Online-only Supplemental Material

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Ziegler AG, Albeer A, Arnolds S, et al. Effect of High Dose Oral Insulin in Children with Stage 1 Type 1 Diabetes: The Fr1da – Insulin – Intervention Randomized Controlled Trial

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Supplemental Methods

Randomization and masking

A computer-generated randomization list was prepared with an allocation ratio of 1:1 (placebo to oral insulin). All investigators and participants were masked to the treatment allocation. Unblinding was not necessary during the study.

Investigational Medicinal Product

Insulin crystals were provided by Eli Lilly and Company, Indianapolis, Indiana, USA. The investigational medicinal products (insulin and placebo) were manufactured as identical capsules containing either insulin crystals (7.5 mg or 67.5 mg) in microcrystalline cellulose (total capsule content 200 mg) or 200 mg microcrystalline cellulose placebo by InPhaSol, Apotheke des Universitätsklinikums Heidelberg, Germany. The drug packages were sequentially numbered according to the randomly allocated treatment. Participants and parents were instructed to administer the contents of one capsule once a day with a small meal, preferably in the morning.

Measurements related to safety

Venous blood glucose concentrations were measured at a central certified laboratory (enzymatic reference method with hexokinase). The families were instructed to note any symptoms of hypoglycemic events such as trembling, sweating, or impaired consciousness that occurred after the child took the study drug. Hypoglycemia was defined as a blood glucose level <50 mg/dL (<2.8 mmol/L). Serum insulin and C-peptide concentrations were measured by fluorescence enzyme immunoassays using an automated immunoassay analyzer (AIA-360, Tosoh Bioscience Inc., South San Francisco, CA). Blood cell counts, blood chemistry, electrolytes, and HbA1c were measured by the central certified laboratory (Medizinisches Dienstleistungszentrum – Medizet, Städtisches Klinikum München GmbH, Munich, Germany).
Islet autoantibodies were measured at the Institute of Diabetes Research, Helmholtz Munich. Autoantibodies to glutamic acid decarboxylase (GADA), islet antigen-2 (IA-2A), and zinc transporter-8 (ZnT8A) were measured using harmonized radio binding methods. GADA and IA-2A were measured by the National Institute of Diabetes and Digestive and Kidney Diseases harmonized assay protocol using ^{35}S-methionine-labeled N-terminally truncated GAD65 (amino acids 96-585) or IA-2ic (amino acids 606-979), as previously described (1). ZnT8A were measured by assays to separately detect autoantibodies to the arginine 325R and tryptophan 325W human ZnT8 variants (ZnT8RA and ZnT8WA, respectively) using ^{35}S-methionine-labeled recombinant ZnT8 (amino acids 268-369), as previously described (2). Samples were classified as ZnT8A positive if they were positive for ZnT8RA and/or ZnT8WA. The assays had sensitivities and specificities of 66% and 99% for GADA, 76% and 100% for IA-2A, 56% and 99% for ZnT8RA, and 50% and 99% for ZnT8WA in the Islet Autoantibody Standardization Program (IASP) 2016 Workshop (3).

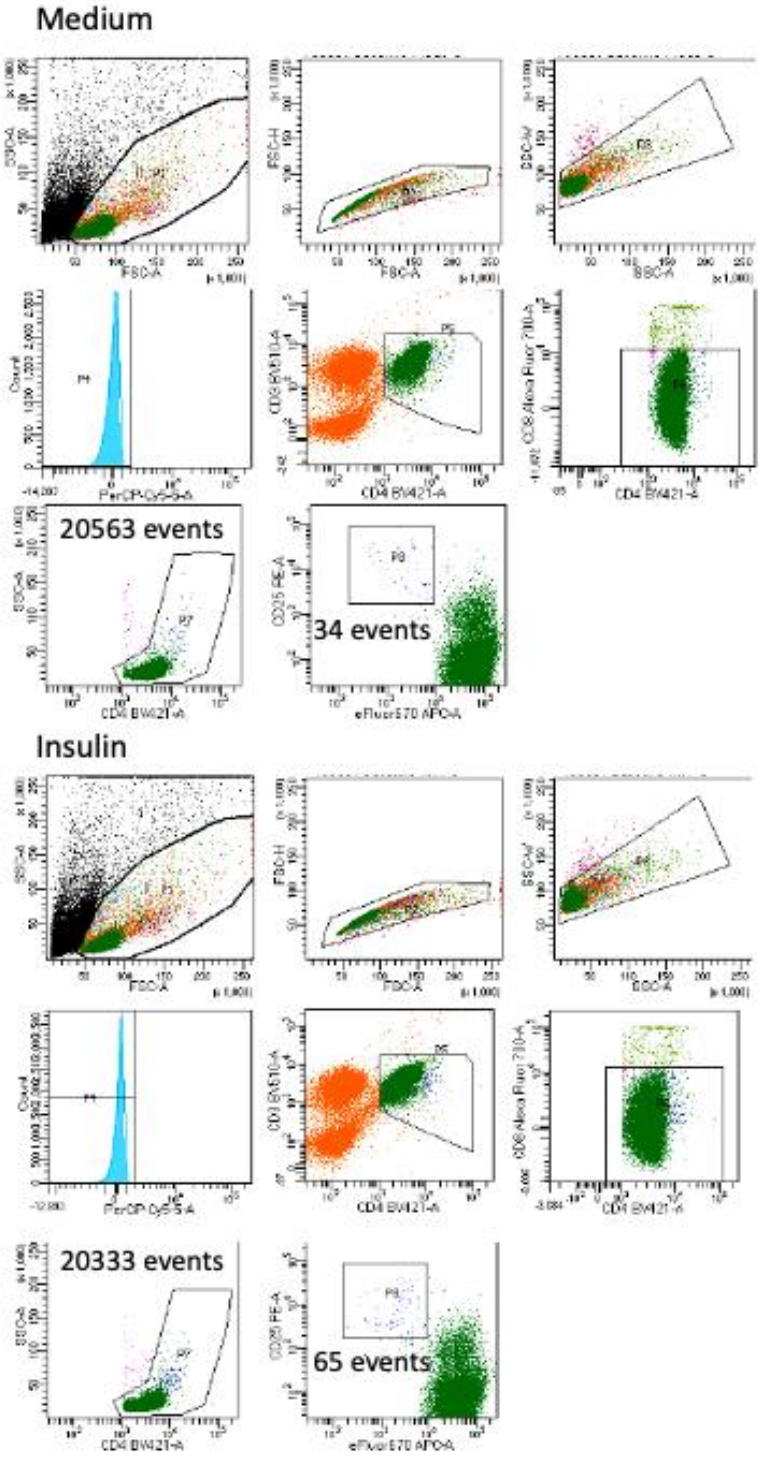
Throughout the study, the investigators recorded any adverse events using an adverse event clinical report form, regardless of the event's severity or relation to the study drug or study procedure.

Measurements of immunological response

The primary immune efficacy outcome was an immune response to insulin, defined as an increase in any one or more of the following: serum insulin autoantibodies (IAA), salivary IgA antibodies to insulin, or a $CD4^+$ T cell response to insulin. IAA levels were measured using a competitive radio binding assay (4). The assay had a sensitivity of 54% and a specificity of 99% in the IASP 2016 Workshop. Salivary IgA binding to insulin was measured using a radio binding assay as previously described (5). $CD4^+$ T cell antigen responses were measured using stored frozen peripheral blood mononuclear cells (PBMCs). Responses were measured using a dye (Cell Proliferation Dye eFluor 670, eBioscience, San Diego, CAL, USA) dilution assay, quantifying proliferation ($eFluor670^{dim}$ cells) and activation ($CD25^+$) after 5 days of culture without or with the antigen insulin that was identical to the insulin administered to the children (50 µg/ml, Eli Lilly and Company, Indianapolis, USA) as previously described (5). The assay included a median of 12 wells containing 200,000 eFlour670 dye-labelled cells in medium plus insulin and 6 wells with cells and medium alone. Cell staining was performed with the following cocktail:

Marker	Fluorochrome	µL/ 100 µL/1x10^6 PBMC	Clone	Provider	Catalog #
CD4	BV421	3	RPA-T4	BD	562424
CD3	BV510	2	SK7	Biolegend	344828
CD8	Alexa fluor 700	1	SK1	Biolegend	344724
CD45RO	PE-Cy7	1	UCHL1	BD	337168
CD45RA	BV650	0.25	HI100	Biolegend	304135
7AAD	PerCP	2.5		BD	559925
CD25	PE	2	M-A251	BD	555432
CD70	BB515	1	Ki-24	BD	565156
CD71	BV786	1	M-A712	BD	563768
CD54	BV711	0,5	HA58	BD	564078
CD49d	APC-Cy7	1	9F10	Biolegend	304328
Proliferation dye	efluor670			eBios	65-0840-90

The cells from batches of three wells (insulin wells 1-3, 4-6, 7-9, 10-12; medium 1-3, 4-6) were pooled and analyzed using a flow cytometer (LSR Fortessa, Becton Dickinson, Franklin Lakes, NJ). The SI was calculated as the total number of $CD4^+eFluor670^{dim}CD25^+$ cells per 50,000 acquired live $CD4^+$ T cells in all wells containing insulin relative to the number of $CD4^+eFluor670^{dim}CD25^+$ cells per 50,000 acquired live $CD4^+$ T cells in all wells containing medium alone. A positive sample was defined as an SI of >3. The gating strategy is shown below for a batch of 3 medium and a batch of three insulin wells. $CD8^+$ T cell proliferation responses to insulin were also measured in the same assay by gating on $CD8^+CD4^-$ T cells.

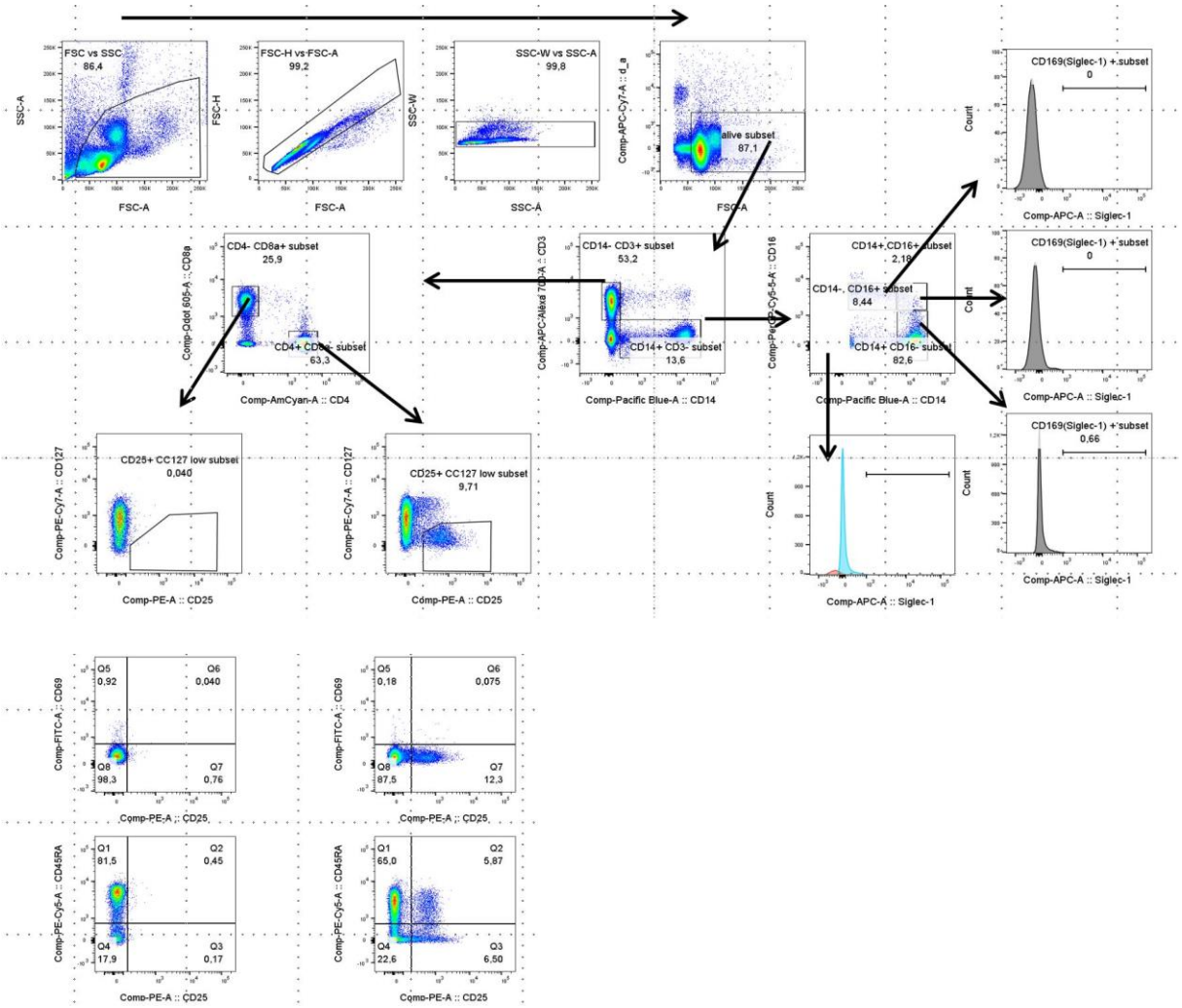


Criteria for the primary outcome “immune responder”: 1) IAA. A positive response was defined as a positive value (>1.5) and an at least 2-fold increase over the baseline value at any of the 3 months, 6 months, 9 months, or 12 months visits. Values below 1.2 were adjusted to 1.2 for the calculation of change. 2) Salivary IgA-insulin. Results were expressed as a standard deviation score (SDS) calculated from the standard deviation of the cpm-binding to insulin of untreated participants (baseline values) and the background corrected cpm for each visit. A positive response was defined as a value of 3 SDS and >3-fold the baseline SDS value (ratio >3) at any of the 3 months, 6 months, 9 months, or 12 months visits. 3) $CD4^+$ T cell response to insulin. A positive response was defined as at least a 2-fold change in the stimulation index (SI) from baseline at any of the 3 months, 9 months, or 12 months follow-up visits. Change could be at least a 2-fold increase in which case the follow-up visit value must be an SI >3.0 or could be at least a 2-fold decrease in which case the baseline visit value must be an SI >3.0. A primary outcome positive response for immune response was defined as a positive response in any of 1), 2), or 3). An “immune responder” was defined as a participant with an antibody or positive T cell response to insulin at any time point during treatment. The number of responders in the insulin treated group was compared with the number of responders in the placebo treated group.
Other secondary outcomes included: 1) $CD4^+$ T cell and $CD8^+$ T cell responses to insulin, to proinsulin (10 μg/ml), and to GAD65 (10 μg/ml). The stimulation index was compared between oral insulin group and placebo group at baseline, 3 months, 9 months, and 12 months using Mann-Whitney U tests. 2) The frequency of circulating Insulin-tetramer positive that had a regulatory T cell phenotype (measured at 6 months) was compared between placebo and study drug treated children using the Mann-Whitney U test. In addition, the secondary outcomes were examined after stratification for HLA DR4-DQ8 haplotype (yes/no) and *INS* genotype at rs689 (susceptible AA vs other). Other covariates investigated for their influence on outcome were sex, age, the presence of a first-degree relative with type 1 diabetes, and SIGLEC1 (CD169) positivity of monocytes (a marker of type 1 interferon response).

Phenotyping of lymphocytes and monocytes

Freshly isolated PBMCs (2.5×10^5 cells) were incubated for 1 min at room temperature with Fc receptor blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany). Cell surface markers were stained for 20 min at 4 °C in phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} ($PBS^{-/-}$; Gibco) containing 0.5% bovine serum albumin using the following mouse anti-human monoclonal antibodies: anti-CD3 Alexa Fluor 700 (clone HIT3α; BioLegend, San Diego, CAL), anti-CD4 Brilliant Violet 510 (SK3; BD Biosciences, San Jose, CAL), anti-CD8a Brilliant Violet 605 (RPA-T8), anti-CD14 Pacific Blue (HCD14), anti-CD16 PerCP-Cy5.5 (3G8; all BioLegend), anti-CD25 PE (M-A251; BD Biosciences), anti-CD45RA PE-Cy5 (HI100; BioLegend), anti-CD69 fluorescein isothiocyanate (FN50; BD Biosciences), anti-CD127 PE-Cy7 (A019D5) and anti-CD169 Alexa Fluor 647 (7-239; both BioLegend).

