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Paper:

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

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One Sentence Summary:

Intradermal administration of up to 12 doses over 6 months of the HLA-DR4 restricted proinsulin peptide epitope, C19-A3, to adults with new onset type 1 diabetes is safe and in some subjects is associated with changes in immune regulation.

Abstract:

Immunotherapy using short immunogenic peptides of disease-related autoantigens restores immune tolerance in preclinical disease models. We studied safety and mechanistic effects of injecting HLA-DR4(*B1*0401*)-restricted immunodominant proinsulin-peptide intradermally 2- or 4-weekly for 6-months in newly-diagnosed type 1 diabetes. 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, while no significant change was seen in the 4-weekly peptide group at these timepoints or the 2-weekly group at 3, 6 and 9-months. Placebo group daily insulin use increased by 50 per cent over 12-months, but remained unchanged in the intervention groups. C-peptide retention in treated subjects associated with proinsulin-stimulated IL-10 production, increased FoxP3 expression by Tregs, low baseline levels of activated β-cell-specific CD8 T-cells as well as 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 non-specific immune modulation.

150 words

Introduction

Type 1 diabetes is a chronic autoimmune disease characterized by progressive, immune-mediated loss of β-cell mass and function. Following 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 hyperglycaemia and hyperglycaemic 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 approximately 4 per cent per year in recent decades, most notably in children and adolescents (*4*). Despite over 25 years of efforts to develop immunomodulatory therapies, 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 pro-inflammatory 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 relate to conventional, FoxP3+CD25^{hi} Tregs, 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*). These findings promote consideration of antigen-specific immunotherapy (ASI) as an approach for type 1 diabetes, since 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 whole antigens that might trigger IgE-mediated hypersensitivity; and it is also under development in autoimmune inflammatory conditions such as coeliac disease and multiple sclerosis (*15*). We have previously described how administration of a peptide representing an immunodominant region of proinsulin presented by the 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*). In the present study, therefore, we examined the proinsulin mono-peptide immunotherapy approach in adults ascertained within 100 days of type 1 diabetes diagnosis and with residual C-peptide in order to examine safety and tolerability in a relevant therapeutic setting, and study early indications of mechanistic and metabolic effects.

Results

Study enrolment and randomization

Of the 233 patients referred to the study sites, 84 were assessed for eligibility and attended screening visits. Of these, 56 subjects either did not possess the *HLA-DRB1*0401* genotype or autoantibodies and 1 subject had stimulated C-peptide <0.2 nmol/L; all were excluded (Supplementary Figure S1). After 24 subjects had been randomized, subjects who did not complete a minimum of 11 out 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 endpoints are shown in **Supplementary Table 1.**

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

Subjects enrolled were treated according to the regime in **Figure 1** and the participant flow is summarized in **Supplementary Figure S1**. Peptide injection was very well tolerated with no serious advents 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 8/9, 10/10, 4/8 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 pre-determined analysis plan, C-peptide AUC was compared between the treatment groups over time (3, 6, 9 and 12 months) by multilevel model repeated measures (MMRM) analysis adjusted for baseline value of AUC, and no significant treatment-related effects were observed. Importantly, 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 which are worthy of discussion. The decline in stimulated C-peptide was different between study groups, and at the 3 month timepoint mean loss of C-peptide in the placebo group exceeded that of the high frequency (p=0.03) and low frequency groups (**Figure 2A**). This difference in C-peptide decline was evident in individual data plots (**Figure 2B and Supplementary Table 2**): compared with baseline, C-peptide levels in subjects receiving placebo showed a decline at every timepoint in every subject (apart from one subject, at 6, 9 and 12 months) and mean values declined significantly 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 mean values were only seen when comparing baseline with 12 month levels in the high frequency group. Thus in this study, patients on placebo manifest an early decline of measurable C-peptide production, whilst this is not seen during administration of proinsulin C19-A3 peptide injections.

Changes in insulin use and HbA1c

Other potential effects of proinsulin peptide immunotherapy on metabolic responses were assessed by changes in insulin use during the study. Mean change in average insulin dose (unit/kg/day) showed a progressive rise in subjects in the placebo arm (**Figure 3A and Supplementary Tables 3-4**). 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 multilevel model repeated measures analysis (p = 0.01).

The study was designed to manage glycaemic control intensively with a target HbA1c of less than 48mmol/mol (6.5%). Differences in HbA1c between study groups would not be expected, therefore, and significant changes were not seen; however, there was a trend for increased HbA1c levels in the placebo group and in the treatment groups initial trends for values to decline and then stabilize/increase after 6 months (**Figure 3B and Supplementary Tables 5-6).** To examine the combined impact of changes in HbA1c and insulin usage on metabolic control, we examined the insulin dose adjusted HbA1c (IDAA1c) according to the formula of Mortensen et al (*17*). IDAA1c increased over 12 months in the placebo group (p=0.04; **Figure 3C**), consistent with a decline in endogenous insulin production, but was maintained at baseline levels in the intervention groups, consistent with C-peptide preservation.

Proinsulin:C-peptide ratio, autoantibodies and T cell responses according to treatment group and responder/non-responders.

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 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; **Figure 4A**). There were no significant differences in CD4 T cell IFN-γ responses to proinsulin, circulating subsets of regulatory T cells (Tregs) or activated CD8 T cells specific for β-cell target peptides between these groups.

