AUTOIMMUNITY

Metabolic and immune effects of immunotherapy with proinsulin peptide in human new-onset type 1 diabetes

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Immunotherapy using short immunogenic peptides of disease-related autoantigens restores immune tolerance in preclinical disease models. We studied safety and mechanistic effects of injecting human leukocyte antigen–DR4(*DRB1*0401*)–restricted immunodominant proinsulin peptide intradermally every 2 or 4 weeks for 6 months in newly diagnosed type 1 diabetes patients. Treatment was well tolerated with no systemic or local hypersensitivity. Placebo subjects showed a significant decline in stimulated C-peptide (measuring insulin reserve) at 3, 6, 9, and 12 months versus baseline, whereas no significant change was seen in the 4-weekly peptide group at these time points or the 2-weekly group at 3, 6, and 9 months. The placebo group's daily insulin use increased by 50% over 12 months but remained unchanged in the intervention groups. C-peptide retention in treated subjects was associated with proinsulin-stimulated interleukin-10 production, increased FoxP3 expression by regulatory T cells, low baseline levels of activated β cell–specific CD8 T cells, and favorable β cell stress markers (proinsulin/C-peptide ratio). Thus, proinsulin peptide immunotherapy is safe, does not accelerate decline in β cell function, and is associated with antigen-specific and nonspecific immune modulation.

INTRODUCTION

Type 1 diabetes is a chronic autoimmune disease characterized by progressive, immune-mediated loss of β cell mass and function. After clinical presentation, most patients undergo continued attrition of remaining functional β cell mass and progress to the point at which residual C-peptide, a surrogate marker for insulin secretion, is absent or present at very low levels in the circulation (*1*, *2*). Two factors compound the clinical burden of type 1 diabetes. First, despite optimized insulin administration regimes, chronic hyperglycemia and hyperglycemic excursions are unavoidable in most patients and result in complications including retinopathy, nephropathy, and neuropathy, which reduce life expectancy by an average of over 10 years (*3*). Second, it is apparent that the incidence of the disease has been increasing by about 4% per year in recent decades, most notably in children and adolescents (*4*).

Despite over 25 years of efforts to develop immunomodulatory therapies to divert the course of type 1 diabetes, no therapeutic has yet emerged that balances robust efficacy with acceptable safety and tolerability for patients. This pharmacopoeial poverty comes at a time when there is increasingly clear evidence that retained C-peptide secretion, even down to the limits of conventional detection, is associated with significantly improved metabolic control and reduced risk of the serious diabetic complications that impact upon quality and duration of life (*5*–*7*).

In the same timeframe, an understanding of the numerous immunological pathways that contribute to β cell loss has emerged. These include delineation of effector pathways, such as autoreactive CD4 T cells secreting proinflammatory cytokines and CD8 T cells with cytotoxic activity upon recognition of β cell targets (*8*–*10*). There is also evidence that immune regulatory pathways may be compromised or unable to adequately control effector responses (*11*, *12*). These findings not only relate to conventional $FoxP3^{+}CD25^{hi}$ regulatory T cells (T_{regs}) but also to regulatory autoreactive CD4 T cells that secrete the immune suppressive cytokine interleukin-10 (IL-10) (*8*, *13*), which have been shown at the clonal level to mediate linked suppression of inflammatory T cells (*14*).

This knowledge promotes consideration of antigen-specific immunotherapy (ASI) as an approach for type 1 diabetes, because it has been shown in preclinical models of inflammation and autoimmunity to limit disease by deletional effects on effector T cells and by promoting cohorts of CD4 T cells with regulatory properties, including those that secrete IL-10 (*15*). One ASI approach involves administration of short peptides, representing epitopes of disease-related autoantigens. This strategy, termed peptide immunotherapy (PIT), has gained considerable traction in clinical allergy, where it avoids the problem of using

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whole antigens that might trigger immunoglobulin E–mediated hypersensitivity; it is also under development in autoimmune inflammatory conditions, such as celiac disease and multiple sclerosis (*15*). We have previously described how administration of a peptide representing an immunodominant region of proinsulin presented by the human leukocyte antigen (HLA) class II diabetes risk molecule HLA-DR4 (*DRB1*0401*) can modulate autoreactive CD4 T cells in patients with long-standing type 1 diabetes, but in that study, circulating C-peptide was absent, and therefore, safety and disease-modifying effects in a clinically relevant target population could not be evaluated (*16*). Therefore, here, we examined the proinsulin mono-PIT approach in adults ascertained within 100 days of type 1 diabetes diagnosis and with residual C-peptide to examine safety and tolerability in a relevant therapeutic setting and study early indications of mechanistic and metabolic effects.

RESULTS

Study enrollment and randomization

Of the 233 patients referred to the study sites, 84 were assessed for eligibility and attended screening visits. Of these, 56 subjects did not have either the *HLA-DRB1*0401* genotype or autoantibodies, and 1 subject had stimulated C-peptide <0.2 nM; all were excluded (fig. S1). After 24 subjects had been randomized, subjects who did not complete a minimum of 11 of 12 treatments ($n = 1$ in the low-frequency and $n = 2$ in the high-frequency groups) were replaced with additional study subjects ($n = 2$ in the low-frequency and $n = 1$ in the high-frequency groups by randomization) to maximize information on treatment exposure, but all subjects ($n = 27$) were retained in the analysis. Four subjects missed follow-up assessments ($n = 3$ in the low-frequency and $n = 1$ in the high-frequency groups; two subjects declined these visits, and two subjects were lost to follow-up). Baseline characteristics are shown in Table 1 and did not differ between groups, except for HbA1c, which was significantly higher in the placebo group compared with high-frequency group ($P = 0.02$). Planned primary and secondary end points are shown in table S1.

