



## Autoantigen-specific regulatory T cells induced in patients with type 1 diabetes mellitus by insulin B-chain immunotherapy

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### ABSTRACT

There is a growing body of evidence to suggest that the autoimmunity observed in type 1 diabetes mellitus (T1DM) is the result of an imbalance between autoaggressive and regulatory cell subsets. Therapeutics that supplement or enhance the existing regulatory subset are therefore a much sought after goal in this indication. Here, we report the results of a double blind, placebo controlled, phase I clinical trial of a novel antigen-specific therapeutic in 12 subjects with recently diagnosed T1DM. Our primary objective was to test its safety. The study drug, human insulin B-chain in incomplete Freund's adjuvant (IFA) was administered as a single intramuscular injection, with subjects followed for 2 years. All subjects completed therapy and all follow-up visits. The therapy was generally safe and well-tolerated. Mixed meal stimulated C-peptide responses, measured every 6 months, showed no statistical differences between arms. All patients vaccinated with the autoantigen, but none who received placebo, developed robust insulin-specific humoral and T cell responses. Up to two years following the single injection, in peripheral blood from subjects in the experimental arm, but not the control arm, insulin B-chain-specific CD4<sup>+</sup> T cells could be isolated and cloned that showed phenotypic and functional characteristics of regulatory T cells. The induction of a lasting, robust immune response generating autoantigen-specific regulatory T cells provides strong justification for further testing of this therapy in type 1 diabetes. ([clinicaltrials.gov](http://clinicaltrials.gov) identifier NCT00057499).

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### 1. Introduction

Type 1 diabetes mellitus (T1DM) is caused by the progressive autoimmune destruction of insulin-producing beta cells [1]. This process is T cell-mediated [2], with CD4<sup>+</sup> cells apparently critical to the process [3–5]. However, a wealth of recent evidence has now highlighted the critical nature of regulatory T cells (Tregs) that are capable of suppressing the autoaggressive T cell population [6]. Tregs can come in several forms, notably as naturally occurring or 'professional' CD4<sup>+</sup>CD25<sup>+</sup> cells, but also as antigen-induced CD4<sup>+</sup> Th2-like regulatory cells [7]. The imbalance between the autoaggressive and

regulatory sets of T cells appears to be at the core of autoimmunity in general, thus implying that successful interventions must delete the autoaggressive cells [8] and/or boost the regulatory population in order to reestablish control and create a healthy balance [9].

It is known that antigen challenge in an autoimmune setting can stimulate beneficial changes in T cell subsets [10] (Th1 vs. Th2) and cytokine production [11]. In practice, antigen-specific therapeutic approaches for autoimmune diseases tend to use putative self-antigens that have been implicated in the disease etiopathogenesis. Self-antigen-based therapy has shown promise in numerous animal models of disease and is being studied in human diseases such as rheumatoid arthritis [12] and multiple sclerosis [13]. A number of self and nonself-antigens have been tested for type 1 diabetes mellitus, such as BCG antigen [14], heat shock protein [15], and a human recombinant GAD65 protein. Although the latter was recently shown to have positive effects on C-peptide production in late onset and

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newly diagnosed T1DM patients [16,17] in general, results have been lackluster. Several earlier attempts to use insulin itself as an immunotherapy [18,19] resulted in failure. In spite of these difficulties, there remains a wealth of data suggesting that insulin is central to autoimmunity in T1DM [20,21], and therefore studies of insulin continue, with new efforts focusing on developing more effective dosing and modes of delivery [18,19,22].

We have addressed this challenge by combining a diabetes-specific antigen – the metabolically inactive insulin B-chain fragment, which contains a major epitope recognized by the immune system [20,23] – and incomplete Freund's adjuvant – a powerful delivery system that promotes regulatory immune responses. We have hypothesized that peripheral reintroduction of the primary autoantigen, insulin in adjuvant, will induce immune tolerance in T1DM patients. Recently published data indicate that insulin given to patients with type 1 diabetes as routine daily treatment induces insulin-specific T regulatory cells [24].

In this study, we examined the safety of this novel insulin B-chain-based therapeutic in patients with newly diagnosed T1DM and analyzed its effects on diabetes control, endogenous insulin production and immune response.

## 2. Research design & methods

### 2.1. Subjects

Twelve subjects between the ages of 18–35 years who had been newly diagnosed with T1DM (within 3 months of enrollment) and who were positive for any of the three diabetes autoantibodies (IAA-if tested within 2 weeks of insulin therapy-, GAD65Ab, IA2Ab) were enrolled at the Joslin Diabetes Clinic in Boston, MA. The protocol was reviewed and approved by the NIAID Data and Safety Monitoring Board and the Institutional Review Board at Harvard Medical School. All patients signed an informed consent. [See [Supplementary Table S1](#) for a complete list of inclusion and exclusion criteria].

### 2.2. Drug preparation

Human insulin B-chain was made as a monocomponent, HPLC-purified peptide, synthesized on a Protein Synthesizer model 433A (Applied Biosystems, Foster City, CA) using amino acid preparations from Peptide International (Louisville, KY). The study drug, IBC-VS01, contained 2 mg insulin B-chain peptide in Montanide ISA51-incomplete Freund's adjuvant (IFA, Seppic Inc., Fairfield, NJ) in a 50/50 (w/w) emulsion. The placebo was a vehicle control (VS02) consisting of buffer solution (4 M urea in phosphate buffer) in Montanide ISA51-incomplete Freund's adjuvant in a 50/50 (w/w) emulsion. Both study drug and placebo were prepared immediately prior to administration using a point-of-use high-pressure syringe mixing method to a volume of 1 ml.

### 2.3. Study design

This was a randomized, double blind, placebo controlled study ([clinicaltrials.gov](http://clinicaltrials.gov) identifier NCT00057499). Subjects were randomly assigned in a 1:1 ratio to receive a single dose of either 2 mg human insulin B-chain in IFA or placebo (IFA) via intramuscular injection. Complete blood counts (CBCs) with differentials, complete laboratory chemistries (including liver and kidney function tests) and urine analyses were performed at follow-up visits scheduled at weeks 1, 2, 3, 4, 8, 12, 24, 52, 78 and 104 post-treatment. At baseline and then at weeks 4, 12, 24, 52, 78 and 104, 100 ml of heparinized blood was collected from each patient. Metabolic control was assessed by recording insulin dose, HbA1c concentrations and stimulated C-peptide values using a mixed meal tolerance test

[MMTT] [25] at entry and at weeks 12, 24, 52, 78 and 104. The primary endpoint of the study was safety, assessed by clinical parameters including adverse events, local reactions, comprehensive physical exams, insulin dose, and laboratory tests. Secondary endpoints included humoral and cellular immune responses to the vaccination.