To provide further mechanistic insight, analyses were also performed 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 group, 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). To examine whether peptide-treated C-peptide responders/non-responders differ by additional metabolic markers, we 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-minute proinsulin/C-peptide ratio are significantly higher compared to baseline at multiple timepoints in peptide-treated C-peptide non-responders (**Figure 5A,C**). No change over time was observed in peptide-treated C-peptide responders, consistent with there being less β-cell stress in this group (**Figure 5B,D**).

We next measured immune changes in peptide-treated C-peptide responders/non-responders. We found a statistically-significant difference between these groups at some of the months (p=0.0029). Higher levels of IL-10 responses appear to have been maintained in the responder group. Subsequent Bonferroni-adjusted, month-wise testing indicates that at 2 months, IL-10

responses against PI were specifically, significantly higher in peptide-treated C-peptide responders (p=0.007) (**Figure 4B and Supplementary Table 7**). Peptide-treated C-peptide responders (but not non-responders) showed a trend for IFN-γ response levels against PI to decline between starting therapy and the first assay performed at 1 month (p=0.08) (**Figure 4C**). Extending these studies to the high-dimensional analysis of Tregs, we noted an increase in levels of Treg expression of FoxP3, the master transcriptional regulator of these cells, during the treatment period (between baseline and 6 months) in peptide-treated C-peptide responders (**Figure 4D**). Levels returned to baseline at 12 months and were unchanged throughout the study in peptide-treated C-peptide non-responders. Of interest, the greatest fold-change in FoxP3 expression was seen in CD45RA- (memory) Treg sub-populations that lacked Helios expression, especially those co-expressing CD39 (**Figure 4E-F**). In contrast, levels of Helios expression by Tregs did not change in either study group (**Figure 4G**). The proportion of CD8 T cells specific for β-cells that expressed the disease-associated activated (CD57+) effector memory phenotype was significantly lower in treated C-peptide responders compared to placebo and non-responders at baseline, and remained lower than placebo at 6 months (**Figure 4H**). As observed in our previous studies in new onset type 1 diabetes patients, 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. There were no treatment- or response-related changes in autoantibodies or ELISPOT responses to the control recall antigen.

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 Ib 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.

Importantly, 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 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; insulin adjusted HbA1c 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, HbA1c stabilization, consistent with a treatment effect. While 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. It is noteworthy that these peptide-treated C-peptide responders/non-responders also differed according to changes in proinsulin:C-peptide (PI:C) 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 PI:C 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 non-responders. 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/non-responder 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} Tregs have also been observed in preclinical models (21). Linked to this, our finding of a higher fold-change in Treg expression of FoxP3 in peptide-treated responders is of considerable interest, since it was most marked in a population of memory Tregs co-expressing CD39, which is associated with controlling inflammation via IL-10 secretion (27). Moreover, the memory subsets markedly upregulating FoxP3 expression were Helios-negative, suggesting that they are peripherally-generated, adaptive Tregs arising post-treatment. 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 cell (APCs) (14). In an extension of this effect, there is also evidence that under these conditions APCs are licensed to induce new cohorts of regulatory T cells ("infectious tolerance") (28). It is tempting to speculate that administration of C19-A3 has resulted in the generation of IL-10+ proinsulin-specific CD4 T cells and/or adaptive Tregs through infectious tolerance and that this response is causally related to the C-peptide retention observed in selected subjects. Why some subjects should respond whilst 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 (29). 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 cements the view 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 normoglycaemia) and in those identified early in life as being at high genetic risk (30). In summary, our study demonstrates that PIT using proinsulin peptide appears safe and well tolerated, even when administered over several months and during the auto-inflammatory process that is associated with the immediate period following diagnosis of type 1 diabetes. Two-weekly dosing does not appear to confer any benefit over 4-weekly dosing. Future studies will need to be powered 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 **Figure 1** and **Supplementary Figure 1**. Five UK centres screened a total of 84 patients. Inclusion criteria were: age 18-45 years; <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 >0.2nmol/L 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 prior to 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 randomised into 3 groups: high frequency (n=9, who received 10µg proinsulin C19-A3 peptide every 2 weeks), low frequency (n=10, 10µg proinsulin C19-A3 every 4 weeks) or placebo (n=8, 50µl 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 50µl 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. Glycaemic control was intensively managed in all subjects with a target HbA1c of less than 48mmol/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 Leiden University Medical Center (Leiden, the Netherlands) and prepared and supplied as lyophilized peptide by Nova Laboratories (Leicester, UK).

Laboratory measures of haematological 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 <1cm in diameter. Safety data were under regular review by an independent Data Safety Monitoring Board.

Primary endpoint was assessment of the safety of proinsulin C19-A3 peptide administration; secondary endpoints were assessments of changes in i) stimulated C-peptide production after MMTT (measured as area under the curve as previously described (31)); 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 U.K. 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), 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 available on https://clinicaltrials.gov/ (NCT01536431).

