Immune and Metabolic Effects of Antigen-Specific Immunotherapy Using Multiple β-Cell Peptides in Type 1 Diabetes

Yuk-Fun Liu,[1,2,3] Jake Powrie,[2] Sefina Arif,[4] Jennie H.M. Yang,[4,5] Evangelia Williams,[4,5] Leena Khatri,[4,5] Mamta Joshi,[2] Loic Lhuillier,[4] Nikolaos Fountoulakis,[2] Emma Smith,[6] Craig Beam,[7] Anna Lorenc,[4] Mark Peakman,[1,2,3,4] and Timothy Tree[4,5]

Diabetes 2022;71:722–732 | https://doi.org/10.2337/db21-0728

Type 1 diabetes is characterized by a loss of tolerance to pancreatic β-cell autoantigens and defects in regulatory T-cell (Treg) function. In preclinical models, immunotherapy with MHC-selective, autoantigenic peptides restores immune tolerance, prevents diabetes, and shows greater potency when multiple peptides are used. To translate this strategy into the clinical setting, we administered a mixture of six *HLA-DRB1*0401*–selective, β-cell peptides intradermally to patients with recent-onset type 1 diabetes possessing this genotype in a randomized placebo-controlled study at monthly doses of 10, 100, and 500 μg for 24 weeks. Stimulated C-peptide (measuring insulin functional reserve) had declined in all placebo subjects at 24 weeks but was maintained at ≥100% baseline levels in one-half of the treated group. Treatment was accompanied by significant changes in islet-specific immune responses and a dose-dependent increase in Treg expression of the canonical transcription factor FOXP3 and changes in Treg gene expression. In this first-in-human study, multiple-peptide immunotherapy shows promise as a strategy to correct immune regulatory defects fundamental to the pathobiology of autoimmune diabetes.

Type 1 diabetes is a chronic autoimmune disease resulting in β-cell death and dependence on exogenous insulin. While insulin therapies are continually improving, the rising incidence of type 1 diabetes, particularly in young children (1) is exposing increasing numbers to the life-long burden of insulin treatment and risk of complications, such as hypoglycemia, ketoacidosis, retinopathy, neuropathy, and nephropathy. It is now well documented that preservation of C-peptide, even down to the limits of detection, is associated with superior metabolic control and protection against these severe complications (2,3). However, no approved immunological therapies that prevent the decline of β-cell function in the setting of this autoimmune disease currently exist, despite success in a small number of phase 2 clinical studies that have demonstrated that decline of β-cell function can be modified by depletion or inhibition of effector T and B cells (4–11). These studies provide important proofs of concept, which thus far have proven difficult to progress to the clinic. There are multiple explanations for this translational gap (12), including considerations of the benefit:risk ratio when immunotherapy is set against the established, relatively safe treatment of insulin replacement, arguing for

[1]Department of Diabetes, School of Life Course Sciences, King's College London, London, U.K.
[2]Department of Diabetes and Endocrinology, Guy's and St. Thomas' NHS Foundation Trust, London, U.K.
[3]Institute of Diabetes, Endocrinology and Obesity, King's Health Partners, London, U.K.
[4]Department of Immunobiology, School of Immunology and Microbial Sciences, King's College London, London, U.K.
[5]National Institute for Health Research Biomedical Research Centre, Guy's and St. Thomas' NHS Foundation Trust and Kings College London, London, U.K.
[6]UCB Celltech, Slough, U.K.
[7]Department of Biomedical Sciences, Homer Stryker MD School of Medicine, Western Michigan University, Kalamazoo, MI

Corresponding authors: Mark Peakman, mark.peakman@kcl.ac.uk, and Timothy Tree, timothy.tree@kcl.ac.uk

Received 16 August 2021 and accepted 15 January 2022

Clinical trial reg. no. NCT02620332, clinicaltrials.gov

This article contains supplementary material online at https://doi.org/10.2337/figshare.18628703.

innovative immune treatments with a high therapeutic index.

There is strong evidence that the loss of immune tolerance and poorly restrained effector T- and B-cell autoimmune attack on β-cells arise on a background of defective immune regulation. In particular, polymorphisms in genes involved in the development and function of $FOXP3^+$ regulatory T cells (Tregs) predispose to type 1 diabetes. In addition, multiple functional Treg defects have been documented in the disease setting (13–19). This understanding has focused attention on immunotherapeutic strategies designed to enhance immune regulation. One prominent example is antigen-specific immunotherapy (ASI) in which β-cell autoantigens are delivered via a route and regimen that favors immunological tolerance. We have developed an ASI approach that uses naturally processed β-cell peptide sequences known to be presented by the HLA class II molecule HLA-DR4, which is highly prevalent in patients with type 1 diabetes. In our previous studies, immunotherapy using a single proinsulin (PI) peptide sequence (C19-A3) was well tolerated and showed hints of efficacy and immune modulation (20), leading us to consider additional strategies to boost efficacy. Using a preclinical, humanized model of peptide immunotherapy, we have shown that combining several different β-cell peptides into a single injectable has a significantly enhanced impact compared with a monopeptide in inducing immune regulation associated with $FOXP3^+$ Treg expansion (21). These findings led us to develop a second-generation peptide immunotherapy drug for type 1 diabetes in which six β-cell peptides (three peptides derived from PI and three from antityrosine phosphatase–like IA2) are combined into a single vial that can be reconstituted to administer up to 0.5 mg in a single injection. We report the findings of the first-in-human study of this drug, which furthers experience to suggest that the peptide immunotherapy approach is safe and well tolerated in type 1 diabetes, as well as extends data that support a mechanism of action in which Tregs undergo treatment-induced changes in phenotype and gene expression.

RESEARCH DESIGN AND METHODS

Study Design

The study was a single-site, placebo-controlled, double-blind, randomized phase 1 study. Inclusion criteria were age 18–45 years, up to 4 years postdiagnosis of type 1 diabetes (classified from date of first insulin injection), *HLA-DRB1*0401* genotype, islet autoantibody positivity (one of GAD antibody, IA2 antibody, or zinc transporter 8 antibody), and stimulated C-peptide >0.2 pmol/mL at any point during a 2-h mixed-meal tolerance test (MMTT) performed within 35 days of random assignment. Main exclusion criteria were use of immunosuppressive or immunomodulatory therapies, immunization with live or killed vaccinations or allergic desensitization procedures <1 month before first treatment, recent participation in other research trials of immunomodulatory agents, pregnancy, and breastfeeding.

The treatment consisted of six naturally processed and presented peptides derived from the β-cell autoantigens PI and IA2 manufactured and supplied according to Good Manufacturing Practice by Almac Group and Symbiosis Pharmaceutical Services. Patients were randomly assigned to one of three arms using drug doses of 10 μg (n = 8), 100 μg (n = 6), and 500 μg (n = 6) or to a placebo arm (water for injection) (n = 6), with a placebo:drug ratio of 2:6 in each cohort. Each cohort had one dose every 4 weeks of drug or placebo administered intradermally for 24 weeks with a further 24-week follow-up period. Subjects were monitored for adverse effects, including hypersensitivity and local injection site reactions, for a minimum of 90 min. Laboratory measures of hematological and biochemistry indices, including full blood count, urea, creatinine, liver function, thyroid-stimulating hormone, calcium, immunoglobulins, and lipid profile, were taken 1 week after each dosing visit. Glycemic control was reviewed at every study visit, with a target HbA_{1c} <53 mmol/mol (7%). Average total insulin dose from the previous 2 days was recorded at treatment and follow-up visits. Stimulated C-peptide was assessed with an MMTT at screening and 24 weeks. An independent data monitoring committee regularly reviewed safety data.