### 2.4. Diabetes autoantibody assays

#### 2.4.1. Insulin autoantibody

A competitive liquid phase radioimmunoassay was used according to previously published method by Vardi et al. [26]. Briefly, 150  $\mu$ l of serum with 50  $\mu$ l of 0.04 M phosphosaline buffer was incubated with another set of serum incubated with 50  $\mu$ l of the same buffer containing human insulin at a concentration of  $9 \times 10^6$  nU/ml of buffer. Two hundred  $\mu$ l of human recombinant  $^{125}$ I labeled insulin (Amersham, GE Healthcare) in a concentration of 7500 nU/ml phosphosaline buffer was added to all tubes for a 7-day incubation. Antibody-bound insulin was precipitated and counted for 10 min in a gamma counter (Cobra II Auto-gamma Counter, Perkin Elmer Inc. [Packard]). All samples were tested in duplicate with a cut-point used of 39 nU/ml (mean  $\pm$  2 SD); based on the 2005 Diabetes Antibody Standardization Program (DASP), the assay had 46% sensitivity and 96% specificity [27].

#### 2.4.2. IA2 and GAD65 assays

Radioimmunoassays were performed according to the method of Grubin et al. [28] and Payton et al. [29], respectively. Briefly, serum samples and negative and positive control samples were incubated with [ $^{35}$ S]methionine-labeled GAD65 or IA2 containing buffer. The next day, 50- $\mu$ l aliquots of the serum samples were incubated on coated Millipore plates with protein A-sepharose CL-4B beads (Amersham, GE Healthcare). Scintillation liquid (Optiphase Supermix, Perkin Elmer Inc.) was added to each well and cpm values were measured. All samples were tested in triplicate. Results are expressed in indices (positive control cpm-sample cpm/positive control cpm-negative control cpm). The cut-point used was 0.1 for both assays (mean  $\pm$  2SD); as per the 2007 DASP, the GAD65 and IA2 assays had sensitivity/specificity of 80/99% and 76/99%, respectively.

### 2.5. Cellular studies

#### 2.5.1. Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were separated from heparinized blood using Ficoll–Paque Plus (Amersham, GE Healthcare). Following counting, they were resuspended at a density of  $1.32 \times 10^7$  cells/ml in culture medium [RPMI-1640, supplemented with 1% 1 M HEPES, 1% Sodium pyruvate, 1% Glutamate, 1% Penicillin/Streptomycin (all from BioWhittaker Cambrex, Walkersville, MD, USA), 1% MEM (non-essential amino acid solution from Gibco, Invitrogen, Grand Island, NY), and 5% heat-inactivated human AB serum (Omega, Tarzana CA, USA)] for the T cell antigen stimulation assays. The remaining cells were frozen in heat-inactivated human AB serum (Omega) with 10% dimethyl-sulfoxide (DMSO, Sigma–Aldrich, St. Louis, MO, USA) and stored at  $-80^\circ\text{C}$  until assayed.

#### 2.5.2. T cell stimulation assay

Freshly isolated PBMCs were pulsed for 2 h at  $37^\circ\text{C}$  with the specific antigens (0.01 and 0.1 LfU/ml tetanus toxoid [TT] for positive control, 25 and 50  $\mu$ g/ml insulin B-chain, 10 and 30  $\mu$ g/ml insulin, 10 and 30  $\mu$ g/ml proinsulin, 1, 5 and 20  $\mu$ g/ml GAD65 and overlapping peptides of proinsulin [OP 1–10 [30], all 40  $\mu$ g/ml]) and were placed into 96-well, round-bottom plates (Corning, USA) at  $1.5 \times 10^5$  cells/well. After 4 days incubation at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ , 100  $\mu$ l culture medium was changed to 100  $\mu$ l fresh medium containing 10 U/ml

recombinant interleukin-2 (IL-2, Tecin™ Teceleukin – bulk Ro 23-6019, NCI). After 7 days stimulation, the supernatant was collected from each well for use in cytokine assays, while the cells were treated overnight with 1  $\mu$ Ci/well  $H^3$  thymidine (GE Healthcare). The next day  $H^3$  thymidine incorporation was assessed by a Wallac 1450 MicroBeta TriLux liquid scintillation and luminescence counter (PerkinElmer Inc.). Stimulation index (SI) values were calculated as the ratio of the normalized count per minute (cpm) values of the stimulated and control cells. An SI value of 3 or above was considered positive.

### 2.5.3. Cytokine production

Cytokine production from the T cell stimulation assay supernatants was evaluated by a sandwich ELISA, using a combination of unlabeled and biotin-labeled monoclonal antibodies to IFN- $\gamma$  and IL-4 (BD Biosciences Pharmingen, USA). Avidin-peroxidase conjugate and TMB substrate (all from BD Biosciences Pharmingen) were used to develop the assay. Interleukin-10 (IL-10) was measured with a Human IL-10 ELISA kit (eBioscience, USA), interleukin-17 (IL-17) with a Human IL-17 ELISA Kit (eBioscience), and TGF- $\beta$  with the Human TGF- $\beta$ 1 DuoSet (R&D Systems, USA), all according to the manufacturers' protocols.

### 2.5.4. iNKT cell frequency [31]

Approximately 9 million PBMCs were thawed, washed in phosphate buffered saline (PBS, pH:7.4) and stained with FITC-labeled anti-6B11 antibody (1:20 dilution; BD Biosciences Pharmingen) and PE-labeled anti-V $\alpha$ 24 antibody (1:50 dilution; Coulter, Marseille, France), in a staining medium of PBS and 0.5% heat-inactivated human AB serum, on ice for 30 min. Following washing with PBS, the cells were resuspended in 0.5 ml fixing medium (2% formalin in PBS) and the frequency of double positive cells was measured by a Beckman Coulter XL FACS analyzer.

### 2.5.5. Tetramer staining, carboxyfluorescein diacetate succinimidyl ester (CFSE) staining for single cell sorting and expansion of the clones

The construction of the expression vectors for generation of soluble DR0401 (DRA\*0101/DRB1\*0401), DR0101 (DRA1\*0101/DRB1\*0101) or DR0301 (DRA1\*0101/DRB1\*0301) tetramer molecules has been described previously [32].

After 8 days of prestimulation with the specific antigens, as per methods described above for the T cell stimulation assay, cells were removed from culture, washed and resuspended in 100  $\mu$ l culture medium containing 10% human AB serum, then split into two 5 ml polystyrene tubes. Next, 1  $\mu$ l peptide-loaded PE-labeled HLA-matched tetramers were added (empty HLA-matched tetramer was used as negative control) and incubated for 4 h at 37 °C, followed by an incubation with FITC-labeled anti-CD4 antibody (BD Biosciences Pharmingen) on ice for 30 min.

For the CFSE assay, PBMCs were incubated at 37 °C for 5 min with 0.5  $\mu$ M CFSE (stock at 0.5 mM in DMSO, Invitrogen, USA). Staining was terminated by adding cRPMI, the cells were washed, resuspended in culture medium and cultured in 96-well plates, as in the T cell stimulation assay, with medium alone, TT (0.1 LfU/ml) or insulin B-chain (50  $\mu$ g/ml). After 7 days of culture, the cells were washed and stained on ice with anti-CD4-PE antibody (BD Biosciences Pharmingen) for 30 min. Prior to sorting propidium-iodide viability staining was applied [33].