Cells were washed twice in $PBS^{-/-}$ and stained for 20 min at room temperature with Zombie NIR (BioLegend) to evaluate cell viability. PBMCs were fixed with 1.5% formalin in $PBS^{-/-}$ and analyzed within 24 h on a flow cytometer (LSR Fortessa, Becton Dickinson, Franklin Lakes, NJ) using FACSDiva acquisition software (Version 7.0; BD Biosciences). FlowJo software (Version 10; TreeStar Inc., Ashland, OR) was used to analyze lymphocyte and monocyte subsets. Typical gating strategies are shown below.

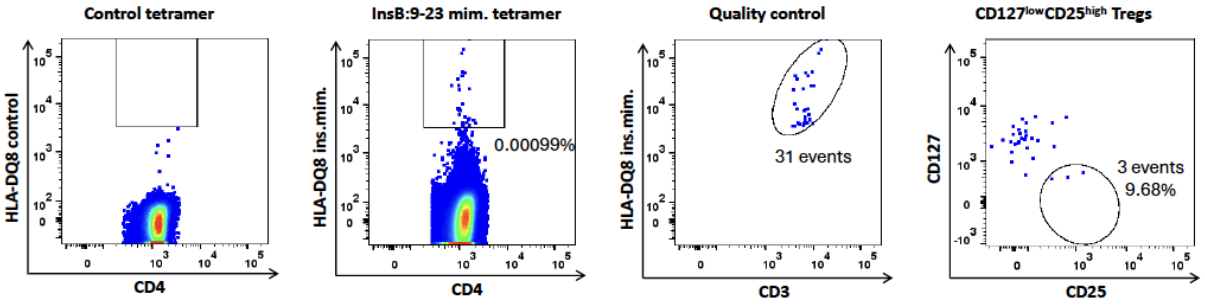


Gating strategy used to derive the frequencies of cell populations shown in Supplemental Table S4.

Insulin-specific HLA-DQ8-restricted tetramer staining

Fluorescent HLA-DQ8-tetramers based on insulin-B-chain-10-23-mimetopes were used to identify human insulin-specific $CD4^+$ T cells as previously described (6). Two insulin-HLA-DQ8-PE-labelled tetramers were combined in staining: a 14E-21E-22E and a 14E-21G-22E-tetramer. For the HLA-DQ8-restricted insulin-specific tetramer staining, PBMCs were used and $CD4^+$ T cells were purified by negative MACS selection and incubated with insulin-specific HLA-DQ8-tetramers for 1 hour at 37 °C in humidified 5% CO_2 with gentle agitation every 20 min followed by direct staining with antibodies for additional surface markers and exclusion of dead cells (Sytox

Blue) for 20 min at 4 °C. A set of exclusion markers (CD8, CD11b, CD19, CD14 and a dead cell exclusion marker (Sytox Blue) was used to increase specificity of the staining. As negative controls, we used a combination of two HLA-DQ8-tetramers fused to irrelevant peptides (PVSKMRMATPLLMQA and QDLELSWNLNGLQADL) and labelled with PE. Virtually no tetramer^{+}CD4^{+}T cells were detected with the control tetramers. An example of the tetramer analysis is shown below.



INS and *HLA DR-DQ* genotyping

INS SNP rs689 typing was performed at a central genotyping laboratory (Life & Brain GmbH, Bonn, Germany) using DNA extracted from dried blood spots or venous blood. Genotyping of *HLA DR-DQ* was performed using SNP data generated with the Infinium® ImmunoArray-24 v2.0 BeadChip (Illumina Inc., San Diego, USA).

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Supplemental Table S1. Immune outcomes of children enrolled in the Fr1da-Insulin-Intervention-Study

	Primary Immune response		Insulin autoantibody response		Saliva IgA-IAA response		$CD4^+$ T cell response to insulin	
	Placebo	Insulin	Placebo	Insulin	Placebo	Insulin	Placebo	Insulin
All tested children	13/42	11/44	9/42	9/44	2/40	2/38	3/38	2/38
INS genotype								
- susceptible	8/27	8/34	5/27	6/34	2/27	2/30	2/26	2/28
- non-susceptible	5/15	3/10	4/15	3/10	0/15	0/10	1/12	0/10
HLA DR4-DQ8								
- present	10/25	7/28	7/25	7/28	1/26	1/24	2/21	1/24
- absent	3/17	3/10	2/17	1/10	1/16	1/10	1/17	1/9
Sex								
- boys	7/27	5/25	4/27	3/25	2/27	1/23	2/24	1/24
- girls	6/15	6/19	5/15	6/19	0/15	1/17	1/14	1/14
SIGLEC1								
- positive	5/22	7/23	5/22	6/23	0/21	1/22	0/19	2/19
- negative	8/20	4/21	4/20	3/21	2/21	1/18	3/19	0/19

Supplemental Table S2. Overview of blood glucose, insulin, C-peptide and insulin/C-peptide ratio values of the study participants before and after taking placebo or oral insulin at baseline and at the 3-month visit

	Time (min)*	n	Placebo	n	Oral Insulin	*P* value
Blood glucose [mg/dl]	-10	110	80.0 (74.0, 87.3)	110	80.0 (74.0, 86.0)	.97
at baseline visit,	+30	109	106.0 (92.0, 120.0)	110	105.5 (91.0, 119.0)	.99
median (IQR)	+60	109	91.0 (80.0, 104.0)	110	93.0 (81.0, 106.0)	.52
	+120	110	90.0 (82.0, 103.0)	110	92.5 (81.0, 102.0)	.79
Blood glucose [mg/dl]	-10	106	80.0 (76.0, 86.0)	108	80.0 (74.5, 86.0)	.91
at 3-month visit,	+30	105	106.0 (92.0, 120.0)	108	106.0 (95.5, 118.0)	.87
median (IQR)	+60	106	88.0 (82.0, 112.0)	107	91.0 (78.0, 110.0)	.93
	+120	106	89.5 (82.0, 103.0)	108	91.5 (81.0, 102.5)	.74
Insulin [µU/ml]	-10	106	3.2 (1.8, 4.6)	106	3.4 (1.8, 5.9)	.17
at baseline visit,	+30	106	17.9 (10.6, 31.1)	108	24.9 (14.3, 33.4)	.022
median (IQR)	+60	106	15.4 (9.0, 29.1)	108	19.0 (12.1, 29.7)	.13
	+120	105	14.10 (9.3, 21.8)	106	16.3 (8.6, 24.7)	.36
Insulin [µU/ml]	-10	97	3.6 (2.4, 5.2)	105	3.9 (2.4, 6.4)	.45
at 3-month visit,	+30	94	19.2 (10.3, 33.2)	103	22.3 (13.5, 41.6)	.15
median (IQR)	+60	95	18.3 (10.2, 28.4)	103	19.0 (10.4, 30.8)	.59
	+120	94	14.4 (7.7, 23.0)	103	17.6 (11.2, 25.7)	.07
C-peptide [ng/ml]	-10	106	0.7 (0.5, 0.8)	106	0.7 (0.5, 1.0)	.20
at baseline visit,	+30	106	2.0 (1.4, 3.0)	107	2.4 (1.8, 3.3)	.09
median (IQR)	+60	106	2.4 (1.6, 3.7)	107	2.5 (2.0, 3.5)	.22
	+120	105	2.4 (1.8, 3.3)	107	2.5 (1.8, 3.3)	.58
C-peptide [ng/ml]	-10	97	0.7 (0.5, 0.9)	105	0.8 (0.6, 1.0)	.28
at 3-month visit,	+30	94	2.2 (1.4, 3.2)	103	2.4 (1.6, 3.6)	.24
median (IQR)	+60	95	2.7 (1.9, 3.6)	103	2.5 (1.9, 3.8)	.89
	+120	94	2.4 (1.7, 3.4)	103	2.8 (1.9, 3.6)	.18
Insulin/C-peptide ratio	-10	106	0.10 (0.08, 0.13)	106	0.11 (0.08, 0.13)	.40
at baseline visit,	+30	106	0.18 (0.13, 0.23)	107	0.20 (0.16, 0.26)	.007
median (IQR)	+60	106	0.14 (0.11, 0.18)	107	0.15 (0.12, 0.19)	.10
	+120	105	0.13 (0.10, 0.15)	105	0.13 (0.10, 0.17)	.72
Insulin/C-peptide ratio	-10	97	0.11 (0.08, 0.13)	105	0.11 (0.08, 0.14)	.98
at 3-month visit,	+30	94	0.20 (0.15, 0.24)	102	0.20 (0.17, 0.25)	.47
median (IQR)	+60	95	0.14 (0.11, 0.18)	103	0.15 (0.12, 0.18)	.38
	+120	94	0.13 (0.09, 0.16)	103	0.14 (0.11, 0.16)	.11