Immunological and metabolic assays

Analysis of autoreactive pro-inflammatory (interferon-γ+ (IFN-γ+)) and regulatory (interleukin-10+, (IL-10+)) CD4 T cells was carried out by enzyme-linked immunospot (ELISPOT) assay using fresh heparinized blood obtained at 1st 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 dosing regimen. Peripheral blood mononuclear cells (PBMCs; 10^6) were cultured with study drug (proinsulin C19-A3 peptide 10µg/ml, recombinant human proinsulin (Biomm, Brazil; 10µg/ml), Pediacel (pentavalent vaccine comprising pertussis, diphtheria, *Haemophilus influenza B*, polio and tetanus toxoid vaccines; Sanofi Pasteur MSD, Maidenhead, UK; 1µl/ml) or control diluent for 48 hours and cytokine secretion measured by indirect assay (U-CyTech, Utrecht, the Netherlands) according to the manufacturer's instructions. Data are expressed as the mean number of spots per triplicate and compared with the mean spot number in the presence of diluent alone (stimulation index; SI). The assay has significant discriminative ability for type 1 diabetes in blinded proficiency testing (*32*) and inter-assay coefficient of variation for the spot number for both the IFN-γ and IL-10 assays were 12.3% and 10.7%, respectively. GADAb, IA-2Ab and ZnT8Ab were measured by ELISA (RSR Ltd, Cardiff, UK) according to the manufacturer's instructions. Stimulated C-peptide was measured using mixed meal tolerance

tests at baseline, 3, 6, 9 and 12 months (*31*). Briefly, Ensure Plus® was administered at 6ml/kg to fasting patients and serum C-peptide levels analyzed using a two-site chemiluminescent assay (Invitron, Monmouth, UK) at -10, 0, 15, 30, 60, 90 and 120 minutes.

Immunophenotyping of Tregs was performed on cryopreserved PBMCs at baseline, 6 and 12 months in batches (each comprising all 3 visit samples from 4 subjects selected at random). Thawed PBMCs were live/dead blue (Thermo Fisher Scientific, London, UK) and then surface stained using anti-CD4-APC-Cy7 (RPA-T4), anti-CD25-PE (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, Oxford, UK unless specified) and intracellularly stained using anti-Ki67-FITC (B56; BD), anti-FOXP3-Alexa Fluor 647 (259D/C7; BD Biosciences) and anti-Helios-Pacific blue (22F6; BioLegend, San Diego) for data acquisition using a BD Biosciences LSR Fortessa. Each data file was randomly subsampled to 12,500 Treg cells 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 (*33*), identifying 20 clusters. Where indicated, independent analysis of manually gated populations was also performed.

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 (*29*) and expressed as a percentage of the parent tetramer population. Identical populations of CD8 T cells specific for common viral peptides CMVpp65 495-503, EBV 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, UK).

Calculation of insulin dose adjusted HbA1c

Insulin dose adjusted HbA1c (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 multilevel model repeated measures (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; 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 V9.4 (SAS Institute, Cary, NC). For comparison of Treg cluster frequencies and mean expression levels between groups and timepoints one-way ANOVA analysis with Tukey post hoc correction was performed in MATLAB® R2016b. P values <0.05 were considered significant.

List of supplementary materials

Supplementary Figure S1. Enrolment, randomization and follow-up of study subjects.
Supplementary Table 1. Trial primary and secondary endpoints
Supplementary Table 2: Normalised C-peptide area under curve (pmol/mL/min) at baseline and 3, 6, 9 and 12 months after initiation of treatment. Percentage change in AUC from baseline is shown in shaded columns. Missing data is indicated by a crossed through cell with subsequent percentage changed marked as not applicable (NA).
Supplementary Table 3: Daily insulin use (unit/Kg) at baseline and 3, 6, 9 and 12 months after initiation of treatment. Percentage change in insulin use from baseline is shown in shaded columns. Missing data is indicated by a crossed through cell with subsequent percentage changed marked as not applicable (NA).
Supplementary Table 4: Daily insulin use (Unit/Kg) at baseline and 3, 6, 9 and 12 months after initiation of treatment. Change in insulin use (Unit/Kg) from baseline is shown in shaded columns. Missing data is indicated by a crossed through cell with subsequent percentage changed marked as not applicable (NA).
SupplementaryTable 5: HbA1c (mmol/mol) at baseline and 3, 6, 9 and 12 months after initiation of treatment. Percentage change in HbA1c from baseline is shown in shaded columns. Missing data is indicated by a crossed through cell with subsequent percentage changed marked as not applicable (NA).
Supplementary Table 6: HbA1c (mmol/mol) at baseline and 3, 6, 9 and 12 months after initiation of treatment. Change in HbA1c (mmol/mol) from baseline is shown in shaded columns. Missing data is indicated by a crossed through cell with subsequent percentage changed marked as not applicable (NA).
Supplementary Table 7. Analysis of in vitro response to proinsulin (or diluent control) according to C-peptide response status in peptide-treated subjects. Data are median spots/million peripheral blood mononuclear cells

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Figure Legends

Figure 1 Legend. 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 placebo (saline injections every 2 weeks), high (10µg proinsulin C19-A3 peptide every 2 weeks), or low (10µg proinsulin C19-A3 peptide every 4 weeks alternating with saline every 4 weeks) frequency active treatment 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.

Figure 2 Legend. Changes in C-peptide.

A. Natural Log of mean change in normalised mixed meal tolerance test (MMTT) stimulated AUC C-peptide from baseline is shown in groups receiving placebo (open squares), low (triangle) and high (inverted triangle) frequency C19-A3 peptide over 12 months. There was decline in stimulated C-peptide in the placebo arm; the change at 3 months significantly exceeded that in the high frequency arm (*; p=0.03). Error bars represent standard error of the mean. **B.** Change in normalised MMTT stimulated AUC C-peptide values from baseline versus level at 3, 6, 9 and 12 months in groups receiving placebo (open squares), low (triangle) and high (inverted triangle) frequency C19-A3 peptide over 12 months. There is a significant reduction in the stimulated C-peptide level in the placebo group at each time point. No significant decline was seen in the low and high frequency groups during the treatment phase; during follow-up a significant change in the high frequency group was seen at 12 months compared to baseline. Comparisons were made using paired t-tests. The mean % change was calculated at each time point for each study group.