The double positive cells from the tetramer staining and propidium-iodide negative, CD4<sup>+</sup>, CFSE<sup>dim</sup> cells from the CFSE assay were single cell sorted using a BD FACS Aria cell sorter into the inner 60 wells of 96-well, round bottom, polystyrene plates with each well containing 150000 irradiated (5000 rad) allogenic feeder cells, 3  $\mu$ g/ml phytohaemagglutinin (Remel Inc., USA) and 20 U/ml IL-2 in 200  $\mu$ l culture medium. The outside wells contained 200  $\mu$ l PBS.

Every second day, 100  $\mu$ l medium was replaced with fresh medium containing 20 U/ml IL-2 for the tetramer stained cells; in the case of CFSE-stained cells, each week 100  $\mu$ l medium was replaced with fresh medium containing 20 U/ml IL-2 and 5 ng/ml IL-4 (PeproTech Inc., USA). After 2 weeks, the wells were screened for growth and growing clones were expanded.

### 2.5.5.1. Characterization of the single cell T clones

**2.5.5.1.1. Testing for antigen specificity by tetramer staining of the clones.** To confirm antigen specificity, clones were stained with the original PE labeled antigen-loaded tetramer and FITC labeled anti-CD4 antibody.

**2.5.5.1.2. Assay for T cell clonal expansion in response to specific antigen.** The cells were left without any stimulation for at least 3 days. The same patient's PBMCs were incubated with the specific antigen, with tetanus toxoid and without antigen for 2 h; they were then irradiated (2000 rad), washed and put out to 96-well plates at a density of 100,000 cells/well using at least 2 wells for each condition. Additional antigens were added to the corresponding conditions and 50,000 cells from the clones were added to the wells. On the 7th day supernatant was collected for cytokine assessment and the cells were treated with 1  $\mu$ Ci/well  $H^3$  thymidine for overnight. The next day  $^3H$ -thymidine incorporation was assessed and stimulation index was calculated (as above).

**2.5.5.1.3. Suppression assay of the T cell clones.** To assess the suppressive capacity of the clones an APC-free suppression assay system was set up [34]. T cell Activation/Expansion Beads (Miltenyi Biotec) were coated with anti-CD2, anti-CD3 and anti-CD28 monoclonal antibodies (5  $\mu$ g/ml) according to the manufacturer's protocol. Responder (CD4<sup>+</sup>CD25<sup>-</sup>) T cells were sorted from the same subject and 10,000 cells were stimulated with 20,000 coated beads alone or with different numbers of cells from the single cell clones (10,000, 5000, 2500; i.e. cell ratios: 1:1, 1:1/2, 1:1/4). Also, 10,000 cells from the clones were stimulated with the beads without responder T cells. The cells were cultured for 5 days and then were treated with 1  $\mu$ Ci/well  $^3H$ -thymidine for overnight. The next day  $^3H$ -thymidine incorporation was assessed. The level of inhibition was expressed as percentage value (100-[cpm of the 1:1 ratio divided by the cpm value of the responder T cells  $\times$  100]).

**2.5.5.1.4. Interleukin-10 and TGF- $\beta$  cytokine production in the suppression assay.** Interleukin-10 (IL-10) was measured with Human IL-10 ELISA kit (eBioscience), TGF- $\beta$  with the Human TGF- $\beta$ 1 DuoSet (R&D Systems) according to the manufacturers' protocols.

**2.5.5.1.5. Intracellular staining (FoxP3).** For intracellular FoxP3 staining a FITC labeled anti-human Foxp3 Staining Set (eBioscience) was used according to the manufacturer's protocol.

**2.5.5.1.6. PCR and Western blot (TGF- $\beta$ , FoxP3, TCR).** RNA extraction was carried out using RNAeasy Microkit<sup>®</sup> (Qiagen, Germany) according to the manufacturer's protocol (including DNase digestion). Matrix bound RNA was resuspended in DEPC-treated water, and quantified at 260 nm. The cDNA was produced from the total RNA with reverse transcriptase with random hexamers following the manufacturer's protocol (Invitrogen). Primers were designed and PCR was performed on the cDNA using Platinum<sup>®</sup> Taq DNA polymerase (Invitrogen) in 25  $\mu$ l reactions according to the manufacturer's protocol. GAPDH or beta-2 microglobulin was used as a housekeeping gene. DNA templates were recovered from PCR products with a QIAquick<sup>™</sup> PCR purification kit (Qiagen). Templates were sequenced with an ABI3100 Analyzer from both sides with forward and reverse primers.

**2.5.5.1.7. TCR sequencing of single cell clones.** TCR alpha and beta chain CDR3 sequences for the insulin B-chain tetramer positive CD4<sup>+</sup> T cell clones were determined by RT-PCR and sequencing analysis. Briefly, total RNA was isolated from CD4<sup>+</sup> T cell clones using an RNeasy Mini Kit (Qiagen) and cDNA was synthesized with

a Taqman Reverse Transcription Kit (Applied Biosystems). A set of five multiplex PCR reactions covering a majority of the human V-beta repertoire were performed as per Akatsuka et al. [35] and two series of amplifications, first, with pooled V-alpha primers and then, with specific V-alpha primers covering the CDR3 regions, were performed as per Seitz et al. [36]. PCR products were visualized on an ethidium bromide stained 2% agarose gel, sequenced using a Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) with either a TCR constant region 3' primer or a specific variable region 5' primer, and then run on an ABI3100 Genetic Analyzer. TCR alpha and beta CDR3 sequence data were analyzed using the IMGT/V-QUEST (<http://imgt.cines.fr>) web-based program from the Université Montpellier, France [37].

## 2.6. Statistical analysis

Statistical analyses were performed using SAS® (SAS Institute, Version 8.2, Cary, NC). Secondary efficacy endpoints (c-peptide, HbA1c, insulin use), autoantibody titers and T cell responses were analyzed by means of repeated measures analysis of variance (ANOVA), utilizing a mixed effects model that incorporates the participant as a random effect across the different visits. The compound symmetry variance structure was specified for the random effect within a participant among the visits (that is, correlations of response levels at any two visits were assumed to be the same). Results are expressed as mean  $\pm$  SEM;  $p < 0.05$  was considered statistically significant.

The analyses of cytokine production were performed using StatView for Windows 5.0.1. (SAS Institute Inc.). Group comparisons for data with normal distribution were performed by Student's *t*-test, except in cases of non-Gaussian distribution where a Mann–Whitney *U* test was used. Results are expressed as mean  $\pm$  SEM;  $p < 0.05$  was considered statistically significant.

## 3. Results

### 3.1. Patient demographics

Twelve subjects diagnosed with T1DM within the previous 3 months were randomized to receive study treatment or placebo between April 2003 and March 2005. All subjects completed all scheduled visits as planned. No significant differences were noted between the two groups with respect to age, BMI, HbA1c, insulin use, autoantibody status and stimulated C-peptide levels. The mean (SEM) ages were  $29.0 \pm 2.5$  years (4 M/2F) and  $27.7 \pm 2.4$  years (5 M/1F) in the insulin B-chain vaccinated and placebo groups, respectively; all BMI values were normal. Table 1 shows a summary of subject demographic data.