*Time relative to treatment application

Supplemental Table S3. Overview of height-, weight-, and body mass index-values for age (z-scores) at each visit during the intervention period for study participants who received placebo or oral insulin

	Visit at	n	Placebo	n	Oral Insulin	*P* value
Height-for-age,	Baseline	106	0.69 (0.09, 1.17)	106	0.59 (-0.04, 1.63)	.99
z-score;	3 months	104	0.73 (0.24, 1.46)	103	0.68 (-0.03, 1.49)	.67
median (IQR)	6 months	102	0.73 (0.26, 1.46)	103	0.76 (0.12, 1.49)	.71
	12 months	93	0.83 (0.32, 1.46)	101	0.67 (0.09, 1.47)	.38
Weight-for-age,	Baseline	105	0.64 (-0.07, 1.15)	99	0.51 (-0.20, 1.20)	.58
z-score;	3 months	101	0.52 (-0.02, 1.04)	95	0.46 (-0.17, 1.21)	.84
median (IQR)	6 months	99	0.55 (-0.10, 1.08)	90	0.57 (-0.13, 1.26)	.96
	12 months	89	0.65 (0.11, 1.16)	88	0.48 (-0.25, 1.12)	.36
BMI-for-age,	Baseline	106	0.23 (-0.27, 0.74)	106	0.13 (-0.36, 0.79)	.95
z-score;	3 months	104	0.07 (-0.47, 0.77)	103	0.25 (-0.50, 0.79)	.48
median (IQR)	6 months	102	0.15 (-0.56, 0.73)	102	0.23 (-0.44, 0.81)	.39
	12 months	93	0.17 (-0.43, 0.56)	101	0.08 (-0.47, 0.86)	.88

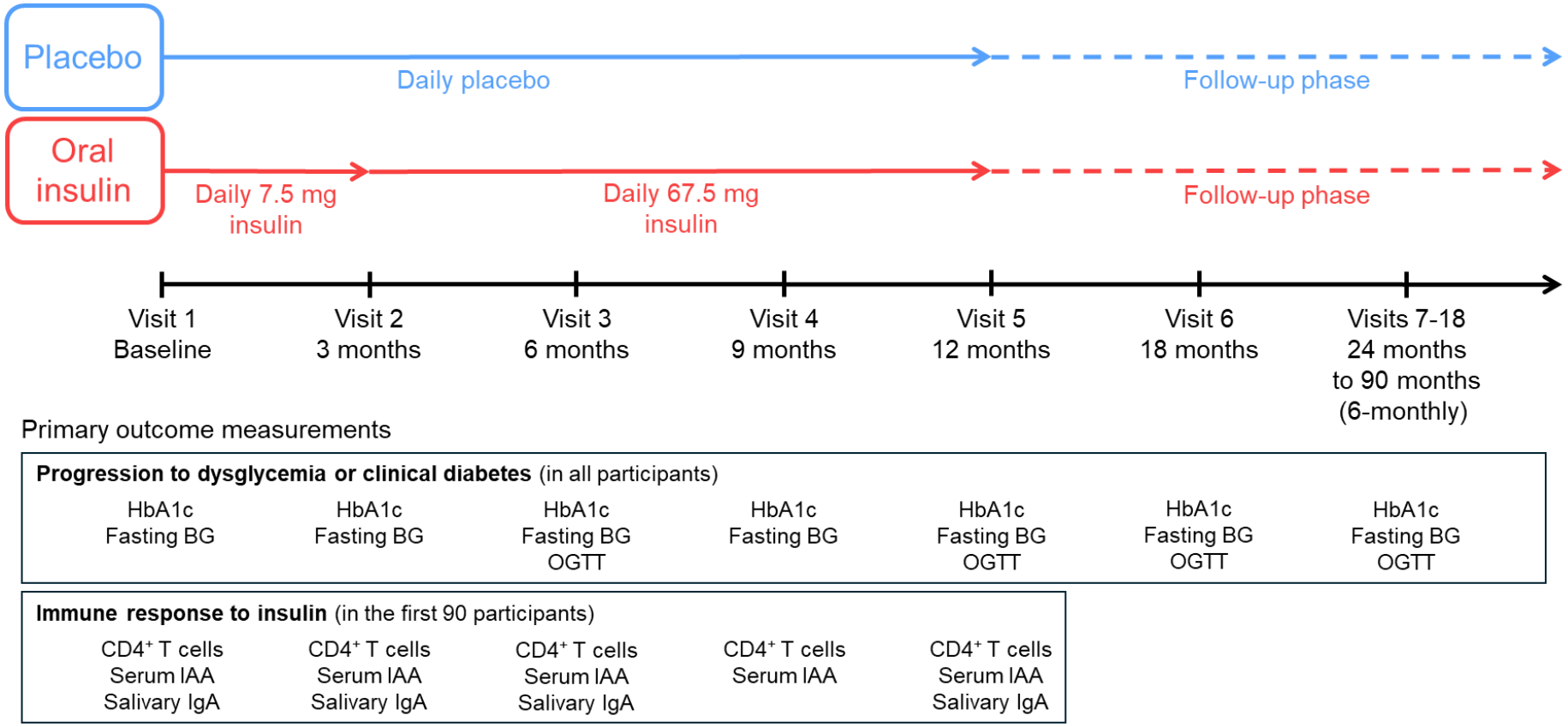
BMI, body mass index

Supplemental Table S4. Frequencies of peripheral blood mononuclear cell (PBMC) subpopulations at the baseline visit and after 12 months of treatment with oral insulin or placebo