Figure 3 Legend. Changes in insulin use and HbA1c.

A. Mean change in average insulin dose from baseline is shown in groups receiving placebo (open squares), low (triangle) and high (inverted triangle) frequency C19-A3 peptide over 12

months. There was a progressive increase in insulin requirement in the placebo arm. In contrast there was a reduction or stabilization in the low and high frequency groups, with significant differences in change compared with placebo in the high frequency group at month 6 (*; p=0.03), month 9 (*; p=0.04) and month 12 (*p=0.01) and low frequency group at month 12 (†; p=0.009). Error bars indicate standard deviations. **B.** Mean change in HbA1c from baseline is shown in groups receiving placebo (open squares), low (triangle) and high (inverted triangle) frequency C19-A3 peptide over 12 months. There were no significant changes within groups and no significant differences between groups. **C.** Insulin dose adjusted HbA1c (IDAA1c) values following treatment were significantly lower in the high frequency arm at baseline, 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=0.05 and p=0.01, respectively) compared with placebo. IDAA1c values increased in the placebo group during the study and were significantly higher at 12 months compared with baseline (‡p=0.04). Error bars indicate standard deviations.

Figure 4. Analysis of T cell responses according to treatment and in peptide-treated C-peptide responders and non-responders.

A. CD4 T cell IL-10 responses to proinsulin stimulation were significantly higher in the blood of the high frequency (open bars) compared with placebo (filled bars; *p=0.015) and low frequency groups (hatched bars; †p=0.003) whilst IFN-γ responses were not different between treatment groups. **B.** In relation to treatment response, across the treatment period IL-10 responses to proinsulin stimulation (after adjusting for baseline) were different between C-peptide responder/non-responder groups at some visits (p=0.0029) and rose and, specifically, were significantly higher in the peptide-treated C-peptide responder group compared with non-responders at month 2 (p=0.007). **(C)** Peptide-treated C-peptide responders (but not non-responders) showed a trend for IFN-γ response levels against PI to decline between starting therapy and the first assay performed at 1 month (*p=0.08), however no significant difference was determined overall (p=0.3385). Bars and symbols represent mean stimulation index at each

timepoint and error bars are the 95% confidence intervals. (**D**) In peptide-treated C-peptide responders expression levels (MFI, mean fluorescence intensity) of FoxP3 across all Treg subsets increased significantly between baseline and 6 months compared with non-responders, in whom levels did not change. (**E**) The most notable change in FoxP3 expression levels was seen in memory (CD45RA-) adaptive Tregs (Helios-negative), (**F**) especially those expressing CD39. (**G**) Helios expression by Tregs did not change in the same period. (**H**) Antigen-experienced (CD57+) CD8 T cells stained with peptide-HLA tetramers loaded with β-cell peptides were significantly lower at baseline in peptide-treated C-peptide responders, compared with placebo and non-responder subjects and remained different to placebo at 6 months. Error bars show mean and SEM. Panels B-H include C-peptide responders/non-responders 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 responder and 10 non-responder subjects (6/3 in the low frequency and 3/7 in the high frequency groups).

Figure 5. Analysis of Proinsulin/C-peptide ratio in peptide-treated C-peptide responders and non-responders.

Proinsulin/C-peptide ratio measured fasting (**A,B**) and at 90 minutes (**B,C**) during the MMTT in peptide-treated subjects who are C-peptide non-responders (**A,C**) and responders (**B,D**). p values are for comparisons against the corresponding baseline. Error bars show mean and SEM.

Table 1. Baseline characteristics of the study subjects

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

Notes: [1] p=0.02 versus placebo; [2] AUC=area under the curve

Inclusion Criteria:
-Age 18-45 years
-HLA-DRB1*0401 genotype
-1 out of 3 autoantibodies
positive (GAD, IA2 or ZnT8)
-Stimulated C-peptide on MMTT
>0.2 nmol/L