The primary end point was assessment of safety and tolerability of drug in subjects with recent-onset type 1 diabetes. Secondary end points were change in metabolic status reflected by stimulated C-peptide areas under the curve (AUCs), HbA_{1c}, average insulin dose, and change in immune function assessed by T-cell biomarkers or islet-cell antibodies. A schematic diagram of the study design, including timing of treatments and evaluation of immunological and metabolic measurements is shown in Fig. 1.

Immune Assays

All immunological samples were coded to blind the laboratory as to the dosing regimen. Antigen-specific autoreactive $CD4^+$ T-cell responses were measured by enzyme-linked immune absorbent spot (ELISpot) assay to detect interferon-γ (IFN-γ), interleukin 17 (IL-17), and IL-10 cytokines. Fresh heparinized blood was obtained at baseline and every 4 weeks thereafter for 24 weeks. Peripheral blood mononuclear cells (PBMCs) (10^6 cells) were cultured with whole PI (10 μg/mL; Biomm) and PI C13-C32 (GGGPGAGSLQPLALEGSLQK), PI C19-A32 (GSLQPLALEGSLQKRGIV), PI C22-A5 (QPLALEGSLQKRGIVEQ), IA2 718–36 (AYQAEPNTCATAQGEGNIK), IA2 752–75 (KLKVESSPSRSDYINASPIIEHDP), and IA2 855–67 (YLKNVQTQETRTL) peptides (10 μg/mL; Thermo Fisher Scientific, representing regions of antigens present in the Multiple Islet Peptide Administration in Type 1 Diabetes [MultiPepT1De] study drug) and as positive assay control, with Infanrix-IPV+Hib (a pentavalent vaccine comprising pertussis, diphtheria, *Haemophilus influenzae* B, polio, and tetanus toxoid vaccines; GlaxoSmithKline UK Ltd.) or diluent

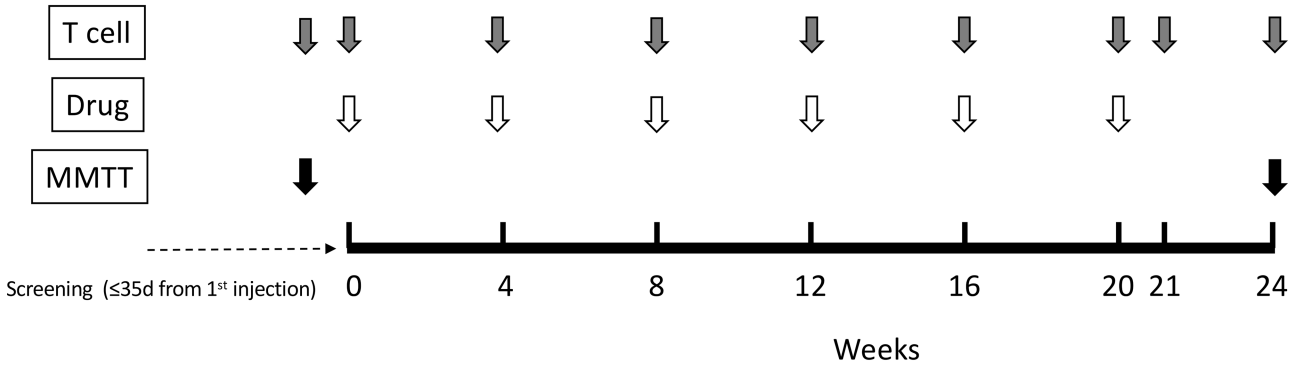

Figure 1—Study design and treatment groups. Graphical representation of study design showing timing of treatments and evaluation of immunological and metabolic measurements. Drug indicates study drug administration, and T cell indicates blood draw for immune readouts.

control for 48 h. Cellular cytokine secretion was measured by indirect ELISpot assay (U-CyTech biosciences) according to the manufacturer's instructions. Data are expressed as SI, which was 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 (22). GAD antibody, IA2 antibody, and zinc transporter 8 antibody were measured by ELISA (RSR Ltd.) according to the manufacturer's instructions. Stimulated C-peptide was measured using MMTTs at baseline and 12, 24, 36, and 48 weeks. 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 (Siemens) at −10, 0, 15, 30, 60, 90, and 120 min.

Analysis of Circulating Tregs

To identify changes in Treg phenotypes, cryopreserved PBMC samples from baseline, 12, and 24 weeks after start of treatment were thawed from all 24 subjects and stained first using live/dead yellow before surface marker staining followed by intracellular marker staining using antibodies and staining conditions detailed in Supplementary Table 2. FOXP3 transcription factor buffer (Thermo Fisher Scientific)–stained samples were acquired on a BD FACSymphony, and data were analyzed using FlowJo software (FlowJo, LCC) (example gating strategy is shown in Supplementary Fig. 1). Cytometer setup and tracking beads were run daily, and the same machine, with the same cytometer configuration and application settings, was used for the measurements of the samples throughout the study.

To study changes in Treg transcriptome, cryopreserved PBMC samples (10^6 cells) from baseline (1 or 2 weeks before first treatment) and 24 weeks after treatment were thawed from 10 subjects (5 MultiPepT1De-treated subjects with >1 and 5 placebo-treated subjects with ≤1 FOXP3 mean fluorescence intensity fold change in Tregs between baseline and 24 weeks; subject and sample details shown in Supplementary Table 3) and stained first using live/dead violet and then surface stained using antibodies listed in Supplementary Table 4. A minimum of 40,000 $CD14^-CD3^+CD4^+CD25^+CD127^{lo}$ Tregs (average 81,600, maximum 158,300) were sorted using BD FACSAria III and frozen at −80°C in minimal PBS before RNA extraction. RNA was extracted using the AllPrep DNA/RNA Micro Kit (QIAGEN), and RNA sequencing libraries were prepared from two-thirds of extracted RNA (91–140 ng) with SMART-Seq v4 and NuGen Ovation Ultralow System V2 and sequenced on a HiSeq 2500 system for 2 × 100 cycles. After quality control, reads were quantified by salmon 0.14.0 in quasi-mapping mode, with human cDNA reference from Ensembl (Homo_sapiens.GRCh38.cdna.all.fa.gz). Transcript-level counts were collapsed to gene level by mappings from the EnsDb.Hsapiens.v86 annotation package. For downstream analysis, we used 13,588 genes that had a mean expression across all individuals of at least five counts and were expressed in at least four individuals. Differentially expressed genes were identified with the package DESeq2, modeling expression as dependent on the individual and whether the sample was acquired after receiving active treatment. False discovery rate (FDR) was controlled with Benjamini-Hochberg procedure. Five genes had an adjusted q-value <10% and 28 <50%.

Ethics

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) 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.