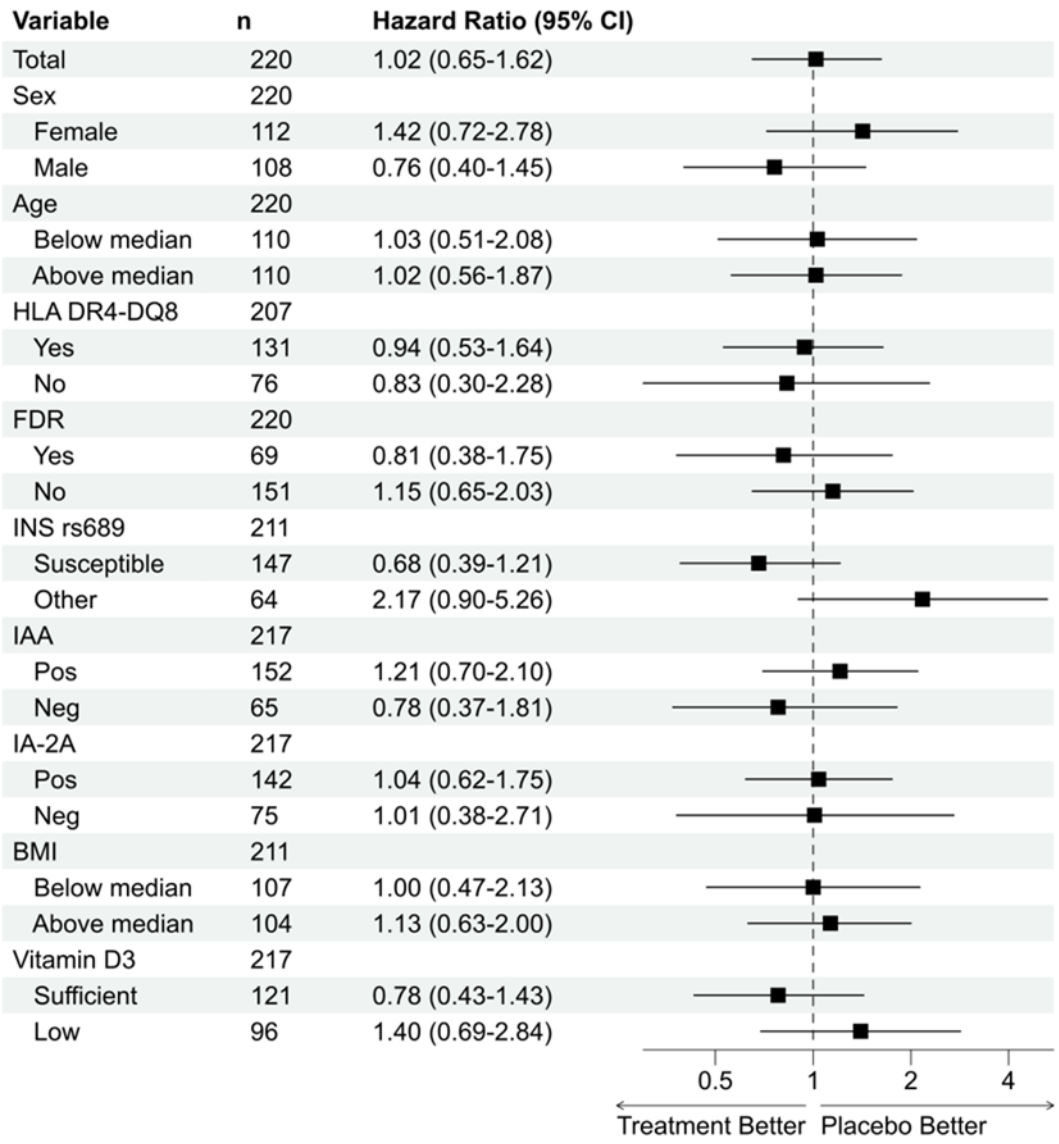
	PBMC samples at baseline					PBMC samples after 12 months of treatment				
	Children treated with oral insulin		Children treated with placebo			Children treated with oral insulin		Children treated with placebo		
PBMC subpopulation	n	Frequency (%), median (IQR)	n	Frequency (%), median (IQR)	*P* value	n	Frequency (%), median (IQR)	n	Frequency (%), median (IQR)	*P* value
CD3+ T lymphocytes	107	68.4 (65.1; 72.9)	108	67.0 (62.4; 73.0)	.32	94	70.3 (64.6; 75.1)	95	68.7 (62.0; 72.6)	.04
CD4+ T lymphocytes	107	62.1 (55.7; 67.3)	108	62.8 (56.1; 65.9)	.98	94	59.5 (55.0; 66.1)	95	59.7 (54.1; 65.4)	.86
CD4+ CD45RA+	107	73.2 (69.5; 80.0)	107	73.2 (68.6; 78.4)	.65	95	74.4 (69.4; 81.1)	95	72.5 (69.3; 78.0)	.25
CD4+ CD69+	106	0.1 (0.1; 0.2)	107	0.2 (0.1; 0.2)	.15	93	0.1 (0.1; 0.2)	95	0.1 (0.1; 0.2)	.87
CD4+ CD25+ $CD127^{low}$	107	6.8 (5.6; 8.3)	108	6.8 (5.3; 7.7)	.24	94	6.7 (5.3; 8.4)	94	6.8 (5.9; 7.9)	.67
CD8a+ T lymphocytes	107	25.7 (21.9; 32.1)	108	27.4 (23.6; 30.8)	.36	94	26.0 (22.1; 32.2)	95	27.9 (23.5; 32.3)	.56
CD8a+ CD45RA+	107	89.0 (84.3; 92.7)	107	87.7 (81.3; 92.7)	.19	93	90.6 (85.0; 93.3)	95	88.5 (80.9; 93.2)	.35
CD8a+ CD69+	107	0.6 (0.3; 0.8)	107	0.5 (0.3; 0.8)	.79	93	0.5 (0.3; 0.8)	95	0.5 (0.4; 0.8)	.36
CD4/CD8 ratio (% / %)	107	2.4 (1.8; 3.0)	108	2.3 (1.8; 2.8)	.46	94	2.2 (1.7; 2.9)	95	2.1 (1.7; 2.7)	.55

Supplemental Table S4 continued

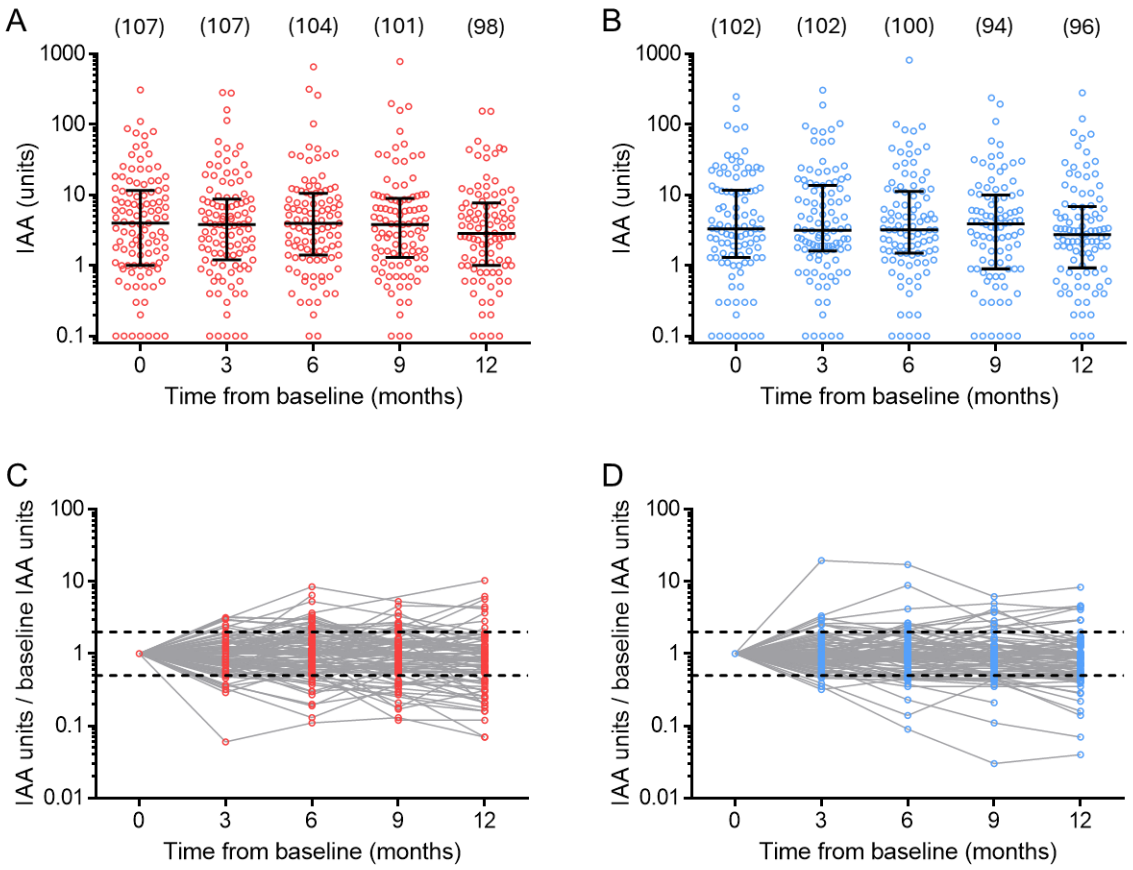
	PBMC samples at baseline					PBMC samples after 12 months of treatment				
	Children treated with oral insulin		Children treated with placebo			Children treated with oral insulin		Children treated with placebo		
PBMC subpopulation	n	Frequency (%), median (IQR)	n	Frequency (%), median (IQR)	*P* value	n	Frequency (%), median (IQR)	n	Frequency (%), median (IQR)	*P* value
CD14+ CD3- (monocytes)	106	6.5 (4.9; 8.2)	108	6.6 (4.9; 8.8)	.42	94	6.7 (4.7; 9.4)	95	6.7 (5.1; 9.0)	.76
CD14+ CD16+	106	1.7 (1.2; 2.6)	108	2.0 (1.0; 3.0)	.37	94	2.0 (1.2; 2.9)	95	1.8 (1.1; 3.0)	.60
CD14+ CD16-	106	85.5 (80.5; 89.0)	108	84.0 (78.8; 87.6)	.20	94	85.7 (80.9; 89.0)	95	85.5 (80.5; 88.9)	.99
CD14+ CD16- / CD169(Siglec-1)+	106	1.2 (0.6; 2.7)	108	1.4 (0.8; 3.3)	.16	94	1.1 (0.6; 2.3)	95	1.2 (0.8; 3.4)	.14
$CD14^{low}$ CD16+	106	6.2 (3.8; 9.0)	108	6.6 (4.7; 9.7)	.28	94	5.7 (4.0; 9.1)	95	5.7 (3.9; 9.5)	.77