High Frequency

Low Frequency

Placebo

100 days from diagnosis

Screening phase

3

6

9

Months
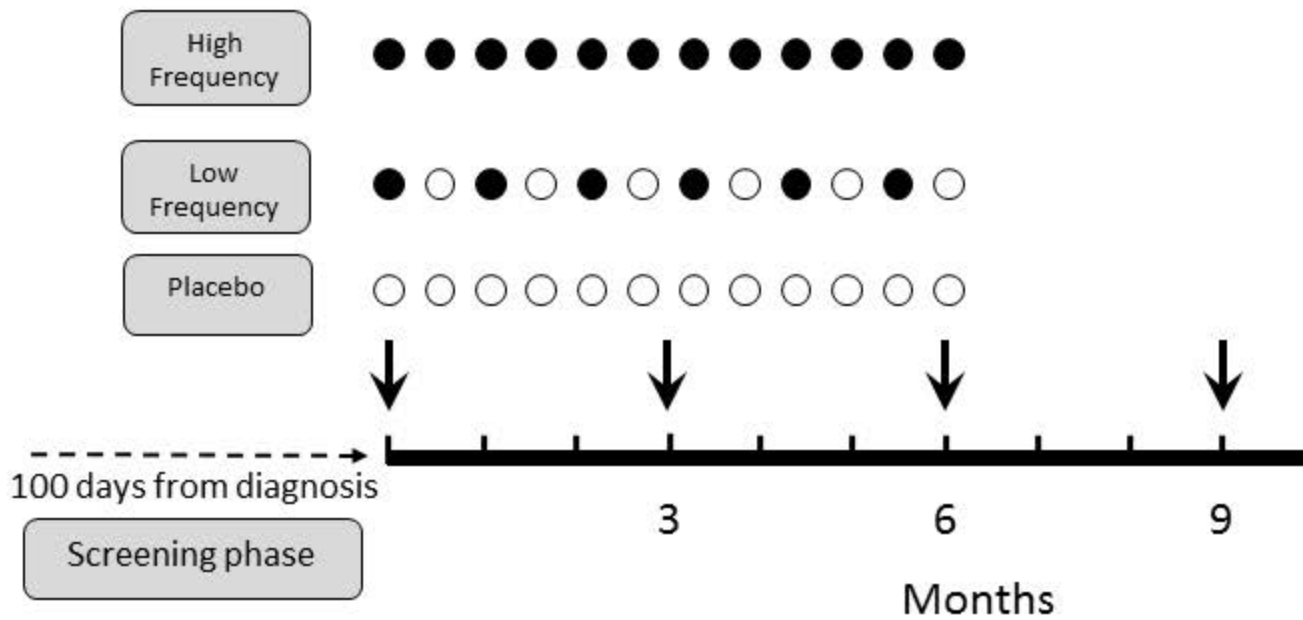
Key:
○ Intradermal placebo injection
● Intradermal C19-A3 injection
↓ Mixed meal tolerance test

A

Mean simulation index (95% CI)

IL-10   IFN-γ

† *

B

Proinsulin IL-10

p=0.007

Mean simulation index

Months of study

C

Proinsulin IFN-γ

*

Months of study

D

Fo...

% change in MFI

Non-Respon...

E

FoxP3 on
CD45RA-/CD39- Tregs

p=0.04

% change in MFI

Non-Responder   Responder

F

Fox P3 on
CD45RA-/CD39+ Tregs

p=0.007

Non-Responder   Responder

G

Helios on all Tregs

p=NS

Non-Responder   Responder

H

All β-cell

p=0.01

p=0.04

% CD57+ PPI+ CD8 T cells

Placebo   Non-Responder   Responder   Pl...

Baseline

Non-responder

A   Fasting

B   Responder — Fasting

C   90 minutes

D   90 minutes

Supplementary Table 1. Trial primary and secondary endpoints

Primary endpoint	• Assessment of the safety of C19-A3 peptide administration in subjects with new-onset Type 1 diabetes.
Secondary endpoints	• Change in stimulated C-peptide production at 3, 6, 9 and 12 months versus baseline and between groups. • Change in level or quality of T lymphocyte biomarkers of β-cell specific immune response at 3, 6, 9 and 12 months versus baseline and between groups. • Change in level or quality of islet cell autoantibody biomarkers of β-cell specific immune response at 3, 6, 9 and 12 months versus baseline and between groups. • Change in glycated haemoglobin (as measured by % HbA1c levels), daily insulin usage, and mean amplitude of glucose excursions[1] at 3, 6, 9 and 12 months versus baseline and between groups.