Statistical Analysis

Measures of changes from each subject's baseline to the key follow-up points of 12 and 24 weeks were calculated using either the (absolute) difference or the (relative) ratio, depending on which represented the subjects' responses, which

were guided by the degree of skewness and variability in the data within and between subjects across time. The 95% CIs were reported for statistical comparisons of mean changes in stimulated C-peptide production, HbA_{1c}, and average daily insulin usage. Differences in change from baseline C-peptide AUC were estimated from an ANCOVA model that included treatment group and baseline as fixed effects. The least squares (LS) mean difference versus placebo and 95% CIs are presented. For analysis of immune changes detected by ELISpot analysis over the treatment period, longitudinal measurements of the SI were transformed using the natural logarithm and 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 (LS means). These statistical analyses were conducted with SAS 9.4 statistical software (SAS Institute). For comparison of Treg cluster frequencies and mean expression levels between groups and time points, one-way ANOVA with Tukey post hoc correction was performed in MATLAB R2016b. $P < 0.05$ was considered significant.

Data and Resource Availability

The data sets generated and/or analyzed during the current study are available from the corresponding authors upon reasonable request.

RESULTS

Screening, Enrollment, and Randomization

Of 100 eligible subjects who completed screening (Fig. 2), 48 negative for *HLA-DRB1*0401*, 3 negative for islet antibody, 7 negative for both genotype and islet antibodies, and 7 failing to reach a peak C-peptide of 0.2 nmol/L were excluded. Subjects enrolled were treated according to the regimen shown in Fig. 1. Three patients did not complete the study, missing at least one dosing visit because of changes in personal/employment circumstances, which did not allow for continued participation; these subjects were replaced with additional subjects. Data analyses excluded only a single subject, who withdrew before any dosing. Baseline characteristics, including insulin dose–adjusted HbA_{1c} and basal/peak-stimulated C-peptide values, are shown in Table 1. We observed no significant differences among any of these measures between treatment groups.

Safety

All patients who received at least one dose of study medication were included in the safety analysis. No serious adverse events were seen in drug treatment groups. Hypoglycemia was the most common adverse event; 270 events were reported in 20 subjects who received drug,

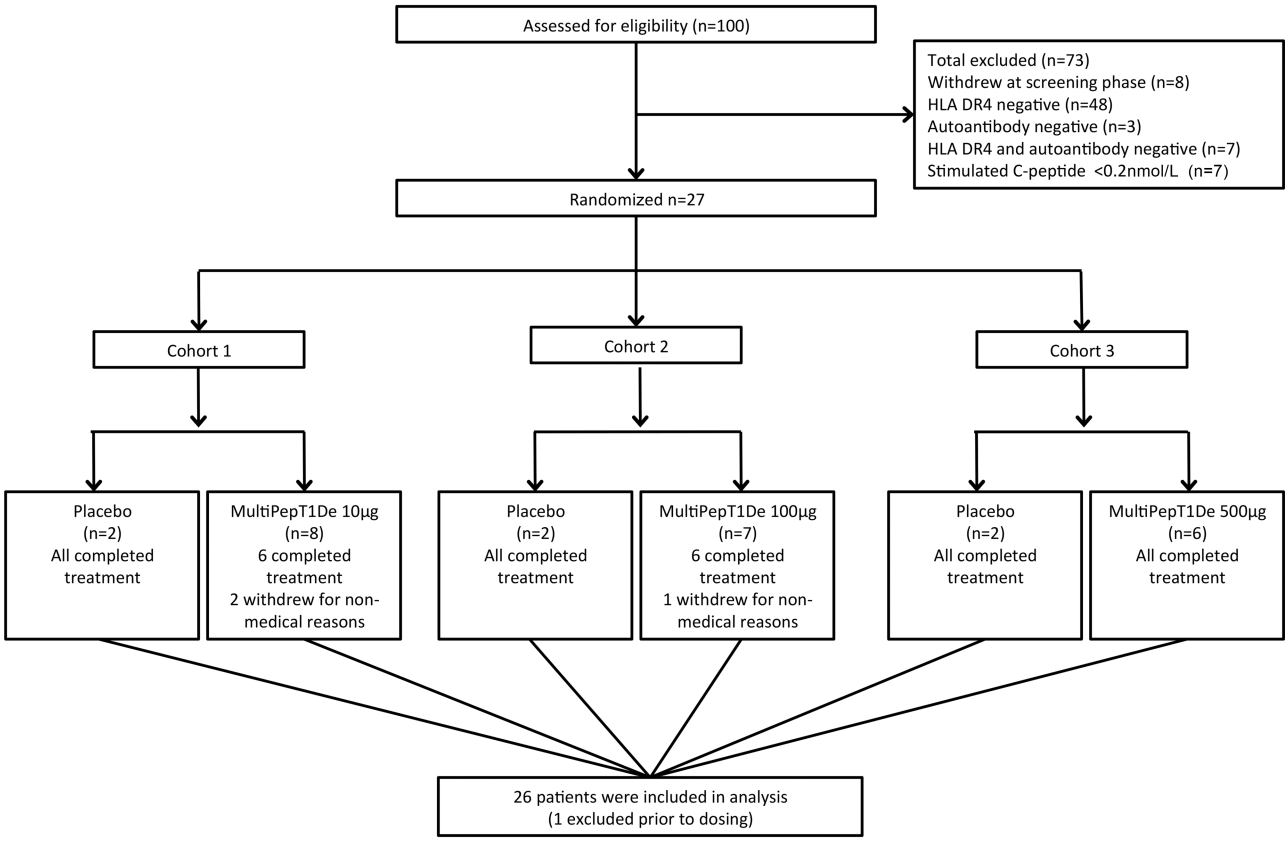

Figure 2—Consolidated Standards of Reporting Trials diagram showing screening and treatment allocation.

Table 1—Baseline demographic data of treatment groups

		Study drug dose			
	Placebo (n = 6)	10 μg (n = 8)	100 μg (n = 6)	500 μg (n = 6)	P
Time since diagnosis (months), mean (SD)	15.79 (13.78)	25.30 (9.84)	21.85 (18.86)	16.15 (14.39)	0.5473†
Sex: male, n (%)	2 (33)	2 (25)	3 (50)	4 (67)	0.6875**
Race: White, n (%)	5 (83)	8 (100)	6 (100)	6 (100)	0.6759**
BMI (kg/m^2), median (range)	23.5 (21.2–33.6)	23.9 (22.0–33.4)	24.3 (19.5–32.3)	23.1 (20.2–32.1)	0.8763§
HbA_{1c} (mmol/mol), mean (SD)	68.7 (27.4)	67.1 (21.9)	57.8* (9.4)	57.5 (17.9)	0.6884 †
Insulin usage (IU/kg/day), mean (SD)	0.35 (0.18)	0.36 (0.14)	0.43 (0.16)	0.42 (0.14)	0.7252†
IDAA1c, mean (SD)	9.85 (2.955)	9.53 (2.322)	9.03 (1.274)	9.07 (1.865)	0.8999†
Fasting C-peptide, mean (SD)	0.231 (0.091)	0.268 (0.233)	0.178 (0.104)	0.304 (0.170)	0.6057†
Peak C-peptide, mean (SD)	0.735 (0.399)	0.787 (0.4887)	0.712 (0.352)	0.933 (0.529)	0.8315

IDAA1c, insulin dose–adjusted HbA_{1c}. *n = 5. **By Fisher exact test. §By Kruskal-Wallis test. †By one-way ANOVA.

and 84 events were reported in the 6 subjects who received placebo. Local skin reactions occurred in both placebo and drug treatment groups, being in general <50 mm in diameter and resolved by 90 min. There were no systemic symptoms of hypersensitivity or anaphylaxis in any of the subjects. We observed no change in the level of autoantibodies directed against IA2 during the study in any of the treatment groups (data not shown). Treatment-emergent adverse events reported by each treatment group are summarized in Supplementary Table 1.