Safety of proinsulin peptide C19-A3 in new-onset type 1 diabetes

Subjects enrolled were treated according to the regimen in Fig. 1, and the participant flow is summarized in fig. S1. Peptide injection was very well tolerated with no serious adverse events considered to be treatment-emergent, and there was no evidence of hypersensitivity reactions at any time during the treatment course. Local erythematous skin reactions without local wheal or swelling have been observed previously with this peptide (*16*) and were seen in 8 of 9, 10 of 10, and 4 of 8 subjects in the high-frequency, low-frequency, and placebo groups, respectively, but did not change in quality or size over time.

C-peptide changes during the study

As specified in the predetermined analysis plan, C-peptide area under the curve (AUC) was compared between the treatment groups over time (3, 6, 9, and 12 months) by multilevel model repeated measures (MMRM) analysis adjusted for the baseline value of AUC, and no significant treatment-related effects were observed. There was no evidence of accelerated C-peptide loss in the treated groups compared to placebo.

However, we noted differences in C-peptide changes during the study that are worthy of discussion. The decline in stimulated C-peptide was different between study groups, and at the 3-month time point, mean loss of C-peptide in the placebo group exceeded that of the high-frequency ($P = 0.03$) and low-frequency groups (Fig. 2A). This difference in C-peptide decline was evident in individual data plots (Fig. 2B and table S2): Compared with baseline, C-peptide levels in subjects receiving placebo showed a decline at every time point in every

Table 1. Baseline characteristics of the study subjects.

		C19-A3 PIT	
Characteristic	Placebo	Low frequency	High frequency
Number of subjects	8	10	9
Mean age (years; ±SD)	28.9 ± 8.2	26.6 ± 5.5	30 ± 5.7
Gender (female:male)	2:6	4:6	3:6
Body mass index (kg/m^2; ±SD)	23.1 ± 2.6	24.2 ± 5.5	25.6 ± 5.4
Number of autoantibodies (GAD65Ab, IA-2Ab, and ZnT8Ab): Single antibody–positive	12.5%	50.0%	11.1%
Double antibody–positive	25.0%	30.0%	11.1%
Triple antibody–positive	62.5%	20.0%	77.8%
Mean time from diagnosis to first dose (days; ±SD)	95 ± 22.8	82.5 ± 16.0	91 ± 15.5
Mean glycated hemoglobin (mmol/mol; ±SD)	62.5 + 13.7	58.4 + 14.9	51.7 + 6.83*
Average total daily insulin dose (IU Kg^{-1} day^{-1}; ±SD)	0.42 + 0.20	0.38 + 0.18	0.30 + 0.07
Stimulated C-peptide AUC (nM/min; ±SD)	0.58 ± 0.25	0.81 ± 0.76	0.99 ± 0.73

*$P = 0.02$ versus placebo.

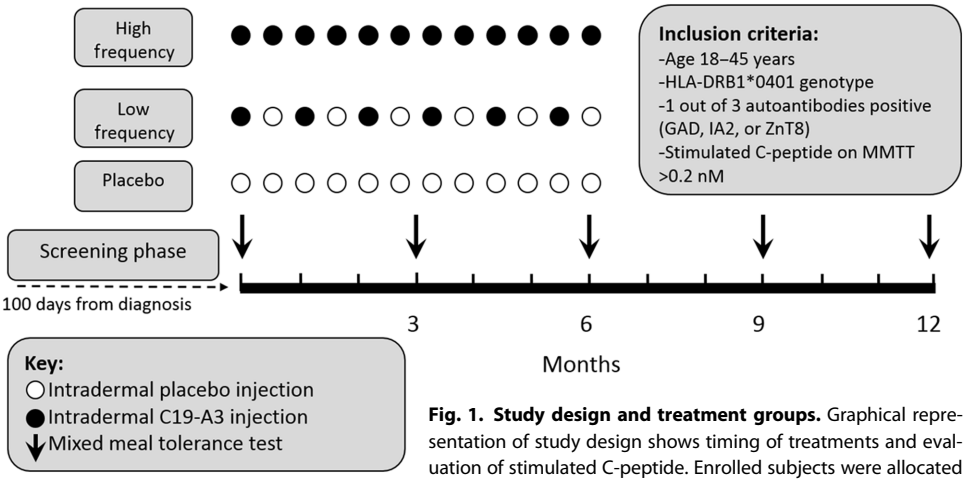

Fig. 1. Study design and treatment groups. Graphical representation of study design shows timing of treatments and evaluation of stimulated C-peptide. Enrolled subjects were allocated randomly with double blinding to receive high-frequency (10-μg proinsulin C19-A3 peptide every 2 weeks) or low-frequency (10-μg proinsulin C19-A3 peptide every 4 weeks alternating with saline every 4 weeks) active treatment or placebo (saline injections every 2 weeks) by intradermal injection for a total of 12 administrations over 6 months. Residual C-peptide production was evaluated at baseline and 3, 6, 9, and 12 months thereafter.

