with T1D suggests that insulin insensitivity was not a precipitating factor for the diagnosis. Even with progression to clinical diabetes, the decline in C-peptide was not significantly less pronounced in the teplizumab group versus placebo ($P = 0.06$), although we suggest that the effects of drug treatment on C-peptide may persist even during and potentially after the clinical diagnosis. There may be waning of the effects of the anti-CD3 antibody on immune cells that we identified previously by tracking the $CD8^+$ memory double-positive cells (*23*). Other observations in the field show that progression to clinical diabetes is associated with acquisition of effector T cell function, but it is possible that in this setting, restored effector function involves waning of the immune effects of teplizumab or even new or regenerated pathologic T cells repopulating the repertoire after a single drug course. The median age at the time of treatment in the TN10 anti-CD3 prevention trial was 13.9 years, and in young children, the thymic output of T cells may be ongoing. In other studies of the long-term outcomes of patients treated with teplizumab, there was an increased frequency of programmed cell death protein 1–positive ($PD\text{-}1^+$) memory $CD8^+$ T cells in responders compared to nonresponders and controls, suggesting that changes in the phenotype and function of the $CD8^+$ memory compartment may occur over time (*50*). Ongoing work tracking T cell receptors and single-cell analyses will help address these hypotheses and may suggest agents that could be used to extend the diabetes-free period possibly by blocking pathways needed for T effector expansion (*50*).

There are limitations to our studies. Our methods do not allow us to distinguish the contribution of maintained beta cell mass versus improved function of beta cells. Our analysis of the total C-peptide AUC suggests the former, but the kinetics of insulin secretion also suggest the latter. Longer-term follow-up of half of the patients who had not been diagnosed with T1D may address this question in a practical way. In addition, studies of beta cell killing are ongoing. The number of subjects in the examined trial was relatively small, and the study was powered to detect differences in diabetes incidence rather than changes in C-peptide AUC, insulin secretion, proinsulin:C-peptide ratio, or immune function. Our analysis of proinsulin:C-peptide could only be done in a subset of participants with samples available after the 6-month time point. In addition, the time-to-event design of the original study had some important implications for the analyses included here. We did not have OGTT analyses for most individuals after diagnosis of T1D, which limited our ability to compare OGTT data between all members of placebo and teplizumab groups over the same time period, particularly for the placebo group, which exhibited more rapid progression to diabetes. The time-to-event design also limited our ability to compare the relationship between metabolic end points and T1D progression because some individuals included in the study that did not progress to diabetes may ultimately develop T1D. Furthermore, given prior results showing that teplizumab treatment preserved C-peptide in patients with recent-onset T1D (*19*–*25*), positive effects on C-peptide might also be expected to occur among individuals who developed diabetes during this study. Participants from both arms of the trial who developed diabetes were enrolled in the TrialNet Long-Term Investigational Follow-up study, which performs longitudinal metabolic testing in participants who have been diagnosed with T1D (*13*).

In summary, we show extended delay in progression to T1D in at-risk subjects treated with teplizumab. Teplizumab treatment changed the biologic course of the disease by enhancing beta cell function as reflected by quantitative and qualitative improvements in insulin secretion. These changes were associated with modulation of the frequency and function of memory $CD8^+$ T cells. The pronounced early efficacy of the drug followed by stabilization of beta cell function also suggests that repeated treatment, which previously has been safely implemented (*19*, *20*, *51*), or the addition of other complimentary agents such as drugs that act directly on beta cells at key time points in the clinical course may be valuable to extend the delay or prevent the diagnosis of T1D. Last, our findings have implications for other autoimmune diseases by showing how immune intervention can change the pathobiology even before disease diagnosis and lead to a clinically meaningful outcome.

MATERIALS AND METHODS

Study design

The design of the TrialNet anti-CD3 prevention trial (TN10), phase 2, randomized, placebo-controlled, double-blind trial (NCT01030861), which tested the effect of a single 14-day course of teplizumab treatment on time to diabetes development, has been previously reported (*26*). Data for this analysis were derived from the phase 2 study, the TrialNet Pathway to Prevention study (TN01), for data before enrollment in the TN10 anti-CD3 prevention trial, and the TrialNet Long Term Investigational Follow-up study for metabolic follow-up (*14*, *52*). Data between July 2011 and March 2020 are included in this analysis. Institutional Review Board approval was obtained at each participating sites for these studies. The participants, their parents, or both provided written informed consent or assent before trial entry. Islet autoantibody testing, HLA genotyping, and OGTT testing were performed at the time of study entry (*4*, *53*). Briefly, eligibility criteria included age ≥8 years at randomization, history of a relative with T1D, positive titers for two or more islet autoantibodies, and dysglycemia on OGTT [fasting glucose, 110 to 125 mg/dl (6.1 to 6.9 mM); a 2-hour postprandial plasma glucose concentration of ≥140 mg/dl (7.8 mM) and <200 mg/dl (11.1 mM); or an intervening postprandial glucose concentration at 30, 60, or 90 min of >200 mg/dl and the absence of a history of diabetes]. For participants who did not have an HbA1c available at the baseline visit, values obtained within the 3 months before treatment were used.