C-Peptide and Metabolic Changes

The study was not powered or designed to detect differences in efficacy markers, such as C-peptide AUC, and given the small numbers of subjects in each group, the results presented here should be considered exploratory in nature. Normalized stimulated C-peptide AUC was analyzed at baseline and 24 weeks, with a decrease in mean values observed over time in the placebo and 10-μg and 500-μg dose groups but with no decrease over time in the 100-μg group (Fig. 3*A*). In post hoc analysis, the mean decline in stimulated C-peptide in subjects who received placebo at 24 weeks versus baseline differed significantly from that of subjects in the combined treatment groups (Fig. 3*B*). C-peptide responders, as defined by Beam et al. (23) as an increase from baseline in normalized C-peptide AUC that was ≥0, were also identified post hoc. There were no C-peptide responders in the placebo group (n = 6), but two (n = 6), four (n = 6), and three (n = 6) responders in the 10-μg, 100-μg, and 500-μg groups, respectively (Fig. 3*C*). Taken together, these findings provide some encouragement for the evaluation of efficacy signals in future, well-powered studies.

There were no treatment-related changes of note in the prespecified or post hoc analyses of HbA_{1c} or insulin dose (Fig. 3*D* and *E* and Supplementary Fig. 2). Two subjects were homozygous for *DRB1*04:01*, precluding meaningful analysis of the impact of HLA homozygosity (one subject in the 100-μg group classified as a C-peptide responder and one non–C-peptide responder in the 10-μg group).

Effect of Multiple Peptide Immunotherapy on Islet-Specific T-Cell Responses and Treg Subpopulations

We examined additional markers representing effector, regulatory, functional, and phenotypic features of global and antigen-specific adaptive immune responses. We assessed cytokine responses to whole PI and component peptides from MultiPepT1De using cytokine ELISpot and report data as model-based estimates of the mean ELISpot response over the course of treatment. CD4 T-cell IL-10 and IL-17 responses to PI stimulation were significantly higher in the blood of all treatment groups compared with placebo (Fig. 4*A*) with a marked increase being observed at 24 weeks in subjects in the 500-μg group (Supplementary Fig. 3). There were no significant differences between these groups for CD4 T-cell IFN-γ responses to PI (Fig. 4*A*) or for any cytokine (IL-10, IL-17, IFN-γ) to any of the component peptides from MultiPepT1De (data not shown). To provide further mechanistic insight, we also performed analyses on MultiPepT1De-treated subjects divided according to the C-peptide response phenotype as defined above. We found statistically significantly higher levels of IL-17 responses when testing against some of the peptide components and whole PI in C-peptide responders compared with non–C-peptide responders (Fig. 4*B*).

We extended these studies to the analysis of Tregs since in a previous study of single-peptide immunotherapy, we had observed changes in expression of FOXP3, the Treg master transcriptional regulator, following treatment (20). Using a similar high-dimensional flow cytometry analysis in the current study, we again observed a significant increase in levels of Treg expression of FOXP3 during the treatment period (comparing baseline with 24 weeks) in MultiPepT1De-treated subjects (Fig. 4*C*). Changes were especially notable in Treg subsets associated with antigen experience (changes in $CD45RA^-$ but not in $CD45RA^+$ Tregs), including those coexpressing CD39 and

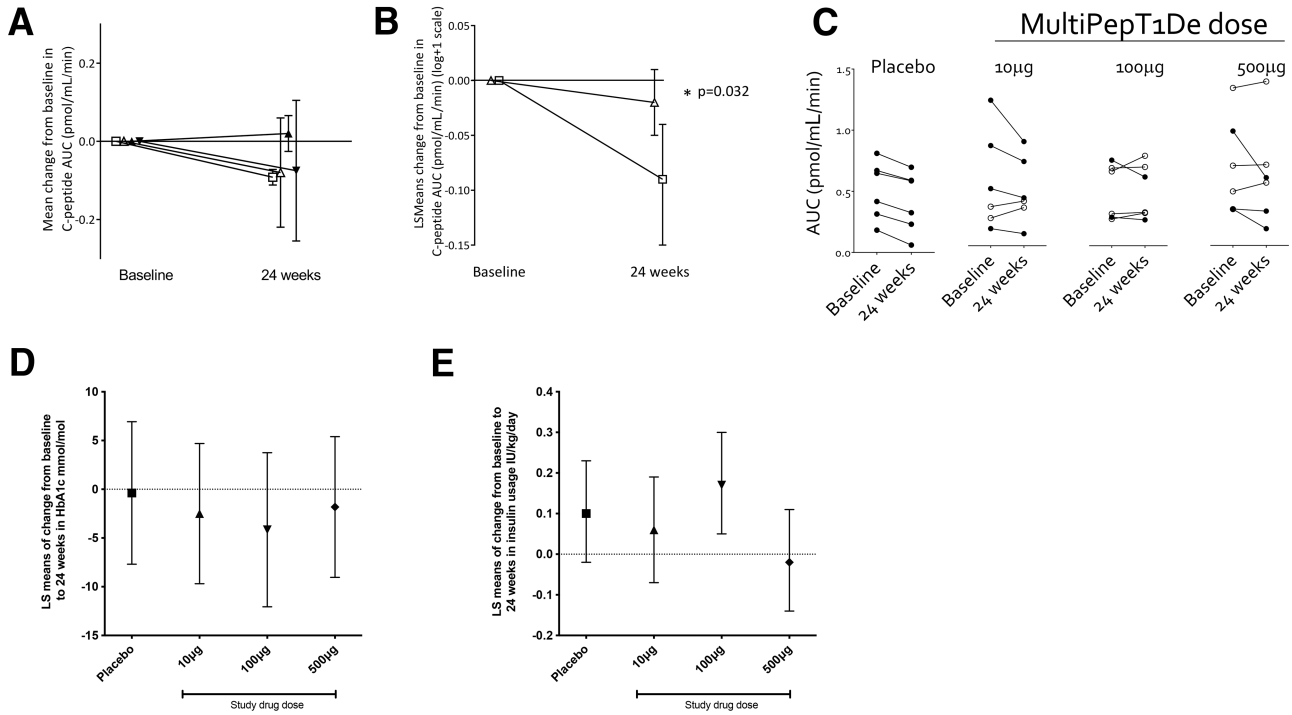

Figure 3—Change in C-peptide, HbA_{1c}, and insulin use during MultiPepT1De treatment from baseline to 24 weeks. *A* and *B*: C-peptide data are shown as mean change from baseline in normalized C-peptide AUC (□, placebo; △, 10-μg dose; ▲, 100-μg dose; ▼, 500-μg dose), LS mean AUC change from baseline comparing placebo (□) and the combined treatment groups (△). *C*: Individual C-peptide AUC data at baseline and 24 weeks (open symbols show C-peptide responders who retained ≥100% of baseline C-peptide level, and closed symbols show C-peptide nonresponders). *D* and *E*: Mean change from baseline in HbA_{1c} and in insulin usage.