### 3.2. Safety

A total of 62 adverse events were reported in the study, of which 61 were deemed unrelated to treatment [See Supplementary Table S2]. One patient experienced mild, transient pain/discomfort at the injection site. No prominent or outstanding pattern of adverse events was noted. The most common adverse event was upper respiratory tract infection. No single type of adverse event occurred in more than 4 participants. A single serious adverse event requiring hospitalization with gastroenteritis was deemed unrelated to treatment.

### 3.3. Efficacy

Endogenous insulin production, as measured by C-peptide during MMTT, showed no statistical difference between the drug (IBC-VS01) and placebo (VS02) groups at any time during the study (Fig. 1). The placebo group tended to have higher C-peptide levels

**Table 1**  
Demographic and baseline characteristics at entry.

	Treatment (IBC-VS01) <i>n</i> = 6	Placebo (VS02) <i>n</i> = 6
Sex	4M 2F	5M 1F
Age – yr	$29.0 \pm 2.5$	$27.7 \pm 2.4$
BMI – kg/m <sup>2</sup>	$21.6 \pm 0.5$	$23.5 \pm 0.5$
DKA – No. present	2	1
Total C-peptide (AUC) (nmol/l)		
Mean	$0.63 \pm 0.07$	$0.73 \pm 0.07$
Median	0.39	0.67
HbA1c -%	8.4	9.1
Daily Insulin Usage – U/kg	$0.32 \pm 0.05$	$0.21 \pm 0.02$
Autoantibodies		
IAA – nU/ml		
mean	$286.6 \pm 61.0$	$54.2 \pm 5.5$
median	70.1	65.9
GAD65 – index	$0.62 \pm 0.04$	$0.52 \pm 0.06$
IA2 – index	$0.69 \pm 0.12$	$2.63 \pm 0.62$

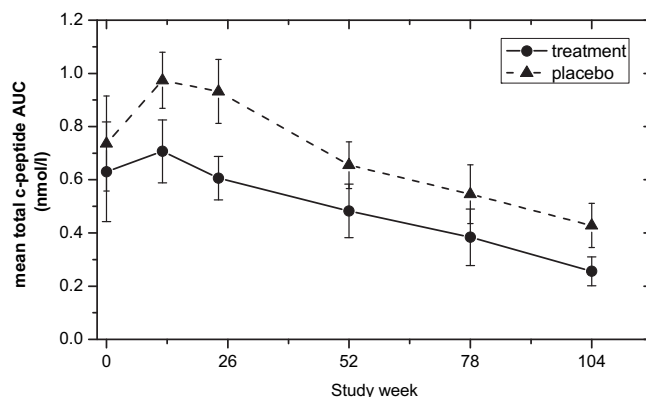
for the duration the study, a reflection of the higher baseline average values in this group at randomization (four of six subjects with mean total C-peptide AUC  $< 0.5$  nmol/l were randomized to the study treatment group). Insulin use and HbA1c were similar in both groups throughout the study (data not shown). One outlier with high HbA1c in the insulin B-chain vaccinated group reported that he intentionally used less insulin to avoid hypoglycemia.

### 3.4. Autoantibody titers

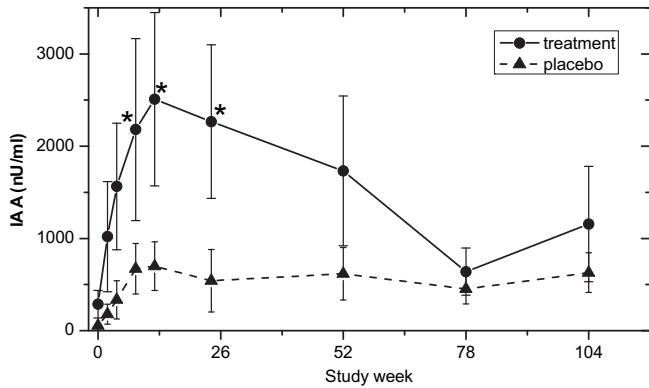
Both groups exhibited increased IAA titers that peaked at 12 weeks post-treatment. Subjects receiving insulin B-chain vaccinations showed a significantly greater increase compared to placebo (Fig. 2;  $p < 0.05$  at 8; 12 and 24 weeks), indicating a specific humoral immune response to insulin in the treatment group [38]. No differences between arms were found for titers of either GAD65 or IA2 autoantibodies.

### 3.5. T cell responses

All patients vaccinated with human insulin B-chain developed a robust T cell response to insulin B-chain. This response peaked at 24 weeks, then slowly declined but remained positive for up-to 2



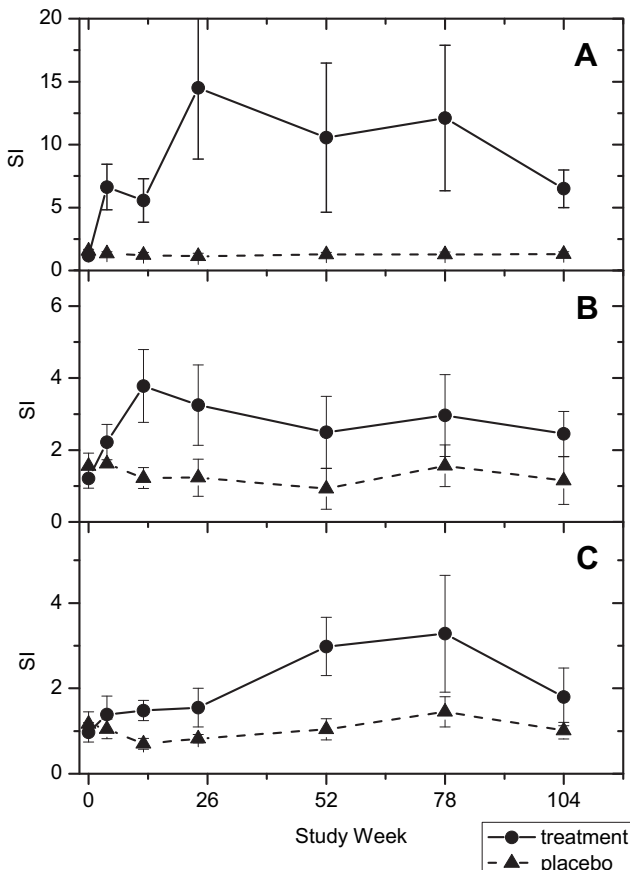
**Fig. 1.** Mean total C-peptide area under the curve (AUC) for the mixed meal tolerance test (MMTT) by group over time. Self-insulin production, as measured by C-peptide during MMTT showed no statistical difference between the drug-treated (IBC-VS01) and placebo (VS02) groups. Four out of six subjects with the lowest stimulated C-peptide (mean total C-peptide AUC  $< 0.5$  nmol/l) were randomized to the insulin B-chain vaccinated group. Even though after 3 months the stimulated C-peptide values declined in both groups, the slope of decline showed a more favorable trend in the insulin B-chain vaccinated group ( $x \pm$  SEM).