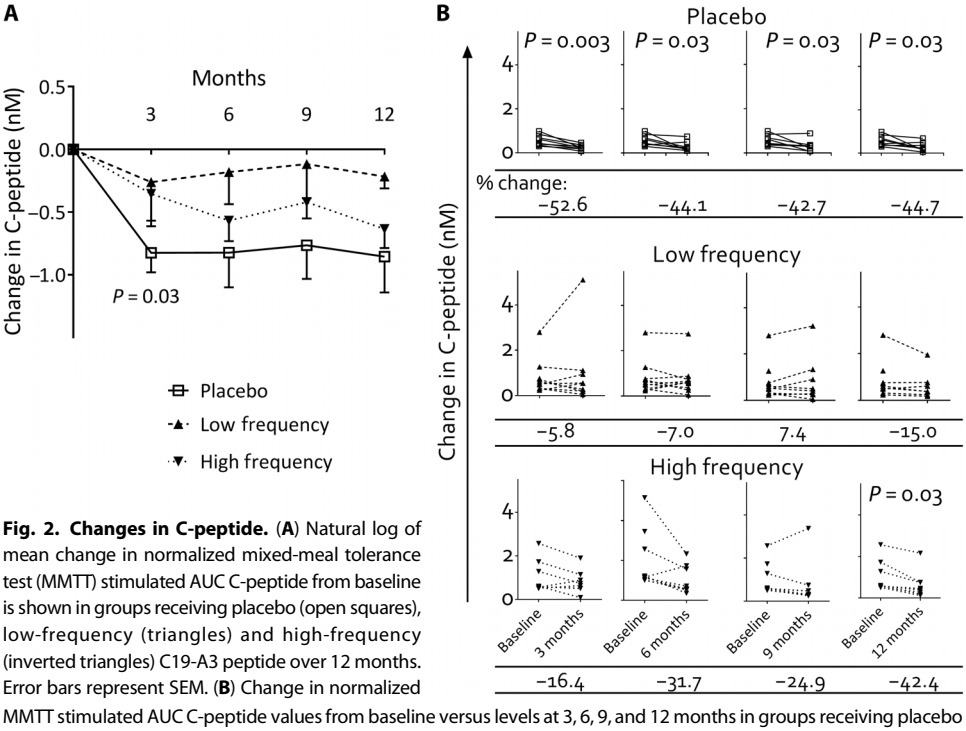

Fig. 2. Changes in C-peptide. (**A**) Natural log of mean change in normalized mixed-meal tolerance test (MMTT) stimulated AUC C-peptide from baseline is shown in groups receiving placebo (open squares), low-frequency (triangles) and high-frequency (inverted triangles) C19-A3 peptide over 12 months. Error bars represent SEM. (**B**) Change in normalized MMTT stimulated AUC C-peptide values from baseline versus levels at 3, 6, 9, and 12 months in groups receiving placebo (open squares), low-frequency (triangles), and high-frequency (inverted triangles) C19-A3 peptide over 12 months. Comparisons were made using paired *t* tests. The mean % change was calculated at each time point for each study group.

subject (apart from one subject, at 6, 9, and 12 months), and means declined significantly ($P = 0.003$, $P = 0.03$, $P = 0.03$, and $P = 0.03$ at 3, 6, 9, and 12 months, respectively) in paired analyses compared to baseline. This contrasted with findings in the treatment groups, in which the mean percent change was more modest, fewer individual subjects showed actual loss of C-peptide, and significant changes in means were only seen when comparing baseline with 12-month levels in the high-frequency group ($P = 0.03$). Thus, in this study, patients on placebo manifest an early decline of measurable C-peptide production, although this is not seen during administration of proinsulin C19-A3 peptide injections.

Changes in insulin use and HbA1c

Other potential effects of proinsulin PIT on metabolic responses were assessed by changes in insulin use during the study. Mean change in average insulin dose (unit kg^{-1} day^{-1}) showed a progressive rise in subjects in the placebo arm (Fig. 3A and tables S3 and S4). In contrast, there was no significant change in average insulin dose in the high- and low-frequency arms of the study. As a result, mean changes in insulin use were significantly lower in the high-frequency arm at 6, 9, and 12 months ($P = 0.03$, $P = 0.04$, and $P = 0.01$, respectively) and significantly lower in the low-frequency arm at 12 months ($P = 0.009$) compared with placebo, with an overall difference between the treatment and placebo groups across all time points in MMRM analysis ($P = 0.01$).

The study was designed to manage glycemic control intensively with a target HbA1c of less than 48 mmol/mol (6.5%). Therefore, differences in HbA1c between study groups would not be expected, and significant changes were not seen; however, there was a trend for HbA1c levels to increase over time in the placebo group, whereas in the treatment groups, there was a trend for values to decline and then stabilize after 6 months (Fig. 3B and tables S5 and S6). To examine the combined impact of changes in HbA1c and insulin usage on metabolic control, we examined the IDAA1c according to the formula of Mortensen *et al.* (*17*). IDAA1c increased significantly over 12 months in the placebo group compared with baseline ($P = 0.04$; Fig. 3C), consistent with a decline in endogenous insulin production, but was maintained at baseline levels in the intervention groups, consistent with C-peptide preservation. IDAA1c values were significantly lower in the high-frequency arm at baseline and 3, 6, 9, and 12 months ($P = 0.02$, $P = 0.001$, $P = 0.003$, $P = 0.01$, and $P = 0.002$, respectively) and significantly lower in the low-frequency arm at 6 and 12 months ($P = 0.047$ and $P = 0.01$, respectively) compared with placebo.

Stratified T cell responses, autoantibodies, and proinsulin/C-peptide ratio

We examined additional markers representing β cell stress and effector, regulatory, functional, and phenotypic features of global and antigen-specific adaptive immune responses. Over the duration