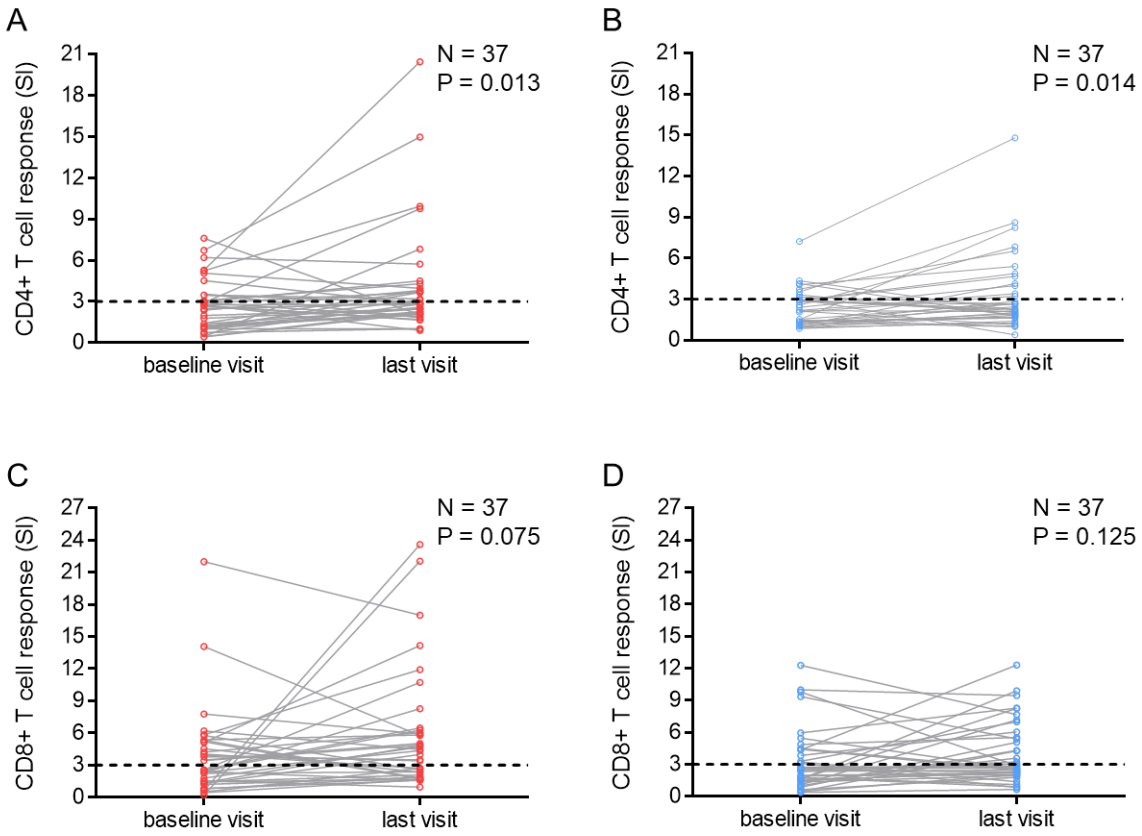
Supplemental Figure S1. Schematic representation of the study design.



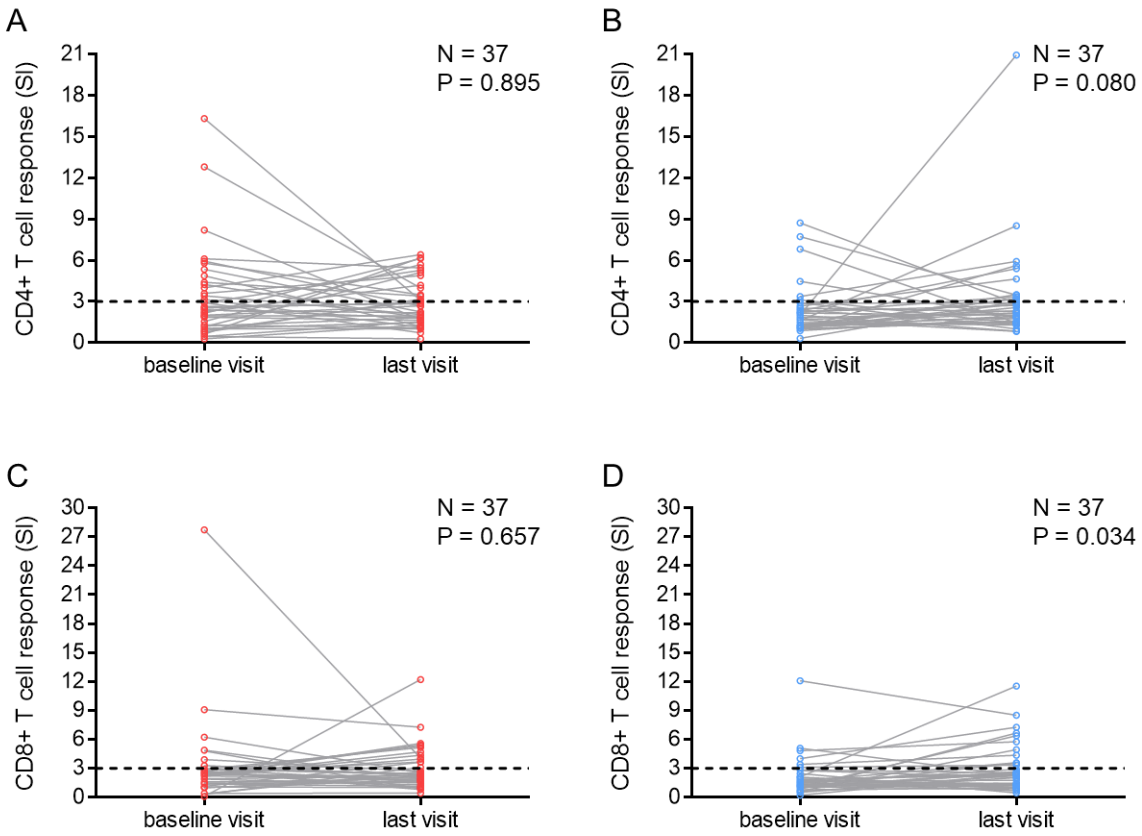
Supplemental Figure S2. Subgroup analyses for the effect of oral insulin vs placebo treatment on the risk of developing clinical diabetes in participants with stage 1 type 1 diabetes. Shown are results of a univariate Cox proportional hazards model of covariates associated with the effect of treatment on the development of the secondary outcome clinical diabetes. Subgroups are defined by the status at baseline.