Supplementary Figure 1

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                           ┌─────────────────────────┐
                           │ 233 potentially eligible│
                           │   patients identified   │
                           └────────────┬────────────┘
                                        │
                                        ▼
                           ┌─────────────────────────┐
                           │ 84 attended screening   │
                           │          visit          │
                           └────────────┬────────────┘
                                        │            ┌──────────────────────────────────────┐
                                        │            │ 57 excluded:                         │
                                        ┆┄┄┄┄┄┄┄┄┄┄▶│ 42 not HLA-DR B1*0401                │
                                        │            │ 6 negative for autoantibodies        │
                                        │            │ 8 neither genotype nor autoantibodies│
                                        ▼            │ 1 peak C-peptide <0.2 nmol/l         │
                           ┌─────────────────────────┐└──────────────────────────────────────┘
                           │     27 randomised       │
                           │     (Including 3        │
                           │     replacements)       │
                           └────────────┬────────────┘
```

8 placebo	10 low frequency	9 high frequency
8 completed all assessments	9 completed all assessments	7 completed all assessments
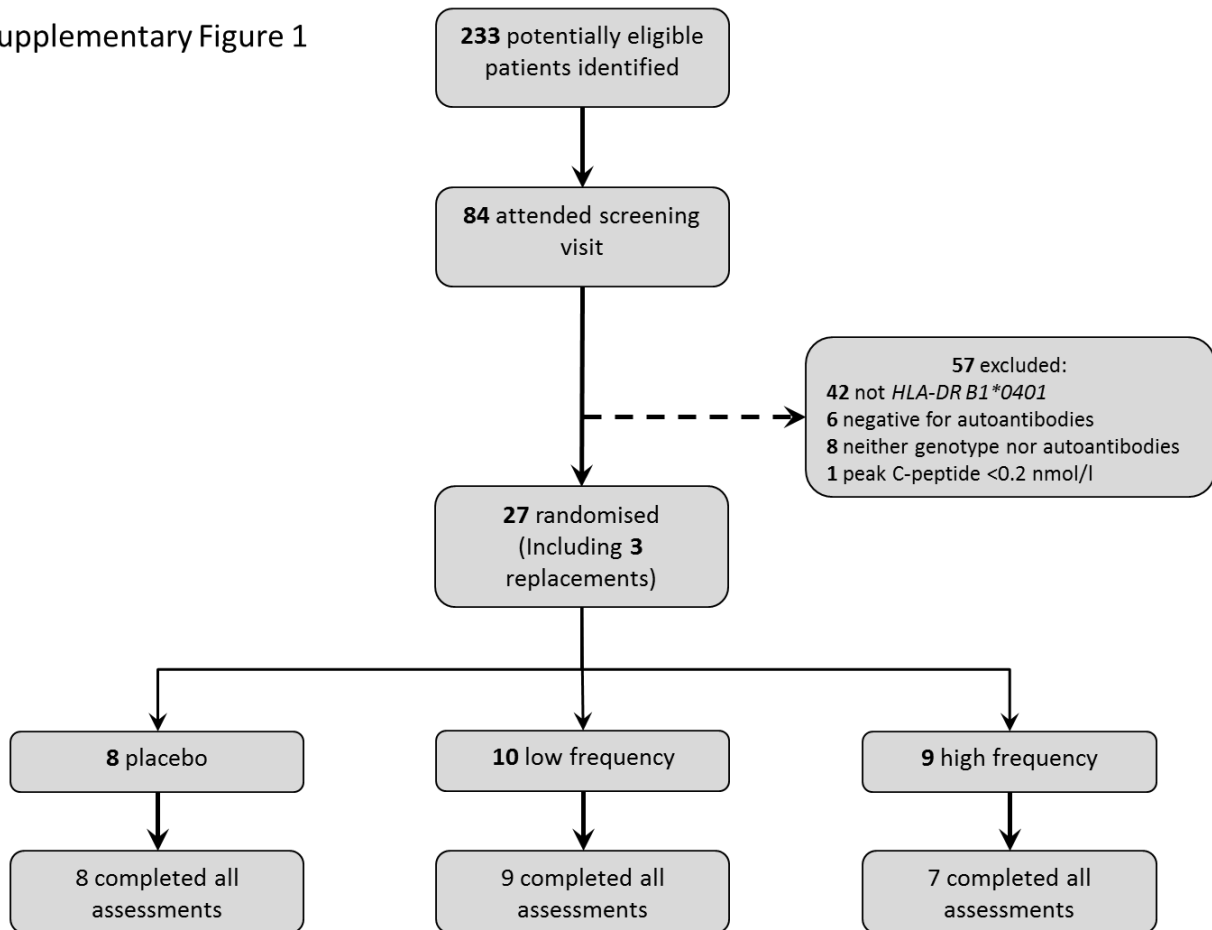
Supplementary Figure S1. Enrolment, randomization and follow-up of study subjects.
Between January 2012 and March 2014, a total of 233 subjects were identified and referred to the study sites. Out of the 233 subjects, 84 attended screening visits. Fifty-seven out of 84 subjects were excluded from the study: 42 did not possess *HLA-DRB1*0401* genotype, 6 subjects lacked autoantibodies; 1 subject had a stimulated C-peptide level <0.2 nmol/L and 8 lacked the *HLA-DRB1*0401* genotype and autoantibodies. Twenty-seven subjects were randomized in 3 study arms and 24 subjects completed all study assessments.

	Patient No.	Baseline C-peptide AUC	Month 3 C-peptide AUC	% change from baseline at Month 3	Month 6 C-peptide AUC	% change from baseline at Month 6	Month 9 C-peptide AUC	% change from baseline...
HIGH-FREQUENCY	1017	0.51	0.64	24.70	0.26	-49.82	0.26	-4
	1019	2.57	1.89	-26.31	1.16	-54.74	3.38	31
	2010	1.28	0.74	-42.19	0.78	-38.87	0.74	-4
	2011	1.72	1.14	-34.02		NA		N
	2017	0.50		NA	0.29	-42.64	0.30	-4
	2021	0.58	0.50	-13.25	0.87	49.30	0.31	-4
	2025	0.58	0.51	-12.20	0.28	-52.47	0.48	-1
	3015	0.56	0.88	57.87	0.36	-36.30	0.48	-1
	4005	0.61	0.08	-86.11	0.18	-70.72	0.25	-5
LOW-FREQUENCY	1024	0.32	0.20	-36.11	0.61	90.50	0.25	-1
	2001	0.62	0.28	-53.90	0.56	-9.32	0.51	-1
	2006	1.27	1.12	-12.46	0.73	-42.74		N
	2016	0.31	0.05	-83.89	0.05	-83.89	0.05	-8
	2018	0.53	0.56	5.38	0.37	-29.95	0.41	-2
	2020	0.72	0.19	-73.52	0.26	-63.28		N
	2027	0.49	0.95	94.03	0.66	36.37	0.91	87
	2031	2.80	5.12	83.06	2.74	-1.97	3.21	14
	4007	0.76		NA	0.88	15.26	1.35	76
	5001	0.24	0.55	129.83	0.62	158.74	0.29	23
PLACEBO	1008	0.30	0.19	-37.20	0.11	-63.10	0.09	-7
	1025	0.36	0.08	-77.30	0.50	39.60	0.30	-1
	2019	0.44	0.20	-53.44	0.19	-56.24	0.30	-3