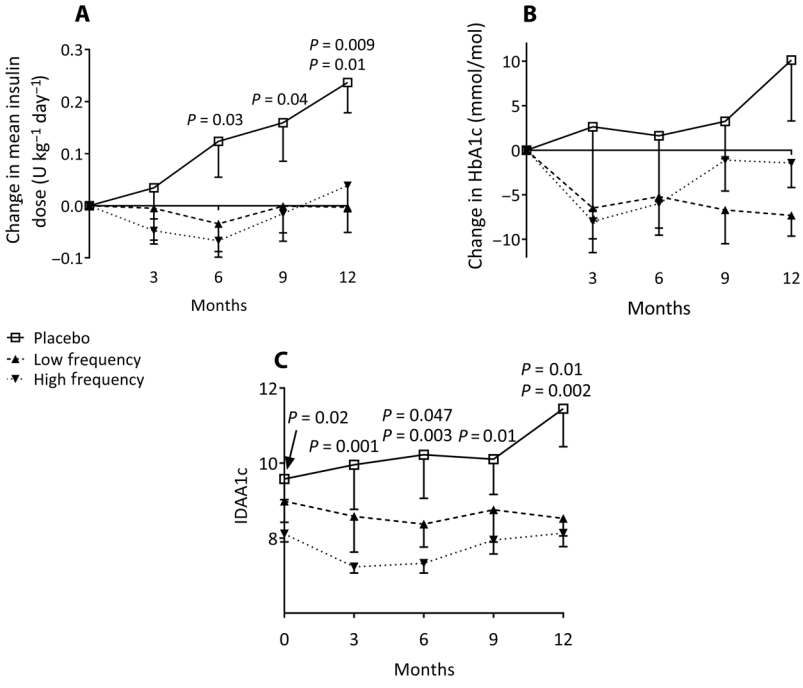

Fig. 3. Changes in insulin use and HbA1c. (**A**) Mean change in average insulin dose, (**B**) mean change in HbA1c, and (**C**) mean change in insulin dose–adjusted HbA1c (IDAA1c) values from baseline over 12 months are shown in groups receiving placebo (open squares), low-frequency (triangles), and high-frequency (inverted triangles) C19-A3 peptide over 12 months. Error bars indicate SDs.

of the treatment period, cumulative CD4 T cell IL-10 responses to proinsulin stimulation were significantly higher in the blood of the high-frequency compared with placebo ($P = 0.015$) and low-frequency groups ($P = 0.003$; Fig. 4A). There were no significant differences in CD4 T cell interferon-γ (IFN-γ) responses to proinsulin, circulating subsets of T_{regs} or activated CD8 T cells specific for β cell target peptides between these groups.

To provide further mechanistic insight, we also performed analyses on subjects divided according to the approach validated by Beam *et al.* (*18*) in which response to treatment is defined as a post-baseline value that is 100% or more of the baseline value of C-peptide AUC. There were 10 such "C-peptide responder" subjects identifiable during the treatment period (6 months)—1 in the placebo, 6 in the low-frequency, and 3 in the high-frequency groups. C-peptide responders were significantly more frequent in the low-frequency group than in the placebo group at 3 months ($P = 0.03$).

We next measured immune changes in peptide-treated C-peptide responders/nonresponders. We found a statistically significant difference between these groups over the duration of the treatment period ($P = 0.0029$). Higher levels of IL-10 responses appear to have been maintained in the responder group, and subsequent Bonferroni-adjusted, month-wise testing indicates that at 2, 5, and 6 months, IL-10 responses against proinsulin were specifically, significantly higher in peptide-treated C-peptide responders ($P = 0.007$, $P = 0.047$, and $P = 0.03$, respectively) (Fig. 4B and table S7). Peptide-treated C-peptide responders (but not nonresponders) showed a trend for IFN-γ response levels against proinsulin to decline between starting therapy and the first assay performed at 1 month ($P = 0.08$) (Fig. 4C).

Extending these studies to the high-dimensional analysis of T_{regs}, we noted an increase in levels of T_{reg} expression of FoxP3, the master transcriptional regulator of these cells, during the treatment period (between baseline and 3 months) in peptide-treated C-peptide responders (Fig. 4D, fig. S2, and table S8). Levels returned to baseline at 12 months and were unchanged throughout the study in peptide-treated C-peptide nonresponders. The greatest fold change in FoxP3 expression was seen in $CD45RA^-$ (memory) T_{reg} subpopulations that lacked Helios expression, especially those coexpressing CD39 (Fig. 4, E and F). In contrast, Helios expression levels on T_{regs} did not change in either study group (Fig. 4G). The proportion of CD8 T cells specific for β cells that expressed the marker of antigen experience ($CD57^+$) was significantly lower in treated C-peptide responders compared to placebo and nonresponders at baseline ($P = 0.01$ and $P = 0.04$, respectively) and remained lower than placebo at 6 months (Fig. 4H, fig. S3, and table S9). As observed in our previous studies in new-onset type 1 diabetes patients (*8*), detectable T cell responses to C19-A3 at baseline were present in a small minority of patients; significant treatment- and responder-related changes were not observed (table S10). There were no treatment- or response-related changes in autoantibodies or enzyme-linked immunospot (ELISPOT) responses to the control recall antigen (tables S11 and S12).

To examine whether peptide-treated C-peptide responders/nonresponders differ by additional metabolic markers, we also measured the proinsulin/C-peptide ratio during the MMTT, high levels of which are an indicator of β cell stress. We observed that both fasting and 90-min proinsulin/C-peptide ratio are significantly higher compared to baseline at multiple time points in peptide-treated C-peptide nonresponders (for fasting, $P = 0.03$, $P = 0.004$, and $P = 0.02$ at 3, 6, and 12 months, respectively; for 90-min, $P = 0.004$, $P = 0.008$, $P = 0.03$, and $P = 0.047$ at 3, 6, 9, and 12 months, respectively) (Fig. 5, A and B, and table S13). No change over time was observed in peptide-treated C-peptide responders, consistent with there being less β cell stress in this group (Fig. 5, C and D).

DISCUSSION

The principle that simple administration of antigens that are targeted in inflammatory diseases, such as autoimmunity and allergy, can have a therapeutic benefit has been borne out by many robust studies in preclinical models, as well as by more recent indications of success in the clinic (*15*, *19*–*21*). Our group has developed a distinctive approach to this in type 1 diabetes, through HLA-guided identification of naturally processed and presented epitopes of major autoantigens, such as proinsulin, that can be developed for PIT (*8*, *22*). The current phase 1b study was designed to explore safety (notably the risks of hypersensitivity and acceleration of loss of β cell function) and examine immunological effects of repeated dosing with such a native peptide sequence at the point of diagnosis of type 1 diabetes. We find that this approach is very well tolerated by patients even with dosing every 2 weeks for 6 months with no evidence of development of hypersensitivity.