**Fig. 2.** Insulin autoantibodies (IAA) by group over time. IAA titers were significantly increased in the insulin B-chain vaccinated subjects compared to the placebo group ( $p < 0.05$  at 8; 12 and 24 weeks) with a peak at 12 weeks, indicating a specific humoral immune response to insulin in the active treatment group ( $x \pm \text{SEM}$ ;  $p < 0.05$ ).

years follow up (Fig. 3A). Less prominent responses were detected to proinsulin (data not shown) and some of the insulin B-chain overlapping peptides (B-chain 5–20 and 9–23 peptides, Fig. 3B and C). No proinsulin, insulin or insulin peptide-specific T cell responses were detected in the placebo group.

The T cell responses to tetanus were similar in both groups.



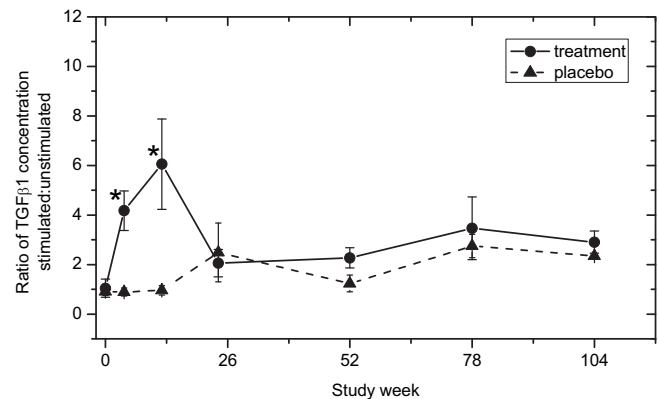
**Fig. 3.** T cell assay stimulation indices (SI) in treatment and placebo groups in response to insulin B-chain (A), B-chain 5–20 peptide (B) and B-chain 9–23 peptide (C) stimulation over time. All patients vaccinated with full human insulin B-chain developed a robust T cell response to insulin B-chain. This response peaked at 24 weeks, then slowly declined but remained positive for up to 2 years during follow up ( $x \pm \text{SEM}$ ;  $n = 6$ , except for B-chain 9–23 peptide treatment group samples, for which only three showed response over time).

In order to characterize the insulin B-chain-specific T cell response, cytokine (TGF- $\beta$ 1, IL-10, IL-17, IL-4, IFN- $\gamma$ ) production was measured in the supernatant of the cell cultures used in the T cell stimulation assay. Higher ratios of TGF- $\beta$ 1 concentration in the supernatant of the B-chain stimulated/unstimulated cells of the T cell stimulation assay were found at week 4 and 12 ( $p = 0.03$  and  $0.02$ , respectively, Fig. 4) in the B-chain vaccinated group compared to the placebo group. No other significant differences were detected in the cytokine production (IL-10, IL-17, IL-4, IFN- $\gamma$ ) of the cell cultures between the arms.

Single cell T cell clones were isolated and expanded from subjects' specimens that had the strongest B-chain-specific T cell responses, taken at the 1.5 and 2 yr time points. Cloning attempts on cells from subjects with no B-chain-specific T cell response did not yield clones. In all, 37 T cell clones were generated [see Supplementary Figures S1 and S2] and tested for cell surface and phenotypic T cell markers such as CD4, CD127, CD45RO, FoxP3 and TGF- $\beta$ 1, as well as for clonal expansion in response to insulin B-chain stimulation. The number of markers tested on each clone was dependent on the number of cells available (Table 2A). Flow cytometric evaluation of insulin B-chain MHC class II tetramer binding confirmed the insulin B-chain specificity of the clones tested (Supplementary Figure S3). Clones were found to be CD4+, CD45RO+, FOXP3 positive (assessed by PCR [Supplementary Figure S4] and FACS methods [Supplementary Figure S5]) and were CD127 negative or low (Table 2A). All clones obtained exhibited one or more phenotypic markers of regulatory T cells. In case of two clones, which exhibited sufficient cell growth to permit functional assays as well (Z and AA; Table 2B) we could demonstrate their suppressive capacity in the T cell suppression assay with 20 and 32% inhibition, as illustrated in Fig. 5. Further characterization of these cells by measurement of IL-10 and TGF- $\beta$ 1 in the supernatants from the inhibition assays revealed that clone Z produced TGF- $\beta$ 1 upon stimulation (34 pg/ml in the supernatant of the cells with 1:1 ratio in the functional assay), but no IL-10; clone AA produced IL-10 (188 pg/ml in the supernatant of the cells with 1:1 ratio in the functional assay), but not TGF- $\beta$ 1.

In order to assess the polyclonality of the clones, T cell receptor alpha and beta chains were sequenced for four of the clones. The CDR3 regions in each were found to be different, indicating that each of the clones was unique (Table 3).

To test whether the cloning procedure itself could produce regulatory T cells, FACS-sorted CD4+CD25- T cells from both



**Fig. 4.** Ratio of the TGF- $\beta$ 1 concentrations of the B-chain stimulated/unstimulated cells in the 7-day assay ( $x \pm \text{SEM}$ ;  $p < 0.05$ ) measured by ELISA. Higher ratios were found at week 4 and 12 ( $p = 0.03$  and  $0.02$ , respectively) in the B-chain vaccinated subjects compared to the placebo group. The mean TGF- $\beta$ 1 concentrations of the B-chain stimulated/unstimulated cells (150000 cells/200  $\mu$ l medium/well) at week 4 were  $127.1 \pm 31.5/23.5 \pm 7.3$  pg/ml in the IBC group and  $49.4 \pm 10.4/36.9 \pm 12.8$  pg/ml in the placebo group and at week 12 were  $363.9 \pm 152.6/55.9 \pm 6.1$  pg/ml in the IBC group and  $112.6 \pm 24.4/170.6 \pm 78.5$  pg/ml in the placebo group.

**Table 2A**

Markers tested on the single T cell clones of the two study subjects. Single cell T cell clones were isolated and expanded from insulin B-chain vaccinated patients using insulin B-chain MHC class II tetramers and CFSE methods, 37 T cell clones were generated that were tested for cell surface and phenotypic T cell markers. The number of markers tested on each clone was dependent on the number of cells available.

	No. tested	Total number of clones 37	
		No. confirmed	% confirmed
CD4 positive	37	37	100.0
CD45RO positive	35	35	100.0
FOXP3/FACS (pos>23%)	15	12	80.0
CD127 negative	31	31	100.0
Clonal expansion to insulin B-chain (pos: SI>3)	37	14	37.8
FOXP3/PCR positive	31	27	87.0
TGFβ PCR positive	26	26	100.0

healthy controls and T1DM patients were subject to the same cloning and expansion process. No regulatory T cells were induced; cells remained CD127 positive and Foxp3 negative by FACS analysis (see [Supplementary Figure S6](#)), while functional assays showed no inhibition.

We monitored iNKT cell frequencies in peripheral blood [31]. The mean percentage value doubled in the insulin B-chain vaccinated group (from 0.047 to 0.085% of total lymphocytes) and from week 4 and afterwards, this increase was sustained compared to the placebo group, however these differences did not reach the level of significance.