Briefly, participants were randomly assigned to teplizumab or saline and treated with a 14-day outpatient course administered as an intravenous infusion in a clinical research center. As per study protocol, OGTTs were performed 3 and 6 months after the infusions and every 6 months thereafter and were used in this analysis. Random screening glucose concentrations were evaluated at 3-month intervals, and an OGTT was performed if the random glucose concentration was >200 mg/dl (11.1 mM). T1D was diagnosed using American Diabetes Association criteria during an OGTT but only after the diabetic OGTT was sequentially confirmed. The date of diagnosis was identified as the time of the first of the two diagnostic tests (*54*). Six participants were clinically diagnosed with T1D outside of OGTT monitoring. The original TN10 trial end date was June 2019. Participants who had not been diagnosed with T1D were transferred into the TrialNet Pathway to Prevention Natural History study (TN01) for follow-up OGTT monitoring (*14*, *52*).

In addition, OGTT data from islet autoantibody–negative relatives obtained through TrialNet testing were also analyzed. Autoantibody-negative relatives selected for analyses were matched to study participants by sex and age. For subjects <19 years, age was matched within 6 months. Subjects ≥19 were matched within ±2 years with the exception of five subjects who required ±6 years. Mean body mass indices for the placebo group, placebo-matched controls, teplizumab group, and teplizumab-matched controls were similar and in the non-overweight range (22.0, 24.1, 22.0, and 22.8, respectively).

Metabolic analyses

OGTT C-peptide and glucose values were tested by Northwest Lipid Research Laboratories using the Tosoh C-peptide immunoassay and Roche glucose assay. Samples for measurement of fasting proinsulin concentrations were available at 6, 12, 18, 24, 30, and 36 months after drug treatment from a subgroup of individuals according to allowable blood volumes. The proinsulin was measured using a TECO intact proinsulin enzyme-linked immunosorbent assay. Proinsulin:C-peptide ratios were calculated by obtaining the equimolar ratio of intact proinsulin:fasting C-peptide ×100 (*55*). OGTT results were assigned to the nearest study visit time point (within 3 months of the official time point assignment). OGTT results were classified as normal, dysglycemic, or diabetic on the basis of above definitions used for study entry. The baseline OGTT was the study at the time or immediately before randomization.

AUC values for insulin secretory rates, C-peptide, and glucose were calculated using the trapezoidal rule, where each of two consecutive timed measurements (for example, 0 and 30 min) forms a trapezoid with a base of 30 min. The formula for the area of a trapezoid is [(Height1 + Height2)/2] × Base. The areas of the four trapezoids are then added together to estimate the AUC. This sum is divided by 120 min to give an AUC mean (*56*). The on-study AUC means for C-peptide, glucose, and HbA1c were calculated by multiplying the AUC means for each OGTT visit and the visit intervals in days (as the trapezoidal base) to calculate a total study AUC and then dividing by the days from the first to the last OGTT (confirmatory diabetic OGTT if developed T1D). Insulin secretory rates were calculated using the Chronobiological Series Analyzer software, which uses a two-compartment model for hormone clearance and standard kinetic parameters for C-peptide (*34*, *57*–*62*). Insulin secretory rate calculations were performed using participant OGTT C-peptide and glucose values, as well as age, sex, height, and weight. The insulin secretion was divided into the amount (in pmol) secreted over the 2-hour OGTT or in the first or second hour of the test.

Flow cytometry analysis

PBMCs were processed and stored at the National Institute of Diabetes and Digestive and Kidney Diseases repository. Cryopreserved vials of PBMCs were sent to the Immune Tolerance Network (ITN) core laboratory at Benaroya Research Institute for analysis by flow cytometry with antibody panels shown in tables S7 and S8. T cell phenotyping was performed on thawed PBMCs, and the frequency of $CD45RO^+CD8^+$ T cells that were $TIGIT^+KLRG1^+CD57^-$ was determined as described previously (*63*). Intracellular cytokine expression was measured after 6 hours with stimulation of PBMCs by plate-bound anti-CD3 (1 μg/ml) and soluble anti-CD28 (10 μg/ml) in the presence of equimolar amounts of GolgiStop. The frequency of $TIGIT^+KLRG1^+CD8^+$ memory ($CD45RA^-$) T cells that produce IFNγ or TNFα was determined at baseline and month 3.

Instrument standardization was performed using eight-peak rainbow calibration beads (Spherotech) adjusting photomultiplier tube voltages for consistent seventh peak mean fluorescent intensities. All samples from the same subject were run on the same day, and an internal control arm from the same subject was run each week. Sample acquisition was performed as previously described on an LSRFortessa (BD Biosciences) with FACSDiva software and analyzed with FlowJo software version 9.5 (Tree Star) (*63*). Quadrants were placed on the basis of staining controls. Gated populations with <100 events were excluded from analysis.