We also find no evidence for accelerated loss of C-peptide secretion as an indicator of augmented β cell damage. Early C-peptide loss after diagnosis was apparent and significant in the placebo group but much less so in either of the treated groups, and C-peptide loss was significantly lower in the high-frequency group at 3 months. These results should be viewed with caution because C-peptide measurements can

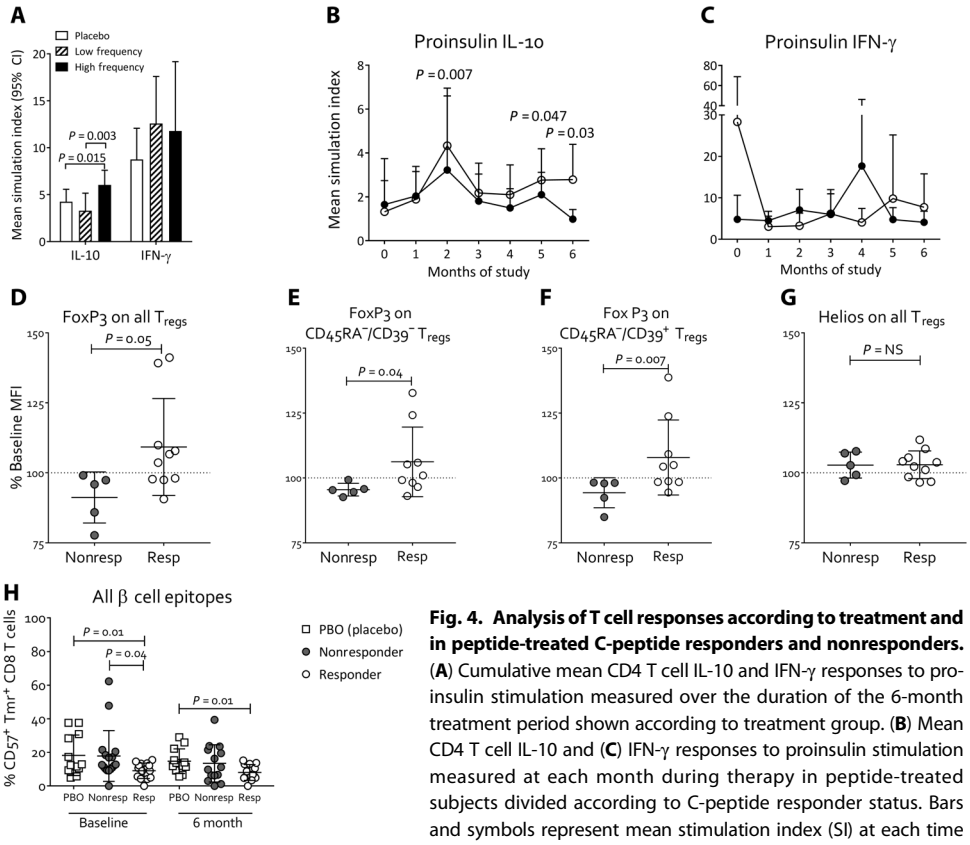

Fig. 4. Analysis of T cell responses according to treatment and in peptide-treated C-peptide responders and nonresponders. (**A**) Cumulative mean CD4 T cell IL-10 and IFN-γ responses to proinsulin stimulation measured over the duration of the 6-month treatment period shown according to treatment group. (**B**) Mean CD4 T cell IL-10 and (**C**) IFN-γ responses to proinsulin stimulation measured at each month during therapy in peptide-treated subjects divided according to C-peptide responder status. Bars and symbols represent mean stimulation index (SI) at each time point, and error bars are the 95% confidence intervals (CI). For analysis over the treatment period, longitudinal measurements of the SI were transformed using the natural logarithm ("Ln") and were analyzed with linear models having visit and treatment as main factors and a repeated-measures error structure. Estimates of the mean SI across visits were computed using model-based estimates (least-squares means). (**D**) Change in FOXP3 expression levels [mean fluorescence intensity (MFI)] on all T_{reg} subsets ($CD4^+CD25^{hi}FOXP3^+$), (**E**) on memory ($CD45RA^-$) adaptive T_{regs}, and (**F**) on memory $CD39^+$ T_{regs} in peptide-treated subjects divided according to C-peptide responder status. (**G**) Change in Helios expression by T_{regs} in the same period and same groups. (**H**) Mean percentage levels of antigen-experienced ($CD57^+$) CD8 T cells stained with peptide-HLA tetramers loaded with β cell peptides at baseline and at 6 months in peptide-treated C-peptide responders, compared with placebo and nonresponder subjects. Error bars show means and SEM. (B to H) C-peptide responders/nonresponders defined as having a post-baseline value that is 100% or more of the baseline value of C-peptide AUC during the treatment period. There were 9 peptide-treated C-peptide responders (6 of 9 subjects in the low-frequency and 3 of 7 in the high-frequency groups) and 10 nonresponders.

be variable, there were small numbers of subjects in each group with some imbalance between groups in baseline metabolic data (Table 1), and the study was not powered to examine efficacy, which would require many more subjects. However, patients receiving proinsulin PIT showed stable daily insulin use, compared with rising use in the placebo group. Stable insulin use in the treatment groups was not associated with poorer glycemic control; IDAA1c levels fell or stabilized, compared with an overall increase in the placebo group. Both treatment groups (high and low frequency) showed similar behavior in relation to C-peptide, insulin use, and HbA1c stabilization, consistent with a treatment effect. Although more frequent dosing was also safe, it did not appear to confer additional effects.