#### 4. Discussion

In this study, we have shown clear indications of an antigen-specific T regulatory cell response arising following treatment of new onset type 1 diabetes with a vaccine comprised of insulin B-chain in IFA. This is a unique response not previously observed in type 1 diabetes using antigen-specific agents. Incomplete Freund's adjuvant has been long overlooked in human diabetes mellitus, even though it has been successfully applied in other indications [39], with a good long-term safety profile [40]. In recent years IFA-enhanced antigen T cell receptor peptide vaccination trials have been successfully conducted in rheumatoid arthritis [41] and in multiple sclerosis [42]. Here, we employed a newer formulation of IFA that has shown excellent tolerability in patients with HIV [43].

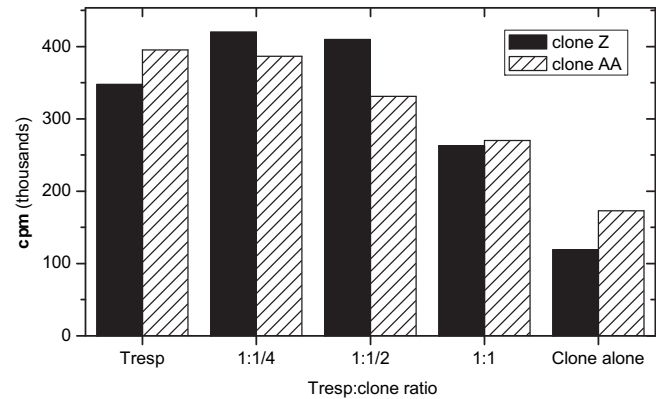
The entry criteria for this study did not require a minimum C-peptide level at baseline for inclusion in this study. This resulted in an imbalance of baseline C-peptide levels between the two groups. The mean total C-peptide level at study entry was higher in

**Table 2B**

Characteristics of two of the T cell clones expanded from subjects with the highest B-chain-specific T cell responses.

	Characteristics of the two inhibitory clones	
	Z*	AA**
CD4	pos	pos
CD45RO	pos	pos
FOXP3/PCR	pos	pos
FOXP3/FACS (pos>23%)	31%	34%
CD127	neg	neg
Clonal expansion to insulin B-chain (pos:SI>3)	NSP	NSP
TGFβ PCR	pos	pos
TGFβ secretion	yes	no
IL-10 secretion	no	yes
Level of inhibition at the 1:1 Resp: T responder ratio	* 20%	** 32%

NSP = No Significant Proliferation.



**Fig. 5.** Suppression assay of two CFSE-B-chain single cell clones with different cell-to-cell ratios of the CD4+CD25- (responder T, 10000) cells and the cells from the clones (2500–5000–10000) of the same subject. The cells were stimulated with bead bound anti-CD-2, anti-CD3 and anti-CD28 (5 μg/ml, Miltenyi Biotec). Responder T cells and cells from the clones were stimulated alone, also. Tritium labeled thymidine (H3 Thymidine) incorporation was assessed after 5 days of culturing. Due to limited cell numbers the cocultures were put together without replicates.

the control group, an imbalance that was sustained throughout the follow-up period, but one that at no time was statistically significant. As this was a pilot study, the sample size was small ( $n = 12$  study participants); the study was not originally powered to detect significant differences between the two groups for changes in mean C-peptide production over time. However, there was a better trend in the stimulated C-peptide decline in the insulin B-chain vaccinated group after three months of the vaccination.

Although it is not possible to draw conclusions concerning the vaccine's clinical efficacy, the vaccine did elicit a clear insulin-specific humoral response. IAA levels, which peaked at 12 weeks then gradually declined, were significantly higher compared to placebo at weeks 8, 12, and 24. Although some elevation of IAA values was noted in the placebo-vaccinated patients, these values stayed within the range typical of subjects receiving exogenous insulin to control diabetes [38]. The increased IAA levels cannot be attributed to differences in insulin dose, as both arms reported similar doses throughout the study, thus increased IAA showed no adverse effect on insulin usage.

We also observed that immune cells from vaccinated subjects responded to insulin B-chain in an antigen-specific manner. All six study participants in the insulin B-chain vaccinated group and none in the control group showed a high level of T cell proliferation in responses to insulin B-chain stimulation. This antigen-specific response reached a zenith at 6 months, then slowly declined, but remained positive for up to 2 years. Interestingly, the antigen-specific response showed a modest positive correlation with the level of decline in stimulated C-peptide response (delta C-peptide and T cell SI at 1 year;  $r = 0.75$ ). Only limited responses were observed to the B-chain fragments (OP2 [B5-20], OP3 [B9-23]) and to proinsulin, and this response was also restricted to the insulin B-chain vaccinated group. Comparing these limited responses to the robust response elicited by the full length B-chain suggests that a combination of the putative epitopes on the B-chain and/or its specific conformational structure is needed to elicit full scale stimulation. The dynamic and the duration of the humoral and cellular responses evoked are quite different. The humoral response peaked at 3 months and reverted to the level of the controls by one year, meanwhile the cellular response reached zenith at 6 months and remained positive throughout the study.

Regulatory T cells are believed to be critical in the maintenance of immune tolerance [44] and there are indications that their suppressive function is defective in T1DM [45]. They exert their

**Table 3**  
CDR3 sequences of the TCR alpha and beta chains from four B-chain specific CD4+ T cell clones. The TCR alpha and beta chain CDR3 sequences of four CD4+ T cell clones specific for the insulin B-chain were analyzed.

Clones	Alpha CDR3		Beta CDR3	
BB			TRBV29-1 TRBJ1-2	TRBD1*01 CSVHSGDGGYTF
BC	TRAV39 TRAJ58	CASRETSGRSLTF	TRBV11-2 TRBJ2-2	TRBD2*02 CASREGVLRPTGELFF
BD			TRBV20-1 TRBJ2-5	TRBD2*01 CSAGRGGALLETQYF
BOB	TRAV35 TRAJ40	CAGREDSGTYKYIF	TRBV4-1 TRBJ2-5	TRBD2*01 CASSRSGAGAQTQYF

effect on other immune cells by cell-to-cell contact as well by a set of powerful cytokines like TGF- $\beta$  and IL-10. They do not represent a homogeneous population and currently there is no single marker regarded as sine qua non in humans. Most carry surface markers alone or in combination, like CD4+CD25 high, CD127 negative/low, and express transcription factor Foxp3.

Peripheral blood T cells of patients who received insulin B-chain vaccine produced a significant amount of TGF- $\beta$  upon stimulation with insulin B-chain. We further characterized and tested single T cell clones isolated from two patients' peripheral blood following insulin B-chain stimulation. These human insulin B-chain T cell clones showed phenotypic characteristics of regulatory T cells: CD127 negative/low, Foxp3+ and TGF- $\beta$  and/or IL-10 positive. As expected, they varied in their capacity to further expand upon insulin B-chain restimulation, some with strong and some with no or little expansion. In case of two clones, we were able to document their inhibitory capacity. The level of inhibition was similar to that observed previously in T1DM subjects [45]. These cells were not produced as a result of the cloning process, as CD4+CD25- cells taken from healthy controls and T1DM patients that were subject to the same cloning procedures showed no such characteristics. Therefore, these data suggest that human insulin B-chain vaccination in patients with T1DM evokes insulin B-chain specific regulatory T cells, which have the capacity to exert their regulatory functions. This response is polyclonal as indicated by the results of the TCR typing.