Statistical analysis

The original trial was designed as a time-to-event analysis, and therefore, participants who were diagnosed with T1D were not followed further in that study. The impact of teplizumab treatment on incidence of T1D after enrollment was performed using a Cox proportional hazards model. For this analysis, metabolic parameters over the entire period of the trial included OGTT data in the visit immediately before and all OGTT data after study drug treatment (confirmatory diabetic OGTT for individuals diagnosed with diabetes or last available OGTT for those remaining diabetes-free). Slopes for changes in glucose and C-peptide before and after enrollment were calculated using linear regression analysis of available OGTT visit data for specified intervals. An impact of treatment on each end point was determined by fitting results to an ANCOVA model, with age, baseline value, and treatment group included as covariates. Wald tests were used to determine whether covariates significantly affected the model. Estimated slopes for changes in the insulin secretion rates were also calculated for each subject on the basis of changes before treatment (time points up to 6 months before baseline) and for after initiation of treatment (time points up to 6 months after baseline) using linear regression models and mixed models for repeated measures. Data were tested for normality, and a parametric or nonparametric test was used for comparisons. Insulin secretion rates were calculated across the overall 2-hour interval and specifically for the first-hour and second-hour intervals of the OGTTs. Differences in these slopes before versus after treatment were compared using Wilcoxon signed-rank tests within and across treatment arms. Differences and percent changes in these slopes before versus after treatment were also evaluated using a generalized linear model to assess the influence of treatment arm.

Flow cytometry data were log-transformed for statistical analysis. Pearson's correlation coefficient was calculated to determine

associations between fold changes in C-peptide AUC and frequency of TIGIT$^+$KLRG1$^+$CD8$^+$ memory T cells. Because our question was focused on the (previously described) TIGIT$^+$KLRG1$^+$CD8$^+$ memory T cells and whether their frequency associated with changes in C-peptide, corrections for multiple comparisons were not performed for this analysis. The frequency of TIGIT$^+$KLRG1$^+$CD8$^+$ memory T cells producing IFNγ or TNFα was analyzed by paired *t* test.

Analysis of proinsulin:C-peptide measures was performed using $\log_2$-transformed measures. The average on-study proinsulin:C-peptide ratios were calculated using a trapezoidal rule calculation to obtain a study-wide AUC of proinsulin:C-peptide ratio over time for the post-baseline time points, divided by the time interval for those measures. If only one post-baseline time point was available (for example, only at 6 months), then the proinsulin:C-peptide ratio at that time point was used.

SUPPLEMENTARY MATERIALS

stm.sciencemag.org/cgi/content/full/13/583/eabc8980/DC1
Fig. S1. Consort diagram.
Fig. S2. Results of OGTT tests over the first 36 months in (A) teplizumab- and (B) placebo-treated participants.
Fig. S3. Average on-study HbA1c values and proinsulin:C-peptide ratios are not significantly different between the treatment groups.
Fig. S4. Relationship of average on-study C-peptide AUC with age.
Fig. S5. C-peptide values are similar between treatment groups at the time of diagnosis.
Fig. S6. Changes in the number of circulating T cells at month 3 and C-peptide AUC at month 6.
Table S1. ANCOVA model of on-study glucose AUC mean.
Table S2. ANCOVA model of on-study A1c AUC mean (ln-ln transform).
Table S3. ANCOVA model of on-study C-peptide AUC mean [ln(x + 1) transform].
Table S4. Comparison of C-peptide AUC means among teplizumab and placebo groups and matched islet autoantibody–negative relatives (nontransformed values).
Table S5. Effect of HLA genotype and ZnT8 positivity on average C-peptide AUC.
Table S6. ANCOVA analysis of C-peptide AUC slope over the first 6 months on study.
Table S7. T cell phenotype flow cytometry panel.
Table S8. Intracellular cytokine staining flow cytometry panel.
Data file S1. Proinsulin:C-peptide ratios (csv).
Data file S2. Insulin secretion data (csv).
Data file S3. OGTT glucose data (Excel).
Data file S4. Participant data (Excel).
Data file S5. C-peptide values (Excel).
Data file S6. Flow data (Excel).
Data file S7. Flow subsets (Excel).
Data file S8. Cytokine data (Excel).

View/request a protocol for this paper from *Bio-protocol*.

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Teplizumab improves and stabilizes beta cell function in antibody-positive high-risk individuals

Emily K. Sims, Brian N. Bundy, Kenneth Stier, Elisavet Serti, Noha Lim, S. Alice Long, Susan M. Geyer, Antoinette Moran, Carla J. Greenbaum, Carmella Evans-Molina, Kevan C. Herold and Type 1 Diabetes TrialNet Study Group

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Prolonged prevention of autoimmune diabetes

Teplizumab was previously shown in a clinical trial to delay onset of type 1 diabetes (T1D) in high-risk relatives of individuals with T1D. Now, Sims *et al.* extend the follow-up analysis of this trial by 12 months, finding that efficacy of the initial 2-week treatment course persisted, with an extended time to T1D diagnosis in the teplizumab-treated group. Clinical benefits associated with reversed C-peptide decline improved beta cell function and partial exhaustion in $CD8^+$ T cells in the treated patients. HbA1c did not differ between placebo and treatment groups. This follow-up study further supports the use of anti-CD3 treatment for the prevention of T1D.