In exploratory analyses, we used validated criteria (*18*) to define a group of clinical responders by their retention of stimulated C-peptide secretion during the treatment period and found such subjects to be enriched in the peptide-treated groups. Note that these peptide-treated C-peptide responders/nonresponders also differed according to changes in proinsulin/C-peptide ratio during the study. Under normal conditions, very small amounts of proinsulin are secreted, but stressed β cells release more relative to mature insulin/C-peptide, due to endoplasmic reticulum dysfunction (*23*). Thus, the circulating proinsulin/C-peptide ratio is a measure of β cell stress, typically showing a rise shortly after diagnosis (*24*, *25*) followed by reduction later in the disease (*26*). Our data can be interpreted as indicating that peptide-treated C-peptide responders have less β cell stress compared to nonresponders. Heterogeneity of response to treatment has been recognized in other intervention studies in type 1 diabetes, and understanding its underlying basis is important for maximizing therapeutic effects. Differences in the T cell response to proinsulin according to treatment group were also observed over the course of the current study, and there was a trend for several important differences in immunological responsiveness to emerge between responder/nonresponder groups. First, in relation to immune regulation, we observed a higher level of IL-10 responses to proinsulin in association with high-frequency treatment and trends for higher IL-10 responses in peptide-treated responders. There have been numerous reports that ASI and PIT induce IL-10 responses and that this is a key component of the therapeutic mechanism, although other mechanisms, including effects on conventional $FoxP3^+CD25^{high}$ T_{regs}, have also been observed in preclinical models (*21*). Linked to this, our finding of a higher fold change in T_{reg} expression of FoxP3 in peptide-treated responders is of considerable interest, because it was most marked in a population of memory T_{regs} coexpressing CD39, which is associated with controlling inflammation via IL-10 secretion (*27*). Moreover, the memory subsets markedly up-regulating FoxP3 expression were Helios-negative, suggesting that they are peripherally generated, adaptive T_{regs} arising after treatment (*28*). It is proposed that autoantigen-specific CD4 T cells with immunoregulatory properties are induced and suppress bystander inflammatory responses to the same epitope, autoantigen, or related autoantigens being presented in cis by the same antigen-presenting cells (APCs) (*14*). In an extension of this effect, there is also evidence that under these conditions APCs are licensed to induce new cohorts of T_{regs} ("infectious tolerance") (*29*). It is tempting to speculate that administration of C19-A3 has resulted in the generation of $IL\text{-}10^+$ proinsulin-specific CD4 T cells and/or adaptive T_{regs} through infectious tolerance and that this response is causally related to the C-peptide retention observed in selected subjects, but this remains to be formally shown. It is also intriguing to note that a single subject in the placebo group is a C-peptide responder by the same criteria. Adequately powered, observational studies on similar subjects, using similar

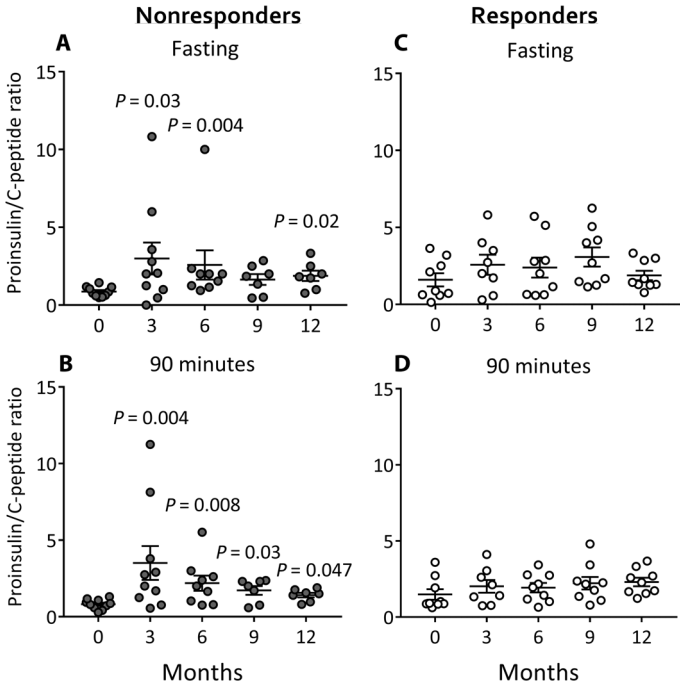

Fig. 5. Analysis of proinsulin/C-peptide ratio in peptide-treated C-peptide responders and nonresponders. Proinsulin/C-peptide ratio measured at fasting and at 90 min during the MMTT in peptide-treated subjects who are C-peptide nonresponders (**A** and **B**) and responders (**C** and **D**). *P* values are for comparisons against the corresponding baseline. Error bars show means and SEM.

immune and metabolic biomarker assays, will be required to explore the underlying basis for this nonprogressor phenotype.

Why some peptide-treated subjects should respond whereas others do not is a common conundrum of the immunotherapy field. We have previously shown that a distinguishing feature of type 1 diabetes is the presence of circulating β cell–specific effector memory CD8 T cells that show evidence (CD57 expression) of recent antigen exposure (*30*). We found that baseline levels of this subset were low in treated C-peptide responders, raising the intriguing possibility that patients with restricted activation of autoreactive cytotoxic T lymphocytes represent a disease stratum that is more permissive to the immune regulatory effects inducible by PIT.

Our study extends experience with ASI and PIT in type 1 diabetes and provides further evidence that it has a very favorable safety profile, especially by comparison with biologic agents that carry the risk of acute toxicities, such as cytokine storm and circulatory compromise, as well as chronic effects, such as increased infection risk. The safety signal in PIT is coupled with strong evidence against any deleterious effect on β cell function. In combination, these two features make this an appealing strategy for prevention, both in stage 1 disease (defined as the presymptomatic presence of β cell autoimmunity evidenced by two or more islet autoantibodies with normoglycemia) and in those identified early in life as being at high genetic risk (*31*).