	3002	0.99	0.23	-76.29	0.10	-89.93	0.21	-7
	3005	0.68	0.36	-46.57	0.26	-61.20	0.37	-4
	3012	0.85	0.47	-44.94	0.75	-11.68	0.89	4
	4001	0.62	0.24	-61.39	0.18	-70.87	0.08	-8
	4012	0.40	0.30	-23.80	0.24	-39.70	0.33	-1

Supplementary Table 2: Normalised C-peptide area under curve (pmol/mL/min) at baseline and 3, 6, 9 and 12 months after initiation of tr... is shown in shaded columns. Missing data is indicated by a crossed through cell with subsequent percentage changed marked as not appl...

	Patient No.	Baseline Insulin use	Month 3 Insulin use	% change from baseline at Month 3	Month 6 Insulin use	% change from baseline at Month 6	Month 9 Insulin use	% chang baseline a 9
HIGH-FREQUENCY	1017	0.29	0.26	-8.67	0.38	31.05	0.31	7.8
	1019	0.40	0.39	-2.62	0.38	-5.38	0.37	-9.1
	2010	0.27	0.20	-26.53	0.12	-54.49	0.14	-47.
	2011	0.39		NA		NA	0.60	55.5
	2017	0.22	0.28	24.36	0.23	3.63	0.28	24.7
	2021	0.28	0.13	-51.89	0.14	-48.22	0.17	-38.
	2025	0.36	0.21	-40.91	0.19	-46.97	0.22	-37.
	3015	0.19	0.20	5.90	0.11	-42.60	0.17	-9.8
	4005	0.33	0.29	-13.05	0.26	-21.19	0.33	1.1
LOW-FREQUENCY	1024	0.47	0.32	-31.89	0.29	-37.73	0.19	-59.
	2001	0.18	0.19	0.58	0.22	21.48	0.29	56.
	2006	0.57	0.36	-37.24	0.24	-57.70		N/
	2016	0.66	1.11	67.25	0.89	35.01	0.94	42.
	2018	0.43	0.48	12.90	0.47	10.31	0.36	-15.
	2020	0.18	0.19	4.46	0.31	69.49		N/
	2027	0.17	0.16	-10.48	0.12	-27.80	0.11	-37.
	2031	0.33	0.16	-50.54	0.17	-48.38	0.19	-40.
	4007	0.55	0.42	-24.71	0.42	-23.75	0.49	-12.
	5001	0.22	0.35	58.44	0.28	27.53	0.44	100.
PLACEBO	1008	0.62	0.58	-5.58	1.02	64.62	1.01	62.6
	1025	0.14	0.12	-14.21	0.20	39.19	0.20	36.6
	2019	0.27	0.24	-11.24	0.32	17.02	0.32	18.2
	3002	0.15	0.40	171.06	0.44	196.01	0.57	282.
	3005	0.5	0.43	-12.90	0.42	-15.62	0.41	-16.
	3012	0.54	0.58	8.50	0.61	13.55	0.57	5.9
	4001	0.65	0.46	-28.91	0.52	-20.02		N/
	4012	0.51	0.83	60.08	0.84	62.12	0.76	47.4

Supplementary Table 3: Daily insulin use (unit/Kg) at baseline and 3, 6, 9 and 12 months after initiation of treatment. Percentage chang
columns. Missing data is indicated by a crossed through cell with subsequent percentage changed marked as not applicable (NA).

	Patient No.	Baseline Insulin use	Month 3 Insulin use	Change from baseline at Month 3	Month 6 Insulin use	Change from baseline at Month 6	Month 9 Insulin use	Change from baseline at 9
HIGH-FREQUENCY	1017	0.29	0.26	-0.02	0.38	0.09	0.31	0.0
	1019	0.40	0.39	-0.01	0.38	-0.02	0.37	-0.0
	2010	0.27	0.20	-0.07	0.12	-0.15	0.14	-0.1
	2011	0.39		NA		NA	0.60	0.2
	2017	0.22	0.28	0.05	0.23	0.008	0.28	0.0
	2021	0.28	0.13	-0.14	0.14	-0.13	0.17	-0.1
	2025	0.36	0.21	-0.14	0.19	-0.17	0.22	-0.1
	3015	0.19	0.20	0.01	0.11	-0.08	0.17	-0.0
	4005	0.33	0.29	-0.04	0.26	-0.07	0.33	0.00
LOW-FREQUENCY	1024	0.47	0.32	-0.15	0.29	-0.17	0.19	-0.2
	2001	0.18	0.19	0.001	0.22	0.04	0.29	0.1
	2006	0.57	0.36	-0.21	0.24	-0.33		NA
	2016	0.66	1.11	0.44	0.89	0.23	0.94	0.2
	2018	0.43	0.48	0.05	0.47	0.04	0.36	-0.0
	2020	0.18	0.19	0.008	0.31	0.13		NA
	2027	0.17	0.16	-0.01	0.12	-0.04	0.11	-0.0
	2031	0.33	0.16	-0.16	0.17	-0.16	0.19	-0.1
	4007	0.55	0.42	-0.13	0.42	-0.13	0.49	-0.0
	5001	0.22	0.35	0.13	0.28	0.06	0.44	0.2
PLACEBO	1008	0.62	0.58	-0.03	1.02	0.40	1.01	0.3
	1025	0.14	0.12	-0.02	0.20	0.05	0.20	0.0
	2019	0.27	0.24	-0.03	0.32	0.04	0.32	0.0
	3002	0.15	0.40	0.25	0.44	0.29	0.57	0.4
	3005	0.5	0.43	-0.06	0.42	-0.07	0.41	-0.0
	3012	0.54	0.58	0.04	0.61	0.07	0.57	0.0
	4001	0.65	0.46	-0.18	0.52	-0.13		NA