It is unknown whether these regulatory T cells are results of the expansion of the existing regulatory repertoire or represent de novo generation of a peripherally induced new regulatory compartment (from insulin B-chain specific effector cells). It is known that effector T cells can be turned into Foxp3+ regulatory T cells [46]. In future trials, our intentions are to further investigate the induced T cell population at the single cell level to understand its ontogenesis and expand on characterization of their function [47].

The antigen specificity of these induced regulatory cells is important as they have the capacity to accumulate at the relevant site of autoimmunity [48]. The regulatory T cells are "multitalented masters of immune regulation" [49] and can function in many ways. They can control by bystander suppression where one antigen-specific regulatory T cell controls effector T cells with different antigen specificity. They can also exert their control by infectious tolerance whereby they change the micro-milieu to foster generation of regulatory T cells with different antigen specificity. Moreover, regulatory T cells can confer suppressor activity to effector T cells [50]. The notion of epitope and antigen spreading in T1DM autoimmunity is well accepted [30], implying that several effector cell populations with different epitope and antigen specificities are present. Thus, one may speculate about the possibility that regulatory T cells generated by this vaccine could control not only insulin B-chain-specific effector T cells, but those specific for the entire panel of islet beta cell antigens involved in the autoimmune process.

Taken together, these data indicate that human insulin B-chain in IFA is a safe intervention and evokes robust, antigen-specific immune response. This vaccine generates type 1 diabetes autoantigen-specific regulatory T cells that appear to retain their functional capacity and

thus have the potential to arrest T1DM autoimmunity. As the first report of the generation of an autoantigen-specific regulatory T cell response in human T1DM, it thus warrants continued clinical investigation to further characterize its mechanism of action and to establish the optimal dose, dose regime, optimal formulation, time and age for intervention.

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### Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jaut.2009.10.005.

### References

- [1] Baecher-Allan C, Hafler DA. Human regulatory T cells and their role in autoimmune disease. *Immunol Rev* 2006;212:203–16.
- [2] Lampeter EF, Homberg M, Quabeck K, Schaefer UW, Wernet P, Bertrams J, et al. Transfer of insulin-dependent diabetes between HLA-identical siblings by bone marrow transplantation. *Lancet* 1993;341:1243–4.
- [3] Orban T, Kis J, Szereday L, Engelmann P, Farkas K, Jalahej H, et al. Reduced CD4+ T-cell-specific gene expression in human type 1 diabetes mellitus. *J Autoimmun* 2007.
- [4] Spatz M, Eibl N, Hink S, Wolf HM, Fischer GF, Mayr WR, et al. Impaired primary immune response in type-1 diabetes. Functional impairment at the level of APCs and T-cells. *Cell Immunol* 2003;221:15–26.
- [5] Eibl N, Spatz M, Fischer GF, Mayr WR, Samstag A, Wolf HM, et al. Impaired primary immune response in type-1 diabetes: results from a controlled vaccination study. *Clin.Immunol.* 2002;103:249–59.
- [6] Filippi C, Bresson D, von Herrath M. Antigen-specific induction of regulatory T cells for type 1 diabetes therapy. *Int Rev Immunol* 2005;24:341–60.
- [7] Yamazaki S, Bonito AJ, Spisek R, Dhodapkar M, Inaba K, Steinman RM. Dendritic cells are specialized accessory cells along with TGF- for the differentiation of Foxp3+ CD4+ regulatory T cells from peripheral Foxp3 precursors. *Blood* 2007;110:4293–302.
- [8] Monti P, Scirpoli M, Rigamonti A, Mayr A, Jaeger A, Bonfanti R, et al. Evidence for in vivo primed and expanded autoreactive T cells as a specific feature of patients with type 1 diabetes. *J Immunol* 2007;179:5785–92.
- [9] Goudy KS, Tisch R. Immunotherapy for the prevention and treatment of type 1 diabetes. *Int Rev Immunol* 2005;24:307–26.
- [10] Avni O, Lee D, Macian F, Szabo SJ, Glimcher LH, Rao A. T(H) cell differentiation is accompanied by dynamic changes in histone acetylation of cytokine genes. *Nat Immunol* 2002;3:643–51.
- [11] Ramiya VK, Shang XZ, Pharis PG, Wasserfall CH, Stabler TV, Muir AB, et al. Antigen based therapies to prevent diabetes in NOD mice. *J Autoimmun.* 1996;9:349–56.
- [12] Trentham DE, Dynesius-Trentham RA, Orav EJ, Combitchi D, Lorenzo C, Sewell KL, et al. Effects of oral administration of type II collagen on rheumatoid arthritis [see comments]. *Science* 1993;261:1727–30.