CD73. Analysis of Treg FOXP3 expression across treatment groups suggests a dose-response relationship, with the highest effect seen in the 100-μg group when tested after 12 and 24 weeks of therapy (Fig. 4*D*). In contrast, we observed no change in the frequency of $FOXP3^+$ Tregs in response to treatment (Supplementary Fig. 4).

Given these consistent peptide-induced changes in this and our previous study, we performed an exploratory Treg transcriptome analysis on a limited number of subjects. To take into account the small sample size, unequal sex representation in the placebo and treated groups, and the known considerable degree of interindividual variation in Treg gene expression (18), we compared subjects without treatment (combined placebo and baseline samples from treated subjects) with subjects with posttreatment samples. Three genes were significantly different between the groups, when FDR was controlled at q-value = 0.01 (*CNKSR2*, *TNFSF8*, *PPT1*), each of which downregulated after treatment. Despite these modest differences in gene expression between baseline and end of treatment, when examining the top 20 differentially expressed genes, we observed that subjects treated with MultiPepT1De clustered according to their treatment time point (Fig. 5), whereas placebo-treated subjects' samples clustered by individual, regardless of time point. We examined gene sets and pathways on which all genes with FDR <0.5 were found; however, we did not detect any significant enrichment, probably because of the low number of genes included. Following this, we examined how gene expression changed over time and how this was related to treatment. While no single gene was significantly different in change of expression between placebo and MultiPepT1De treatment (when FDR is controlled at q-value <0.5), again we observed clear clustering of subjects by treatment status (Fig. 6).

DISCUSSION

Simple administration of antigens that are targeted in inflammatory diseases, such as autoimmunity and allergy, has been demonstrated to have a therapeutic benefit in many robust studies in preclinical models, as well as has shown increasing success in the clinic (21,24–26). 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 PI and IA2, that can be developed for peptide immunotherapy (20,27–29). In previous studies, we explored the safety effects of repeated dosing with a single native peptide sequence at the point of diagnosis of type 1 diabetes (20). We found this approach to be very well tolerated, even with dosing as frequent as every 2 weeks for 6 months. Moreover, in patients receiving single PI peptide immunotherapy, the loss of C-peptide after

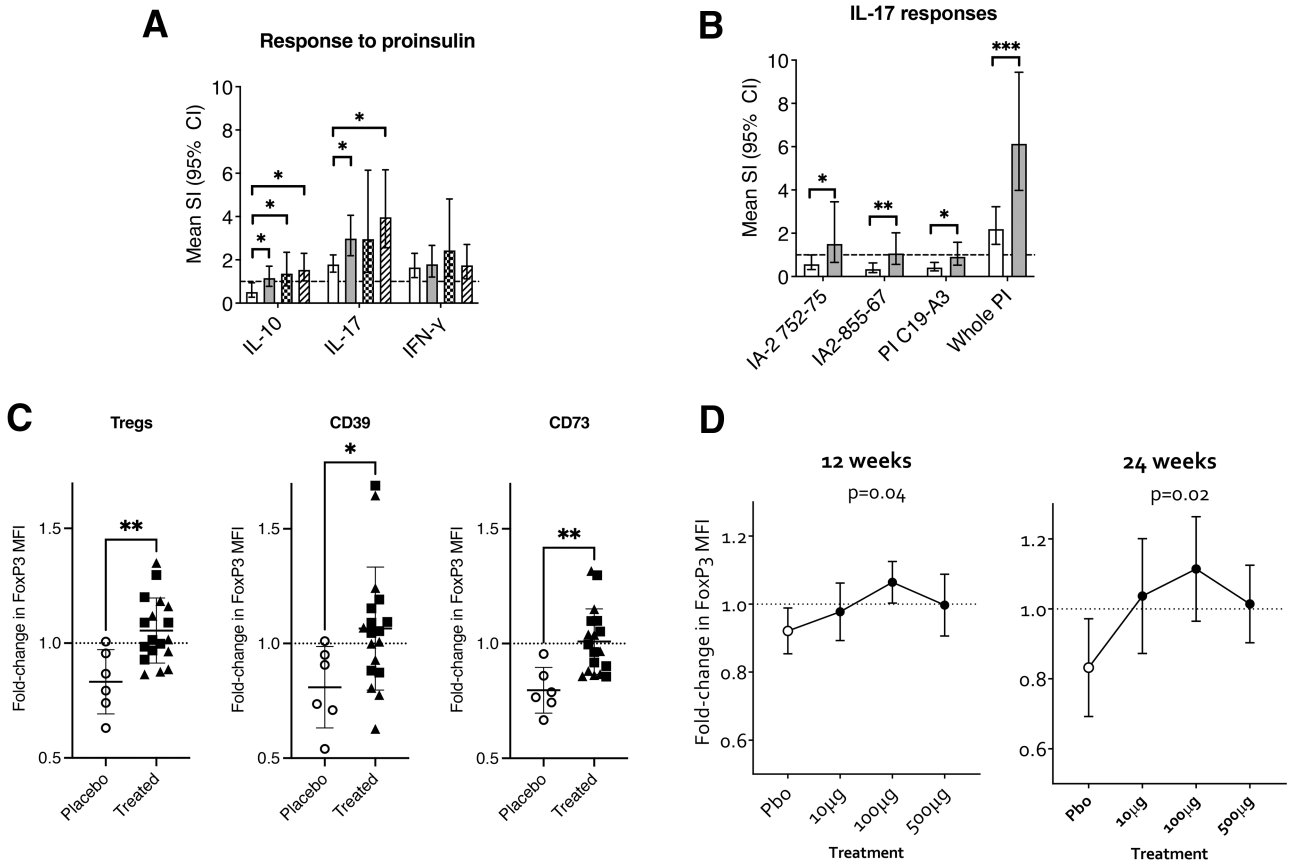

Figure 4— Islet-specific cytokine responses and Treg phenotype changes. *A*: Cumulative IFN-γ, IL-10, and IL-17 responses to PI across all treatment groups, as measured by ELISpot (open bars, placebo group; shaded bars, 10-μg dose group; hatched bars, 100-μg group; striped bars, 500-μg dose group). *B*: IL-17 responses to specified antigenic stimuli, as measured by ELISpot comparing C-peptide responders (shaded bars) and nonresponders (open bars). *C*: Analysis of Treg changes in expression of FOXP3. Change in FOXP3 expression levels (mean fluorescence intensity [MFI] at week 24 relative to baseline) on all Treg subsets ($CD4^+CD25^{hi}FOXP3^+$) and antigen-experienced ($CD45RA^-$) subsets coexpressing CD39 and CD73 comparing subjects treated with peptide and placebo. ■, C-peptide responders; ▲, C-peptide nonresponders. *D*: Dose-response relationship of FOXP3 MFI change and MultiPepT1De in all Treg subsets. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Pbo, placebo.

diagnosis was less apparent than in the control group, and treatment was accompanied by immune changes, such as induction of IL-10 responses to PI and higher FOXP3 expression that are consistent with an immune modulatory effect of the treatment. Although the current study was conducted in a different phase of the disease (eligible patients could be up to 4 years from diagnosis), the findings show a remarkable alignment with our previous work, with similar effects on C-peptide preservation observed in some subjects, modulatory changes detected in T-cell responses to relevant peptides and autoantigens, and alterations in the antigen-experienced Treg proteome. Taken with the observations in preclinical models that the approach of using multiple peptides from more than a single antigen may be more potent than a monopeptide (21,30), these findings serve to justify a future, fully powered clinical trial designed to examine the efficacy of the peptide-based approach in limiting loss of β-cell function in newly diagnosed type 1 diabetes.