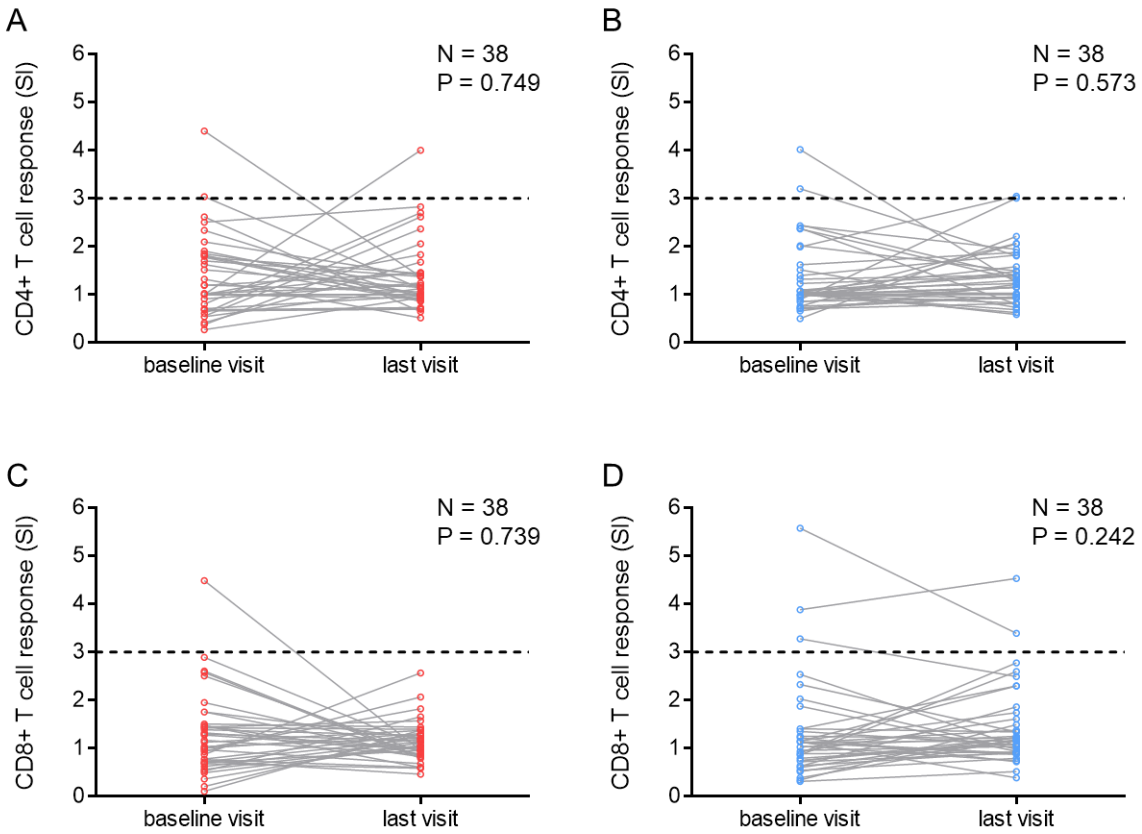
Supplemental Figure S3. Insulin autoantibody (IAA) response over time. IAA titers were determined in sera collected at visit 1 at baseline and 3, 6, 9 and 12 months after baseline from participants receiving oral insulin (**A, C**) or placebo (**B, D**). IAA units are plotted for each time point, with the number of participants with available measurements indicated in parentheses above the respective plots (**A, B**). The median values (IQR) are indicated. To illustrate the extent of change in IAA titers during the treatment phase, the IAA units of each participant for each time point are presented as a quotient relative to the IAA units at baseline (**C, D**). The ratios for the individual participants are connected by lines. The upper and lower dashed lines show the threshold values for a two-fold change in IAA units compared to the baseline value. The frequency of an at least two-fold increase or decrease in IAA units compared to baseline did not differ significantly in participants receiving oral insulin (**C**) or placebo (**D**).



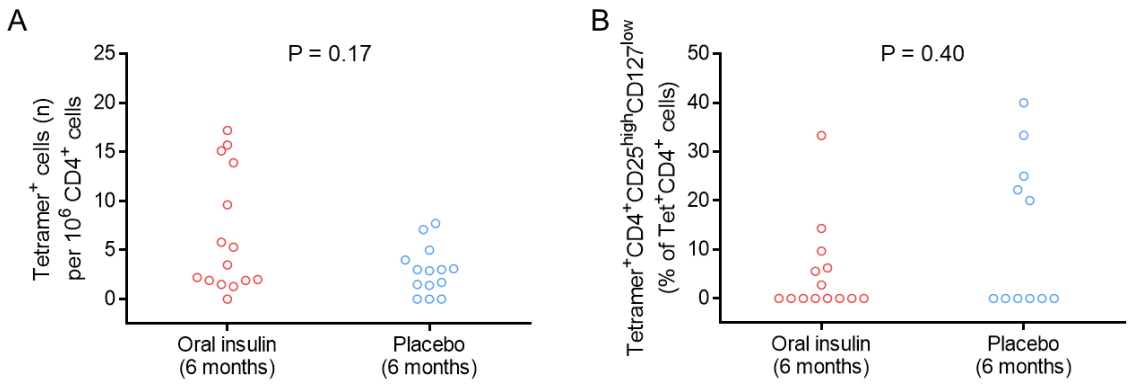
Supplemental Figure S4. Analysis of changes in the T cell response to GAD_{65} during the treatment phase. Shown are T cell responses of $CD4^+$ T cells (**A, B**) and $CD8^+$ T cells (**C, D**) to human GAD_{65} autoantigen, expressed as stimulation index (SI), in participants receiving oral insulin (**A, C**) or placebo (**B, D**). T cell response values of individual participants at baseline and at the last visit at which a T cell assay was performed are connected by lines. The number of participants for whom sufficient sample material was available at both time points to perform the respective T cell tests is indicated. The dashed line represents the threshold value (SI >3) for a GAD_{65}-specific positive T cell response. Significant increases in the T cell response to GAD_{65} over time were observed in both treatment groups for $CD4^+$ T cells (Wilcoxon matched pairs signed rank test).