- [13] Weiner HL, Mackin GA, Matsui M, Orav EJ, Khoury SJ, Dawson DM, et al. Double-blind pilot trial of oral tolerization with myelin antigens in multiple sclerosis [see comments]. *Science* 1993;259:1321–4.
- [14] Allen HF, Klingensmith GJ, Jensen P, Simoes E, Hayward A, Chase HP. Effect of Bacillus Calmette-Guerin vaccination on new-onset type 1 diabetes. A randomized clinical study. *Diabetes Care* 1999;22:1703–7.
- [15] Raz I, Elias D, Avron A, Tamir M, Metzger M, Cohen IR. Beta-cell function in new-onset type 1 diabetes and immunomodulation with a heat-shock protein peptide (DiaPep277): a randomised, double-blind, phase II trial. *Lancet* 2001;358:1749–53.
- [16] Agardh CD, Cilio CM, Lethagen A, Lynch K, Leslie RD, Palmer M, et al. Clinical evidence for the safety of GAD65 immunomodulation in adult-onset autoimmune diabetes. *J Diabetes Complications* 2005;19:238–46.
- [17] Ludvigsson J, Faresjo M, Hjorth M, Axelsson S, Cheramy M, Pihl M, et al. GAD treatment and insulin secretion in recent-onset type 1 diabetes. *N Engl J Med* 2008.
- [18] Effects of insulin in relatives of patients with type 1 diabetes mellitus. *N Engl J Med* 2002;346:1685–91.
- [19] Skyler JS, Krischer JP, Wolfsdorf J, Cowie C, Palmer JP, Greenbaum C, et al. Effects of oral insulin in relatives of patients with type 1 diabetes: the Diabetes Prevention Trial—Type 1. *Diabetes Care* 2005;28:1068–76.
- [20] Nakayama M, Abiru N, Moriyama H, Babaya N, Liu E, Miao D, et al. Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice. *Nature* 2005;435:220–3.
- [21] Kent SC, Chen Y, Bregoli L, Clemmings SM, Kenyon NS, Ricordi C, et al. Expanded T cells from pancreatic lymph nodes of type 1 diabetic subjects recognize an insulin epitope. *Nature* 2005;435:224–8.
- [22] Kupila A, Sipila J, Keskinen P, Simell T, Knip M, Pulkki K, et al. Intranasally administered insulin intended for prevention of type 1 diabetes—a safety study in healthy adults. *Diabetes Metab Res Rev* 2003;19:415–20.
- [23] Alleva DG, Gaur A, Jin L, Wegmann D, Gottlieb PA, Pahuja A, et al. Immunological characterization and therapeutic activity of an altered-peptide ligand, NBI-6024, based on the immunodominant type 1 diabetes autoantigen insulin B-chain (9–23) peptide. *Diabetes* 2002;51:2126–34.
- [24] Tiittanen M, Huupponen JT, Knip M, Vaarala O. Insulin treatment in patients with type 1 diabetes induces upregulation of regulatory T-cell markers in peripheral blood mononuclear cells stimulated with insulin in vitro. *Diabetes* 2006;55:3446–54.
- [25] Schatz D, Cuthbertson D, Atkinson M, Salzlner MC, Winter W, Muir A, et al. Preservation of C-peptide secretion in subjects at high risk of developing type 1 diabetes mellitus—a new surrogate measure of non-progression? *Pediatr Diabetes* 2004;5:72–9.
- [26] Vardi P, Dib SA, Tuttleman M, Connelly JE, Grinbergs M, Rabizadeh A, et al. Competitive insulin autoantibody RIA: prospective evaluation of subjects at high risk for development of Type 1 diabetes mellitus. *Diabetes* 1987;36:1286–91.
- [27] Torn C, Mueller PW, Schlosser M, Bonifacio E, Bingley PJ. Diabetes antibody standardization program: evaluation of assays for autoantibodies to glutamic acid decarboxylase and islet antigen-2. *Diabetologia* 2008;51:846–52.
- [28] Grubin CE, Daniels T, Toivola B, Landin-Olsson M, Hagopian WA, Li L, et al. A novel radioligand binding assay to determine diagnostic accuracy of isoform-specific glutamic acid decarboxylase antibodies in childhood IDDM. *Diabetologia* 1994;37:344–50.
- [29] Payton MA, Hawkes CJ, Christie MR. Relationship of 37,000 and 40,000-M triptic fragment of islet antigens in insulin dependent diabetes to the protein tyrosine phosphatase-like molecule IA-2(ICA 512). *JCI* 1995;96:1506–11.
- [30] Durinovic-Bello I, Boehm BO, Ziegler AG. Predominantly recognized proinsulin T helper cell epitopes in individuals with and without islet cell autoimmunity. *J Autoimmun* 2002;18:55–66.
- [31] Kis J, Engelmann P, Farkas K, Richman G, Eck S, Lolley J, et al. Reduced CD4+ subset and Th1 bias of the human iNKT cells in Type 1 diabetes mellitus. *J Leukoc Biol* 2007;81:654–62.
- [32] Novak EJ, Liu AW, Nepom GT, Kwok WW. MHC class II tetramers identify peptide-specific human CD4(+) T cells proliferating in response to influenza A antigen. *J Clin Invest* 1999;104:R63–7.
- [33] Mannering SI, Dromey JA, Morris JS, Thearle DJ, Jensen KP, Harrison LC. An efficient method for cloning human autoantigen-specific T cells. *J Immunol Methods* 2005;298:83–92.
- [34] Oberg HH, Wesch D, Lenke J, Kabelitz D. An optimized method for the functional analysis of human regulatory T cells. *Scand J Immunol* 2006;64:353–60.
- [35] Akatsuka Y, Martin EG, Madonik A, Barsoukov AA, Hansen JA. Rapid screening of T-cell receptor (TCR) variable gene usage by multiplex PCR: application for assessment of clonal composition. *Tissue Antigens*. 1999;53:122–34.
- [36] Seitz S, Schneider CK, Malotka J, Nong X, Engel AG, Wekerle H, et al. Reconstitution of paired T cell receptor alpha- and beta-chains from microdissected single cells of human inflammatory tissues. *Proc Natl Acad Sci U S A*. 2006;103:12057–62.
- [37] Giudicelli V, Chaume D, Lefranc MP. IMGT/V-QUEST, an integrated software program for immunoglobulin and T cell receptor V-J and V-D-J rearrangement analysis. *Nucleic Acids Res* 2004;32:W435–40.
- [38] Fineberg SE, Kawabata TT, Finco-Kent D, Fountaine RJ, Finch GL, Krasner AS. Immunological responses to exogenous insulin. *Endocr Rev* 2007;28:625–52.
- [39] Salk J, Salk D. Control of influenza and poliomyelitis with killed virus vaccines. *Science* 1977;195:834–47.
- [40] Beebe GW, Simon AH, Vivona S. Long-term mortality follow-up of army recruits who received adjuvant influenza virus vaccine in 1951–1953. *Am J Epidemiol* 1972;95:337–46.
- [41] Moreland LW, Morgan EE, Adamson TC, Fronck Z, Calabrese LH, Cash JM, et al. T cell receptor peptide vaccination in rheumatoid arthritis: a placebo-controlled trial using a combination of Vbeta3, Vbeta14, and Vbeta17 peptides [see comments]. *Arthritis Rheum* 1998;41:1919–29.
- [42] Gold DP, Smith RA, Golding AB, Morgan EE, Dafashy T, Nelson J, et al. Results of a phase I clinical trial of a T-cell receptor vaccine in patients with multiple sclerosis. II. Comparative analysis of TCR utilization in CSF T-cell populations before and after vaccination with a TCRV beta 6 CDR2 peptide. *J Neuroimmunol* 1997;76:29–38.
- [43] Trauger RJ, Ferre F, Daigle AE, Jensen FC, Moss RB, Mueller SH, et al. Effect of immunization with inactivated gp120-depleted human immunodeficiency virus type 1 (HIV-1) immunogen on HIV-1 immunity, viral DNA, and percentage of CD4 cells. *J Infect Dis* 1994;169:1256–64.
- [44] Roep BO. T-cell responses to autoantigens in IDDM. The search for the Holy Grail. *Diabetes* 1996;45:1147–56.
- [45] Lindley S, Dayan CM, Bishop A, Roep BO, Peakman M, Tree TI. Defective suppressor function in CD4(+)CD25(+) T-cells from patients with type 1 diabetes. *Diabetes* 2005;54:92–9.
- [46] Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med* 2007;204:1757–64.
- [47] Bradshaw EM, Kent SC, Tripuraneni V, Orban T, Ploegh HL, Hafler DA, et al. Concurrent detection of secreted products from human lymphocytes by microengraving: cytokines and antigen-reactive antibodies. *Clin Immunol* 2008.
- [48] You S, Thiebtemont N, Alyanakian MA, Bach JF, Chatenoud L. Transforming growth factor-beta and T-cell-mediated immunoregulation in the control of autoimmune diabetes. *Immunol Rev* 2006;212:185–202.
- [49] Tang Q, Bluestone JA. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nat Immunol* 2008;9:239–44.
- [50] Jonuleit H, Schmitt E, Kakirman H, Stassen M, Knop J, Enk AH. Infectious tolerance: human CD25(+) regulatory T cells convey suppressor activity to conventional CD4(+) T helper cells. *J Exp Med* 2002;196:255–60.