A notable finding in these studies has been the higher mean IL-10 responses to PI observed in the treatment compared with the placebo group. IL-10 is an anti-inflammatory cytokine with immune modulatory/suppressive effects. It has multiple, pleiotropic effects in immunoregulation and inflammation achieved via downregulation of expression of proinflammatory type 1 cytokines, such as IFN-γ and tumor necrosis factor-α, HLA and costimulatory molecules on antigen-presenting cells, and blockade of nuclear factor-κB activity. Interestingly, analysis of C-peptide responders (23) showed that all nine subjects with preserved C-peptide were found in the treatment groups with four of the nine in the 100-μg treatment group. Within these responders, a higher IL-17 response was seen at baseline than in nonresponders, and overall, the PI-stimulated IL-17 response increased in treatment groups. IL-17 is often viewed as a proinflammatory cytokine; however, its production is also associated with cells with an immunoregulatory phenotype. $CD4^+$ T cells that

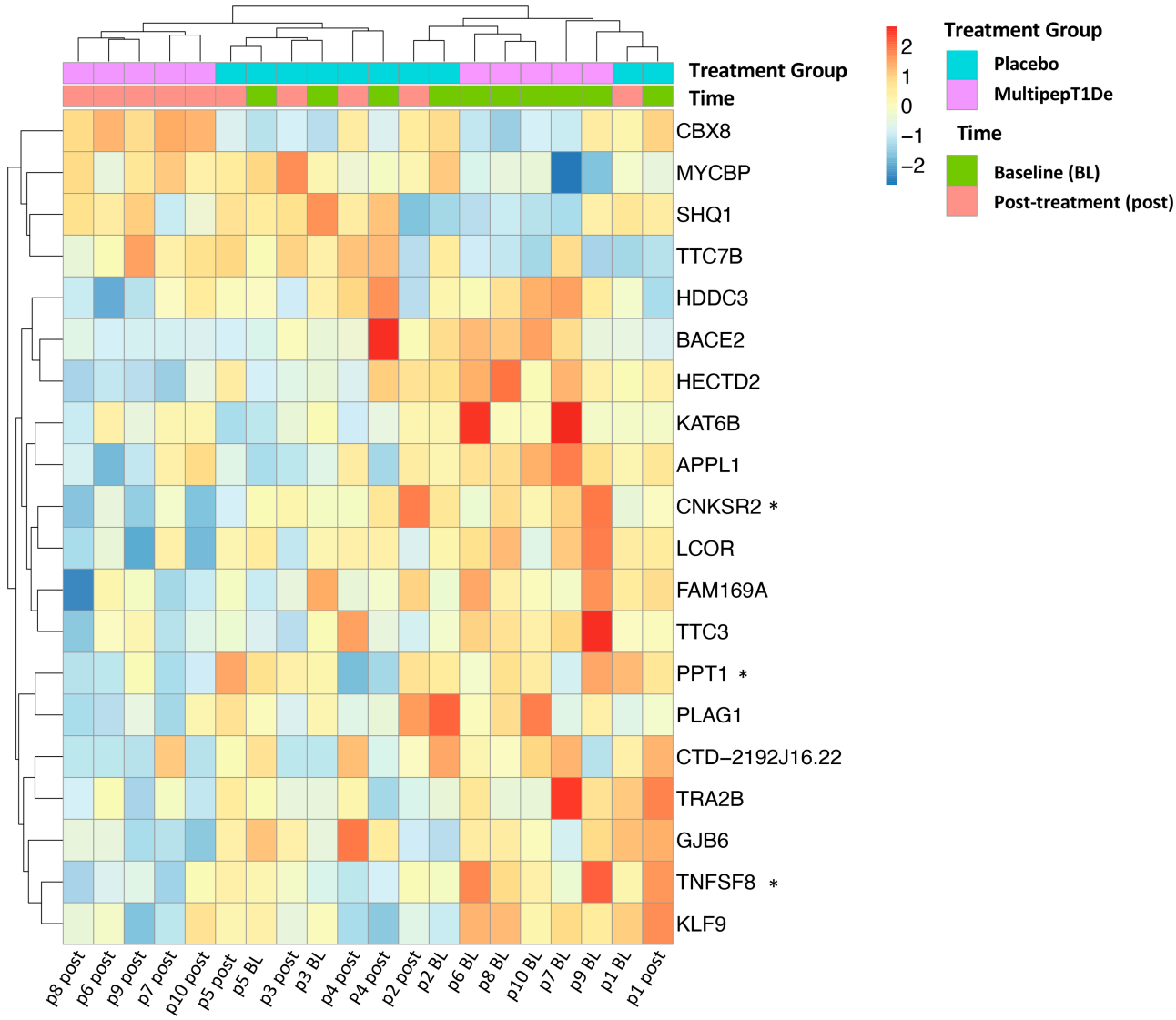

Figure 5—Expression of genes differentially expressed in MultiPepT1De-treated samples. The top 20 differentially expressed genes identified in this analysis were normalized genewise and hierarchically clustered by genes and samples. Participant numbers and time points are indicated at the bottom of the plot. Genes that differed significantly (FDR controlled at q = 0.01) between groups (combined placebo and baseline samples from treated individuals vs. posttreatment samples) are indicated by an asterisk.

coproduce IL-17 and IL-10 have been shown to limit T helper 17 cell–mediated disease (31–33), and it has been shown that IL-17 is produced by FOXP3$^+$ Tregs when activated in an inflammatory milieu (34). Moreover, IL-17$^+$/FOXP3$^+$ Tregs retain suppressive function and FOXP3 expression and exhibit the plasticity to secrete IL-17 or suppress it, depending on the nature of the stimulus provided.

While numerous reports have linked ASI to IL-10 responses, relatively few have reported effects on conventional FOXP3$^+$CD25^{hi} Tregs, including preclinical models. The consistent finding across our peptide immunotherapy studies of a higher fold change in Treg expression of FOXP3 with therapy is of considerable interest. As previously reported, changes are seen in populations of memory Tregs coexpressing markers of antigen experience, such as CD39, which is associated with enhanced Treg stability under inflammatory conditions and increased IL-10 secretion (35). It has been proposed that peptide immunotherapy induces autoantigen-specific CD4 T cells that exert immunoregulatory properties via effects on antigen-presenting cells coincident with the presentation of other autoantigens in *cis* to effector T cells, providing an opportunity for bystander suppression and infectious tolerance (36–39). Our exploratory analysis of the Treg transcriptome further supports the hypothesis that treatment targets FOXP3$^+$CD25^{hi} Tregs, with treated individuals demonstrating a distinct change in expression profile following therapy. These changes were detected despite samples being taken 4 weeks after the last