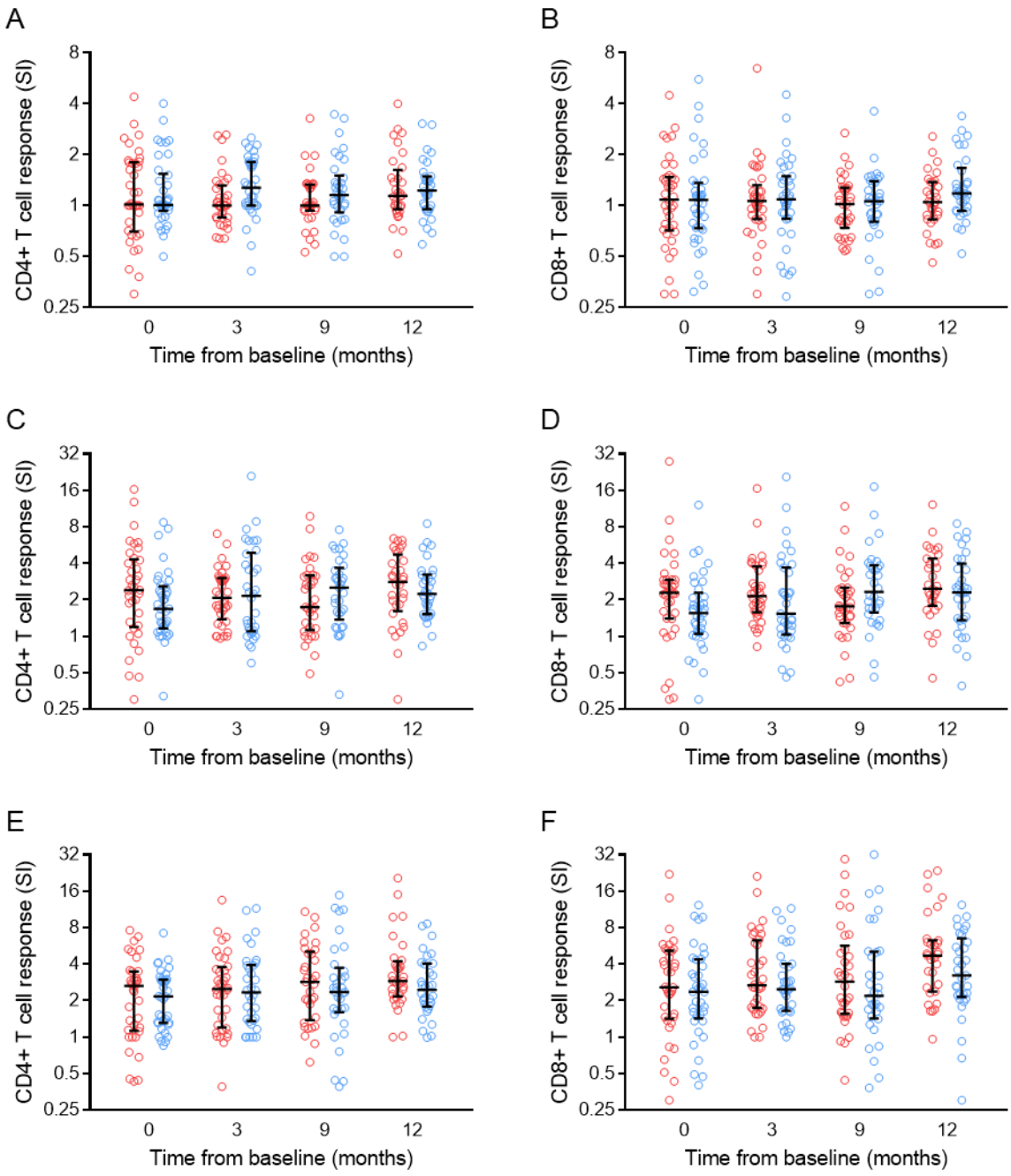
Supplemental Figure S5. Analysis of changes in the T cell response to proinsulin during the treatment phase. Shown are T cell responses of $CD4^+$ T cells (**A, B**) and $CD8^+$ T cells (**C, D**) to human proinsulin autoantigen, expressed as stimulation index (SI), in participants receiving oral insulin (**A, C**) or placebo (**B, D**). T cell response values of individual participants at baseline and at the last visit at which a T cell assay was performed are connected by lines. The number of participants for whom sufficient sample material was available at both time points to perform the respective T cell tests is indicated. The dashed lines represent the threshold value (SI >3) for a proinsulin-specific positive T cell response. Significant increases in the T cell response to proinsulin over time were observed for $CD8^+$ T cells in the placebo group (Wilcoxon matched pairs signed rank test).



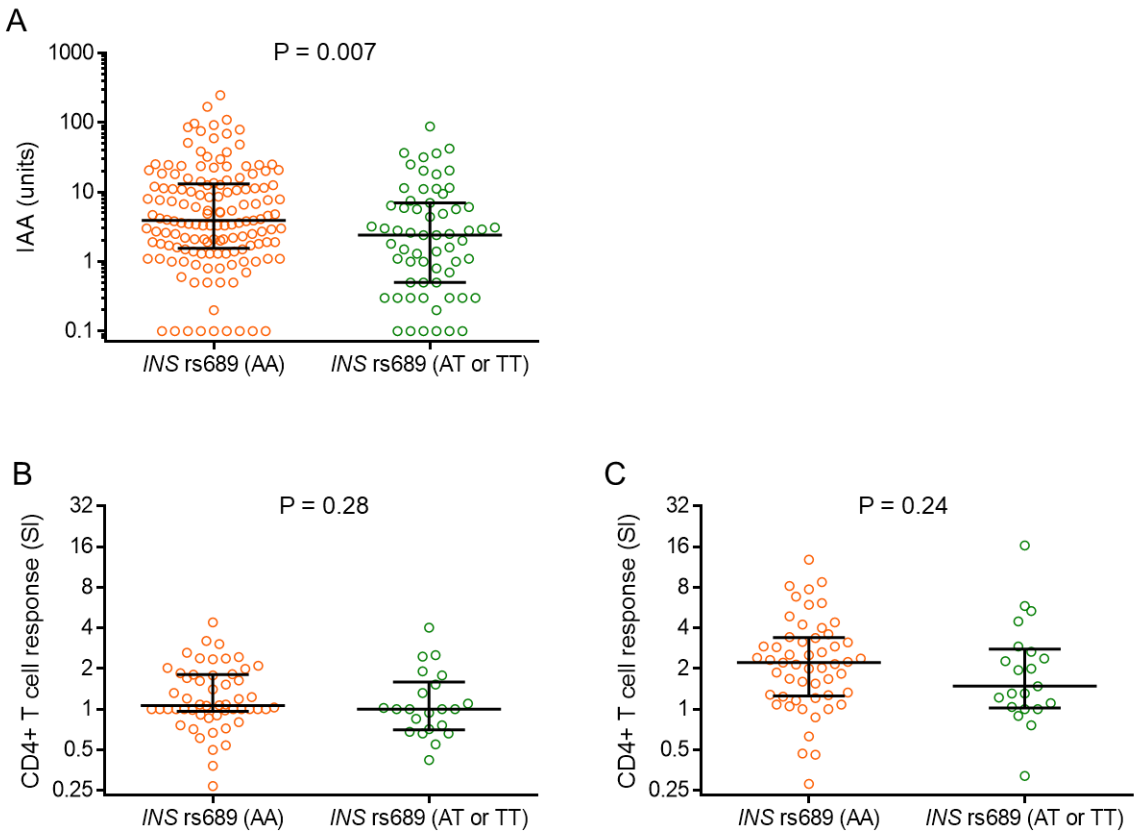
Supplemental Figure S6. Analysis of changes in the T cell response to insulin during the treatment phase. Shown are T cell responses of $CD4^+$ T cells (**A, B**) and $CD8^+$ T cells (**C, D**) to human insulin autoantigen, expressed as stimulation index (SI), in participants receiving oral insulin (**A, C**) or placebo (**B, D**). T cell response values of individual participants at baseline and at the last visit at which a T cell assay was performed are connected by lines. The number of participants for whom sufficient sample material was available at both time points to perform the respective T cell tests is indicated. The dashed lines represent the threshold value (SI >3) for an insulin-specific positive T cell response. No significant changes in the T cell response to insulin were observed over time in either treatment group (Wilcoxon matched pairs signed rank test).



Supplemental Figure S7. Insulin tetramer-positive Treg cells. Shown is the number of tetramer^{+} cells per 10^{6} CD4^{+} T cells (**A**) and the proportion of tetramer^{+}CD4^{+}CD25^{high}CD127^{low} regulatory T cells as a percentage of tetramer^{+}CD4^{+} cells (**B**) in participants receiving oral insulin or placebo 6 months after the start of treatment. There are no significant differences between the treatment groups.



Supplemental Figure S8. Analysis of T cell responses during the treatment phase. Shown are T cell responses of $CD4^+$ T cells (**A, C, E**) and $CD8^+$ T cells (**B, D, F**) to human insulin (**A, B**), proinsulin (**C, D**), and GAD_{65} (**E, F**) at visit 1 at baseline and 3, 6, 9 and 12 months after baseline, expressed as stimulation index (SI), from participants receiving oral insulin (red circles) or placebo (blue circles). The median values (IQR) are indicated. There are no significant differences in T cell responses between the treatment groups at the respective time points during treatment.



Supplemental Figure S9. Immune responses to insulin and proinsulin with respect to *INS* rs689 genotype at baseline. Shown are insulin autoantibody (IAA) titers (**A**) and T cell responses of $CD4^+$ T cells to human insulin (**B**) and proinsulin (**C**), expressed as stimulation index (SI), for participants with the type 1 diabetes-susceptible *INS* rs689 (AA) genotype (orange circles) as compared to children with non-susceptible *INS* rs689 (AT or TT) genotypes (green circles). The median values (IQR) are indicated. IAA titers are significantly higher in participants with the susceptible *INS* rs689 genotype. There are no significant differences in T cell responses between the groups.