	4012	0.51	0.83	0.31	0.84	0.32	0.76	0.2

Supplementary Table 4: Daily insulin use (Unit/Kg) at baseline and 3, 6, 9 and 12 months after initiation of treatment. Change in insulin columns. Missing data is indicated by a crossed through cell with subsequent percentage changed marked as not applicable (NA).

	Patient No.	Baseline HbA1c	Month 3 HbA1c	% change from baseline at Month 3 (mmol/mol)	Month 6 HbA1c	% change from baseline at Month 6 (mmol/mol)	Month 9 HbA1c	% change from baseline a (mmo
HIGH-FREQUENCY	1017	50	43	-14	55	10	62	2
	1019	56	36	-35.71	35	-37.5	37	-33
	2010	64	52	-18.75	54	-15.62	56	-12
	2011	45	36	-20		NA	55	22
	2017	48	45	-6.25	47	-2.08	50	4.
	2021	52	44	-15.38	42	-19.23	39	-2
	2025	41	42	2.43	39	-4.87	44	7.
	3015	52	43	-17.30	49	-5.76	50	-3.
	4005	57	52	-8.77	51	-10.52	62	8.
LOW-FREQUENCY	1024		48	NA	49	NA	49	N
	2001	52	40	-23.07	54	3.84	45	-13
	2006	38	43	13.15	51	34.21		N
	2016	77	99	28.57	82	6.49	90	16
	2018	75	53	-29.33	54	-28	56	-25
	2020	46		NA	55	19.56		N
	2027	53	41	-22.64	47	-11.32	49	-7.
	2031	47	39	-17.02	35	-25.53	41	-12
	4007	60	54	-10	39	-35	48	-2
	5001	78	59	-24.35	62	-20.51	66	-15
PLACEBO	1008	86	148	72.09	129	50	110	27
	1025	68	51	-25	47	-30.88	57	-16
	2019	61	62	1.63	67	9.83	67	9.
	3002	60	63	5	67	11.66	59	-1.
	3005	37	51	37.83	52	40.54	50	35
	3012	69	58	-15.94	55	-20.28	58	-15
	4001	58	47	-18.96	50	-13.79	65	12
	4012	61	41	-32.78	46	-24.59	60	-1.

SupplementaryTable 5: HbA1c (mmol/mol) at baseline and 3, 6, 9 and 12 months after initiation of treatment. Percentage change in H
Missing data is indicated by a crossed through cell with subsequent percentage changed marked as not applicable (NA).

	Patient No.	Baseline HbA1c	Month 3 HbA1c	Change from baseline at Month 3 (mmol/mol)	Month 6 HbA1c	Change from baseline at Month 6 (mmol/mol)	Month 9 HbA1c	Chang... baseline a... (mmo...
HIGH-FREQUENCY	1017	50	43	-7	55	5	62	1
	1019	56	36	-20	35	-21	37	-1
	2010	64	52	-12	54	-10	56	-
	2011	45	36	-9		NA	55	1
	2017	48	45	-3	47	-1	50	2
	2021	52	44	-8	42	-10	39	-1
	2025	41	42	1	39	-2	44	3
	3015	52	43	-9	49	-3	50	-
	4005	57	52	-5	51	-6	62	5
LOW-FREQUENCY	1024		48	NA	49	NA	49	N
	2001	52	40	-12	54	2	45	-
	2006	38	43	5	51	13		N
	2016	77	99	22	82	5	90	1
	2018	75	53	-22	54	-21	56	-1
	2020	46		NA	55	9		N
	2027	53	41	-12	47	-6	49	-
	2031	47	39	-8	35	-12	41	-
	4007	60	54	-6	39	-21	48	-1
	5001	78	59	-19	62	-16	66	-1
PLACEBO	1008	86	148	62	129	43	110	2
	1025	68	51	-17	47	-21	57	-1
	2019	61	62	1	67	6	67	6
	3002	60	63	3	67	7	59	-
	3005	37	51	14	52	15	50	1
	3012	69	58	-11	55	-14	58	-1
	4001	58	47	-11	50	-8	65	7
	4012	61	41	-20	46	-15	60	-

Supplementary Table 6: HbA1c (mmol/mol) at baseline and 3, 6, 9 and 12 months after initiation of treatment. Change in HbA1c (mmol/...
Missing data is indicated by a crossed through cell with subsequent percentage changed marked as not applicable (NA).

Month of study	C-peptide non-responder				C-peptide responder			
	IFN-γ		IL-10		IFN-γ		IL-10	
	Proinsulin	Control	Proinsulin	Control	Proinsulin	Control	Proinsulin	Control
0	4	2	4	7	5	2	3	4
1	5	11	2	4.5	14	37	7	5
2	6	5	3	3	10	6	9	3
3	3	5	0	1	3	2	2	3
4	31	2	1.5	4.5	3	3	2	9
5	4	4	5.5	12.5	4	4	3	9
6	3	6	0.5	5	10	6	3	5

Supplementary Table 7. Analysis of in vitro response to proinsulin (or diluent control) according to C-peptide response status in peptide-treated subjects. Data are median spots/million peripheral blood mononuclear cells