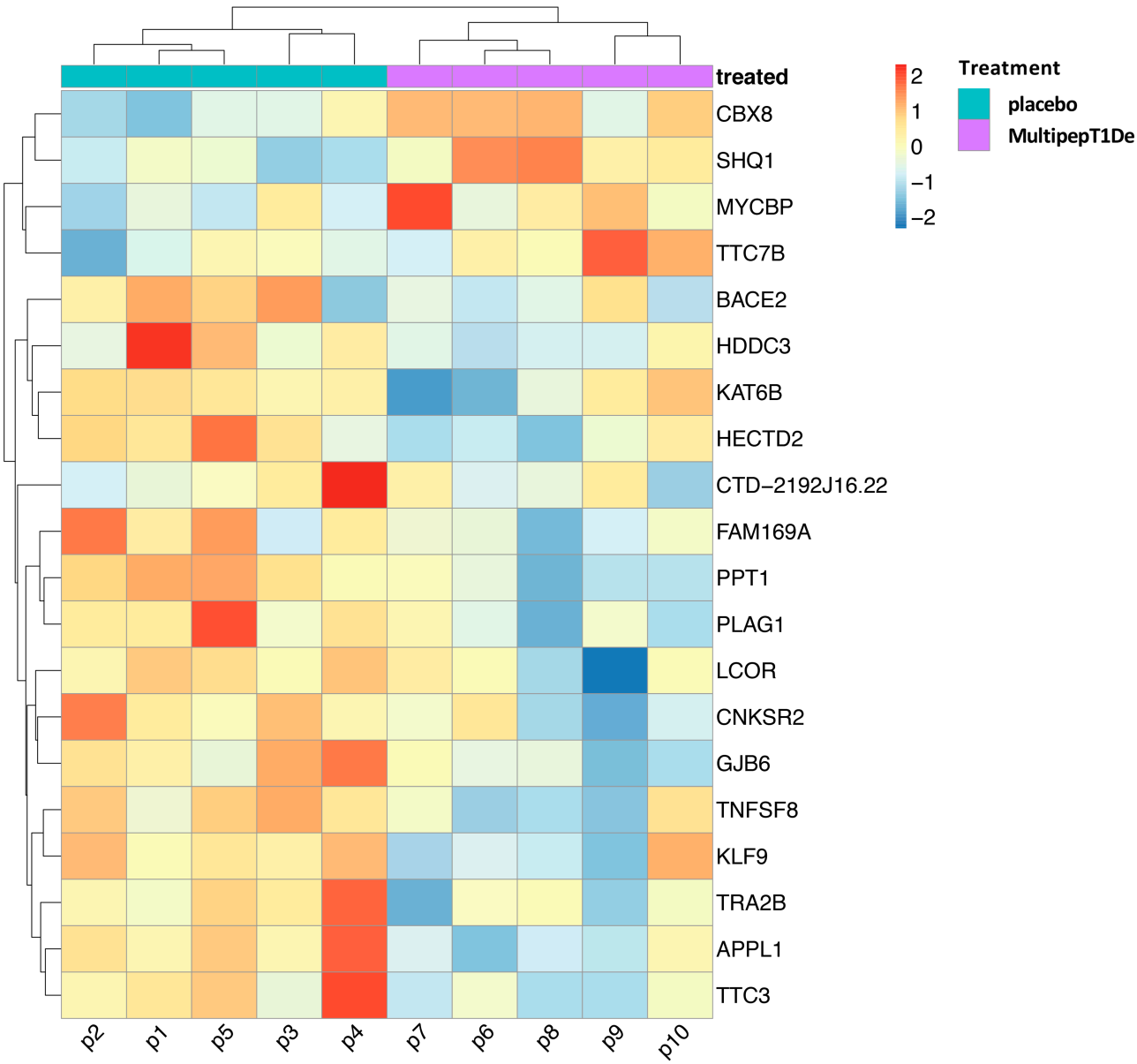

Figure 6—Change in Treg expression after MultiPepT1De treatment. Difference between pre- and posttreatment expression of the top 20 differentially expressed genes from Fig. 5, normalized genewise and hierarchically clustered by genes and samples. Participant numbers are indicated at the bottom of the plot.

drug administration, raising the possibility that an even clearer signal would have been detected in samples taken closer to therapy. Unfortunately, the low number of individuals analyzed and the high degree of interindividual variation in transcriptional profiles at baseline limited our power to detect differential expression and to identify enriched specific pathways targeted by treatment. However, some of these preliminary findings are intriguing and could be the basis for extension studies. For example, expression of TNFSF8 by Tregs (which is downregulated following MultiPepT1De treatment) is known to be promoted by IFN-I signaling and is associated with a decrease in Treg activation status and a decrease in their suppressive function in vivo (40). Future studies, powered to examine clinical efficacy, will allow a more thorough dissection of the therapeutic potential of peptide immunotherapy and can be complemented by immune studies at single-cell resolution on $FOXP3^{+}CD25^{hi}$ Tregs to better define the mechanism of action.

Funding. This research was funded/supported by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Guy's and St. Thomas' NHS Foundation Trust and King's College London and/or the NIHR Clinical Research Facility. Flow cytometry studies were supported by the T1DUK Immunotherapy Consortium funded by Diabetes UK (grants 15/0005232 and 15/0005233). The study was cosponsored by King's College London and Guy's and St. Thomas' NHS Foundation Trust. UCB Pharma

provided grant funding and input into the study design, outcome analysis, and data interpretation.

The views expressed are those of the authors and not necessarily those of the National Health Service, NIHR, or the Department of Health and Social Care.

Duality of Interest. Y.-F.L. has consulted for UCB Pharma. M.P. has consulted for UCB Pharma and benefits from a license agreement with King's College London. T.T. has received speaker fees from UCB Pharma. No other potential conflicts of interest relevant to this article were reported.

Author Contributions. Y.-F.L., J.P., S.A., J.H.M.Y., E.W., L.K., M.J., N.F., A.L., and T.T. contributed to the acquisition and analysis of data. Y.-F.L., M.P., and T.T. wrote the manuscript. L.L., E.S, and M.P. contributed to the conception and design of the study. E.S., C.B., and A.L. performed the formal statistical analysis. M.P. and T.T. were responsible for funding acquisition. All authors reviewed the manuscript and gave approval for the final draft. M.P. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References

1. Patterson CC, Gyürüs E, Rosenbauer J, et al. Trends in childhood type 1 diabetes incidence in Europe during 1989-2008: evidence of non-uniformity over time in rates of increase. Diabetologia 2012;55:2142–2147

2. Lachin JM, McGee P; DCCT/EDIC Research Group. Impact of C-peptide preservation on metabolic and clinical outcomes in the Diabetes Control and Complications Trial. Diabetes 2014;63:739–748

3. Sørensen JS, Johannesen J, Pociot F, et al.; Danish Society for Diabetes in Childhood and Adolescence. Residual β-cell function 3-6 years after onset of type 1 diabetes reduces risk of severe hypoglycemia in children and adolescents. Diabetes Care 2013;36:3454–3459

4. Killestein J. Anti-CD3 monoclonal antibody in new-onset type 1 diabetes mellitus. N Engl J Med 2002;347:1116–1117

5. Keymeulen B, Vandemeulebroucke E, Ziegler AG, et al. Insulin needs after CD3-antibody therapy in new-onset type 1 diabetes. N Engl J Med 2005;352:2598–2608

6. Herold KC, Gitelman SE, Willi SM, et al. Teplizumab treatment may improve C-peptide responses in participants with type 1 diabetes after the new-onset period: a randomised controlled trial. Diabetologia 2013;56:391–400