In summary, our study demonstrates that PIT using proinsulin peptide appears safe and well tolerated, even when administered over several months and during the autoinflammatory process that is associated with the immediate period after diagnosis of type 1 diabetes. Two-weekly dosing does not appear to confer any benefit over 4-weekly dosing. However, the major restriction of our study is the small number of subjects enrolled. Combined with disease heterogeneity, this limits opportunities to better understand dosing and identify robust immunological effects and biomarkers. Future studies will need to be powered for these and for efficacy, should examine whether children are similarly responsive, and begin to explore opportunities for prevention.

MATERIALS AND METHODS

Study design

A schematic representation of this randomized, double-blind, placebo-controlled phase 1b study is shown in Fig. 1 and fig. S1. Five UK centers screened a total of 84 patients. Inclusion criteria were as follows: age (18 to 45 years of age), <100 days from diagnosis of type 1 diabetes (dated from day of first insulin injection), *HLA-DRB1*0401* genotype, islet autoantibody positivity [one of glutamic acid decarboxylase antibody (GADAb), insulinoma-associated antigen-2 antibody (IA-2Ab), or zinc transporter 8 antibody (ZnT8Ab)], and stimulated C-peptide >0.2 nM at any point during a 2-hour MMTT. Main exclusion criteria were use of immunosuppressive or immunomodulatory therapies, immunization with live or killed vaccinations or allergic desensitization procedures less than 1 month before first treatment, recent participation in other research trials of immunomodulatory agents, pregnancy, and breast feeding.

The three-group study design aimed to provide at least 16 patients on active treatment and 8 subjects in the placebo group to achieve sufficient data to inform future study designs and future sample size calculations. The study was not intended to show a statistically significant difference between the control and treatment groups and was not powered to do so. Twenty-seven subjects were randomized into three groups: high frequency (n = 9, who received 10-μg proinsulin C19-A3 peptide every 2 weeks), low frequency (n = 10, 10-μg proinsulin C19-A3 every 4 weeks), or placebo (n = 8, 50-μl 0.9% saline every 2 weeks). To ensure subject and physician blinding, the low-frequency group received 0.9% saline injections at 2-week intervals between peptide dosing. C19-A3 or saline was delivered as a 50-μl intradermal injection in the upper arm.

Subjects received a total of 12 injections over a 6-month period followed by a 6-month observation period. Patients were routinely monitored for a minimum of 1 hour after each injection for acute adverse effects. Glycemic control was intensively managed in all subjects with a target HbA1c of less than 48 mmol/mol (6.5%), with a record of average total daily insulin use in the previous 2 days documented at each visit.

Proinsulin C19-A3 peptide (GSLQPLALEGSLQKRGIV) was manufactured to good manufacturing practice (GMP) standards by the interdivisional GMP facility of the Leiden University Medical Center and prepared and supplied as lyophilized peptide by Nova Laboratories.

Laboratory measures of hematological indices, liver function, thyroid-stimulating hormone, urea, creatinine, calcium, lipid levels, and immunoglobulin levels were performed at baseline, 3, 6, 9, and 12 months. Any local skin reactions to dose administration were monitored until resolving and <1 cm in diameter. Safety data were under regular review by an independent Data Safety Monitoring Board.

Primary end point was an assessment of the safety of proinsulin C19-A3 peptide administration; secondary end points were assessments of changes in (i) stimulated C-peptide production after MMTT [measured as AUC as previously described (*32*)], (ii) level or quality of T lymphocyte biomarkers of β cell–specific immune response, (iii) level or quality of islet cell autoantibody biomarkers of β cell–specific immune response, and (iv) insulin use and HbA1c, at 3, 6, 9, and 12 months versus baseline and between groups.

Ethics statement

This study was carried out with the approval of the UK National Research Ethics Service, and written informed consent was obtained from all participants. The trial was conducted in compliance with the principles of the Declaration of Helsinki (1996) and the principles of Good Clinical Practice and in accordance with all applicable regulatory requirements including but not limited to the Research Governance Framework and the Medicines for Human Use (Clinical Trial) Regulations 2004, as amended in 2006. Further details are available on https://clinicaltrials.gov/ (NCT01536431).

Immunological and metabolic assays

Analysis of autoreactive proinflammatory (IFN-γ^+) and regulatory (IL-10^+) CD4 T cells was carried out by ELISPOT assay using fresh heparinized blood obtained at first injection and monthly thereafter until the last assay was performed 2 weeks after the last injection. Samples were coded to blind the laboratory as to the dosing regimen. Peripheral blood mononuclear cells (PBMCs; 10^6) were cultured with study drug [proinsulin C19-A3 peptide (10 μg/ml)], recombinant human proinsulin (Biomm; 10 μg/ml), Pediacel (a pentavalent vaccine comprising pertussis, diphtheria, *Haemophilus influenzae B*, polio, and tetanus toxoid vaccines; Sanofi Pasteur MSD; 1 μl/ml), or control diluent for 48 hours, and cytokine secretion measured by indirect assay (U-CyTech) according to the manufacturer's instructions. Data are expressed as SI calculated as the mean number of spots per triplicate for test condition divided by the mean number of spots per triplicate in the presence of diluent alone. The assay has significant discriminative ability for type 1 diabetes in blinded proficiency testing (*33*). GADAb, IA-2Ab, and ZnT8Ab were measured by enzyme-linked immunosorbent assay (RSR Ltd.) according to the manufacturer's instructions. Stimulated C-peptide was measured using MMTTs at baseline and 3, 6, 9, and 12 months (*32*). Briefly, Ensure Plus was administered at 6 ml/kg to fasting patients, and serum C-peptide levels were analyzed using a two-site chemiluminescent assay (Invitron) at −10, 0, 15, 30, 60, 90, and 120 min.