7. Sherry N, Hagopian W, Ludvigsson J, et al.; Protégé Trial Investigators. Teplizumab for treatment of type 1 diabetes (Protégé study): 1-year results from a randomised, placebo-controlled trial. Lancet 2011;378:487–497

8. Rigby MR, Harris KM, Pinckney A, et al. Alefacept provides sustained clinical and immunological effects in new-onset type 1 diabetes patients. J Clin Invest 2015;125:3285–3296

9. Pescovitz MD, Greenbaum CJ, Krause-Steinrauf H, et al.; Type 1 Diabetes TrialNet Anti-CD20 Study Group. Rituximab, B-lymphocyte depletion, and preservation of beta-cell function. N Engl J Med 2009;361:2143–2152

10. Orban T, Bundy B, Becker DJ, et al.; Type 1 Diabetes TrialNet Abatacept Study Group. Co-stimulation modulation with abatacept in patients with recent-onset type 1 diabetes: a randomised, double-blind, placebo-controlled trial. Lancet 2011;378:412–419

11. Feutren G, Papoz L, Assan R, et al. Cyclosporin increases the rate and length of remissions in insulin-dependent diabetes of recent onset. Results of a multicentre double-blind trial. Lancet 1986;2:119–124

12. Atkinson MA, Roep BO, Posgai A, Wheeler DCS, Peakman M. The challenge of modulating β-cell autoimmunity in type 1 diabetes. Lancet Diabetes Endocrinol 2019;7:52–64

13. Lindley S, Dayan CM, Bishop A, Roep BO, Peakman M, Tree TIM. Defective suppressor function in CD4($^{+}$)CD25($^{+}$) T-cells from patients with type 1 diabetes. Diabetes 2005;54:92–99

14. Garg G, Tyler JR, Yang JHM, et al. Type 1 diabetes-associated IL2RA variation lowers IL-2 signaling and contributes to diminished CD4+CD25+ regulatory T cell function. J Immunol 2012;188:4644–4653

15. Long SA, Cerosaletti K, Bollyky PL, et al. Defects in IL-2R signaling contribute to diminished maintenance of FOXP3 expression in CD4(+)CD25(+) regulatory T-cells of type 1 diabetic subjects. Diabetes 2010;59:407–415

16. Glisic-Milosavljevic S, Waukau J, Jailwala P, et al. At-risk and recent-onset type 1 diabetic subjects have increased apoptosis in the CD4+CD25+ T-cell fraction. PLoS One 2007;2:e146

17. Pesenacker AM, Wang AY, Singh A, et al. A Treg gene signature is a specific and sensitive biomarker to identify children with new onset type 1 diabetes. Diabetes 2016;65:db150572

18. Ferraro A, D'Alise AM, Raj T, et al. Interindividual variation in human T regulatory cells. Proc Natl Acad Sci U S A 2014;111:E1111–E1120

19. Brusko TM, Wasserfall CH, Clare-Salzler MJ, Schatz DA, Atkinson MA. Functional defects and the influence of age on the frequency of CD4+ CD25+ T-cells in type 1 diabetes. Diabetes 2005;54:1407–1414

20. Alhadj Ali M, Liu YF, Arif S, et al. Metabolic and immune effects of immunotherapy with proinsulin peptide in human new-onset type 1 diabetes. Sci Transl Med 2017;9:eaaf7779

21. Gibson VB, Nikolic T, Pearce VQ, Demengeot J, Roep BO, Peakman M. Proinsulin multi-peptide immunotherapy induces antigen-specific regulatory T cells and limits autoimmunity in a humanized model. Clin Exp Immunol 2015;182:251–260

22. Herold KC, Brooks-Worrell B, Palmer J, et al.; Type 1 Diabetes TrialNet Research Group. Validity and reproducibility of measurement of islet autoreactivity by T-cell assays in subjects with early type 1 diabetes. Diabetes 2009;58: 2588–2595

23. Beam CA, Gitelman SE; Type 1 Diabetes TrialNet Study Group. Recommendations for the definition of clinical responder in insulin preservation studies. Diabetes 2014;63:3120–3127

24. Couroux P, Patel D, Armstrong K, Larché M, Hafner RP. Fel d 1-derived synthetic peptide immuno-regulatory epitopes show a long-term treatment effect in cat allergic subjects. Clin Exp Allergy 2015;45:974–981

25. Patel D, Couroux P, Hickey P, et al. Fel d 1-derived peptide antigen desensitization shows a persistent treatment effect 1 year after the start of dosing: a randomized, placebo-controlled study. J Allergy Clin Immunol 2013;131:103–109.e1–7

26. Larché M, Wraith DC. Peptide-based therapeutic vaccines for allergic and autoimmune diseases. Nat Med 2005;11(Suppl.):S69–S76

27. Peakman M, Stevens EJ, Lohmann T, et al. Naturally processed and presented epitopes of the islet cell autoantigen IA-2 eluted from HLA-DR4. J Clin Invest 1999;104:1449–1457

28. Arif S, Tree TI, Astill TP, et al. Autoreactive T cell responses show proinflammatory polarization in diabetes but a regulatory phenotype in health. J Clin Invest 2004;113:451–463

29. Thrower SL, James L, Hall W, et al. Proinsulin peptide immunotherapy in type 1 diabetes: report of a first-in-man phase I safety study. Clin Exp Immunol 2009;155:156–165

30. Smith CE, Miller SD. Multi-peptide coupled-cell tolerance ameliorates ongoing relapsing EAE associated with multiple pathogenic autoreactivities. J Autoimmun 2006;27:218–231

31. Gagliani N, Amezcua Vesely MC, Iseppon A, et al. Th17 cells transdifferentiate into regulatory T cells during resolution of inflammation. Nature 2015;523:221–225

32. Ghoreschi K, Laurence A, Yang XP, et al. Generation of pathogenic T(H)17 cells in the absence of TGF-β signalling. Nature 2010;467:967–971

33. McGeachy MJ, Bak-Jensen KS, Chen Y, et al. TGF-β and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology. Nat Immunol 2007;8:1390–1397

34. Beriou G, Costantino CM, Ashley CW, et al. IL-17-producing human peripheral regulatory T cells retain suppressive function. Blood 2009;113:4240–4249
35. Gu J, Ni X, Pan X, et al. Human CD39^{hi} regulatory T cells present stronger stability and function under inflammatory conditions. Cell Mol Immunol 2017;14:521–528
36. Richardson N, Wraith DC. Advancement of antigen-specific immunotherapy: knowledge transfer between allergy and autoimmunity. Immunother Adv 2021; 1:ltab009
37. Akdis CA, Akdis M. Mechanisms of immune tolerance to allergens: role of IL-10 and Tregs. J Clin Invest 2014;124:4678–4680
38. Campbell JD, Buckland KF, McMillan SJ, et al. Peptide immunotherapy in allergic asthma generates IL-10-dependent immunological tolerance associated with linked epitope suppression. J Exp Med 2009;206:1535–1547
39. Tree TIM, Lawson J, Edwards H, et al. Naturally arising human CD4 T-cells that recognize islet autoantigens and secrete interleukin-10 regulate proinflammatory T-cell responses via linked suppression. Diabetes 2010;59:1451–1460
40. Gangaplara A, Martens C, Dahlstrom E, et al. Type I interferon signaling attenuates regulatory T cell function in viral infection and in the tumor microenvironment. PLoS Pathog 2018;14:e1006985