Immunophenotyping of T_{regs} was performed on cryopreserved PBMCs at baseline and 6 and 12 months in batches (each comprising all three visit samples from four subjects selected at random). Thawed PBMCs were stained with LIVE/DEAD Blue (Thermo Fisher Scientific) and then surface-stained using anti-CD4–APC-Cy7 (RPA-T4), anti-CD25–PE (phycoerythrin) (2A3 and M-A251), anti-CD27–BV605 (L128), anti-CD39–PE-Cy7 (A1; BioLegend), anti-CD45RA–PE-CF594 (HI100), anti-CD278–BV711 (DX29), and anti-HLA-DR–BV786 (G46-6; all BD Biosciences unless specified) and intracellularly stained using anti-Ki67–fluorescein isothiocyanate (B56; BD Biosciences), anti-FOXP3–Alexa Fluor 647 (259D/C7; BD Biosciences), and anti-Helios–Pacific Blue (22F6; BioLegend) for data acquisition using a BD Biosciences LSR Fortessa. Each data file was randomly subsampled to 12,500 T_{regs} and scaled using inverse hyperbolic sine (arcsinh) transformation with a cofactor of 150. Automated, unsupervised clustering analysis with Euclidean distance metric and $k = 100$ was performed for CD25, CD45RA, CD27, HLA-DR, CD39, ICOS, Ki67, FOXP3, and Helios using the PhenoGraph algorithm (*34*), identifying 20 clusters. Where indicated, independent analysis of manually gated populations was also performed using Cytobank software version 5.6.0 or FlowJo version 10.

$CD57^+$ (antigen-experienced) effector memory β cell peptide–specific CD8 T cells were detected using peptide-HLA-A*0201 tetramers loaded with preproinsulin 15–24, insulin B chain 10–18, and IA-2 797–805, as previously described (*30*), and expressed as a percentage of the parent tetramer population. Identical populations of CD8 T cells specific for common viral peptides CMVpp65 495–503, Epstein-Barr virus BMLF-1 280–288, and influenza matrix 58–66 were measured as controls. For calculation of the proinsulin/C-peptide ratio, serum analytes were measured by chemiluminescence (Invitron Ltd.).

Calculation of IDAA1c

IDAA1c is a surrogate measure of β cell function (*17*) and is calculated according to the following formula: HbA1c (%) + [4 × insulin dose (units per kilogram per day)].

Statistical analysis

For analysis of metabolic changes, comparisons against baseline and between treatment groups were made initially using MMRM analysis adjusted for baseline value for all data points, followed by exploratory analyses using Student's *t* tests for paired and unpaired samples, Mann-Whitney *U* test, and Wilcoxon matched pairs test, which were also used for immune marker comparisons. For analysis of immune changes detected by ELISPOT analysis over the treatment period, longitudinal measurements of the SI were transformed using the natural logarithm (Ln) and were analyzed with linear models having visit and treatment as main factors and a repeated-measures error structure. Estimates of the mean SI across visits were computed using model-based estimates ("least-squares means"). These statistical analyses were conducted with SAS version 9.4 (SAS Institute). For comparison of T_{reg} cluster frequencies and mean expression levels between groups and time points, one-way ANOVA (analysis of variance) with Tukey post hoc correction was performed in MATLAB R2016b. $P < 0.05$ was considered significant.

SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/9/402/eaaf7779/DC1

Fig. S1. Enrollment, randomization, and follow-up of study subjects.

Fig. S2. Representative flow cytometry gating strategy for T_{reg} subsets.

Fig. S3. Representative flow cytometry gating strategy for antigen-specific CD8 T cells.

Table S1. Trial primary and secondary end points.

Table S2. Normalized C-peptide AUC at baseline and 3, 6, 9, and 12 months after initiation of treatment.

Table S3. Percentage change in daily insulin use (unit/kilogram) at baseline and 3, 6, 9, and 12 months after initiation of treatment.

Table S4. Change in daily insulin use (unit/kilogram) at baseline and 3, 6, 9, and 12 months after initiation of treatment.

Table S5. Percentage change in HbA1c (mmol/mol) at baseline and 3, 6, 9, and 12 months after initiation of treatment.

Table S6. Change in HbA1c (mmol/mol) at baseline and 3, 6, 9, and 12 months after initiation of treatment.

Table S7. In vitro response to proinsulin (or diluent control) according to C-peptide response status in peptide-treated subjects.

Table S8. Percentage change in T_{reg} FOXP3 and Helios MFI from baseline at months 3 and 12.

Table S9. Percentage of $CD57^+$ β cell–specific CD8 T cells at baseline and 6 months after initiation of treatment.

Table S10. In vitro response to proinsulin peptide C19-A3.

Table S11. Autoantibody levels at baseline and 6 and 12 months.

Table S12. In vitro response to recall antigen according to treatment group and C-peptide response.

Table S13. Analysis of proinsulin/C-peptide ratio in peptide-treated C-peptide responders and nonresponders.

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Metabolic and immune effects of immunotherapy with proinsulin peptide in human new-onset type 1 diabetes

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Peptide therapy prompts responses in diabetes

Immunotherapy using peptides has been successful for some patients with allergies, but has not yet been deployed in autoimmune diseases, which may involve greater safety risks. Alhadj Ali *et al*. designed a placebo-controlled trial to determine whether a proinsulin peptide could safely elicit immune and metabolic responses in people recently diagnosed with type 1 diabetes without accelerating disease. This small trial showed that treatment seemed to modify T cell responses and did not interfere with residual β cell function. In contrast to subjects in the placebo arm, treated subjects did not need to increase their insulin use. These encouraging results support a larger trial to investigate efficacy of the peptide therapy for treating disease.