Increased Interleukin-35 Levels in Patients With Type 1 Diabetes With Remaining C-Peptide

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OBJECTIVE

Many patients with long-standing type 1 diabetes have remaining functional β-cells. This study investigated immunological differences between patients with or without measurable remaining endogenous insulin production after ≥10 years duration of disease.

RESEARCH DESIGN AND METHODS

Patients (*n* = 113; ≥18 years of age) with type 1 diabetes and with disease duration of ≥10 years were recruited at Uppsala University Hospital. Residual β-cell function was determined with an ultrasensitive C-peptide ELISA. Circulating cytokines, including interleukin-35 (IL-35), were determined in plasma. Additional blood samples were collected from 14 of the identified C-peptide–positive patients and 12 of the C-peptide–negative patients, as well as from 15 healthy control subjects, and were used for immediate investigation of peripheral blood mononuclear cells.

RESULTS

The blood concentration of the cytokine IL-35 was markedly lower in C-peptide–negative patients, and this was associated with a simultaneous decrease in the proportion of IL-35$^+$ regulatory T cells (Tregs), IL-35$^+$ regulatory B cells, and IL-35–producing CD8$^+$Foxp3$^+$ cells. IL-35 has previously been shown to maintain the phenotype of Tregs, block the differentiation of T-helper 17 cells, and thereby dampen immune assaults to β-cells. We found that the proportions of IL-17a$^+$ cells among the Tregs, CD4$^+$ T cells, and CD8$^+$ T cells were lower in the C-peptide–positive patients.

CONCLUSIONS

Patients with remaining endogenous β-cell function after >10 years duration of type 1 diabetes differ immunologically from other patients with long-standing type 1 diabetes. In particular, they have a much higher IL-35 production.

Type 1 diabetes is characterized by an immune-mediated progressive destruction of the insulin-producing β-cells. Recent improvements in the sensitivity of C-peptide assays have, however, revealed that many patients even after long-standing type 1 diabetes have remaining low C-peptide concentrations in blood (1–4), which reflect functional, glucose-responsive β-cells (3). This suggests that in these individuals, some of the β-cells are resistant to immune destruction and thereby a suitable target for new protocols aiming to expand β-cell mass. Residual insulin secretion, albeit at higher levels, is known to contribute to lower mean blood glucose concentrations, less blood glucose fluctuations, and diminished risk of ketoacidosis (5). Stimulated C-peptide

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levels >200 pmol/L are also associated with decreased risks of hypoglycemic events and late complications (6).

Presently, there is scarce knowledge on the determinants for a long-term residual β-cell mass in type 1 diabetes. A previous study showed a correlation of remaining C-peptide concentrations to disease duration and level of zinc transporter-8 autoantibodies, but then almost half of the studied patients were investigated between 0 and 10 years after diagnosis (2). In another study, only nonfasting C-peptide concentrations were analyzed in patients with type 1 diabetes for >50 years, which made correlations between residual C-peptide levels and other parameters difficult (1). Nevertheless, 2.5% of these patients had sustained residual C-peptide production (>200 pmol/L) and were characterized by lower HbA_{1c}, older age of onset of disease, and higher frequency of the HLA DR3 genotype.

The current study focused on this gap in knowledge and tested the hypothesis that differences in cell-mediated immunity explain the maintenance of endogenous insulin production in some patients with long-standing type 1 diabetes.

RESEARCH DESIGN AND METHODS

This study was approved by the Uppsala County ethics board, and the reported investigations were carried out in accordance with the principles of the Declaration of Helsinki as revised in 2000. All participants were provided oral and written information and signed a written consent prior to inclusion in the study.

Study Participants, Clinical Parameters, and Blood Sampling

Patients (≥18 years of age) with type 1 diabetes and with disease duration of ≥10 years were recruited at Uppsala University Hospital. Fasting blood samples were collected under standardized conditions in all patients. None of the patients were, or had recently been, ill from infectious diseases. Routine lab parameters were analyzed at our central clinical chemistry laboratory, whereas HLA class II alleles were determined at our central clinical immunology laboratory. Separate blood plasma was obtained in EDTA tubes by centrifugation and immediately frozen at −80°C for later determination of C-peptide and cytokine concentrations. Additional blood samples were collected from 14 of the identified C-peptide–positive patients and 12 of the C-peptide–negative patients, as well as from 15 healthy control subjects, and were used for immediate investigation of peripheral blood mononuclear cells (PBMCs) (see below). The selection of C-peptide–negative and –positive patients for the cellular studies was based on their willingness and availability for further blood sampling on requested dates. Healthy control subjects were matched to the patients with type 1 diabetes for age, sex, and BMI. None of the healthy control subjects had a first-degree relative diagnosed with type 1 diabetes.

For 18 of the C-peptide–positive patients, a follow-up visit with new C-peptide measurement was performed 12–18 months after the first visit. Similarly, 24 of the C-peptide–negative patients were investigated for fasting and stimulated C-peptide concentrations.

Determination of C-Peptide and Cytokine Concentrations in Plasma

Plasma C-peptide concentrations were analyzed blinded by Mercodia AB (Uppsala, Sweden) using an ultrasensitive ELISA (catalog no. 10-1141-01; Mercodia) (2). The assay was calibrated against the international reference reagent for C-peptide, C-peptide 84/510 (a World Health Organization standard), and is listed with the U.S. Food and Drug Administration as class I in vitro diagnostic devices. The ultrasensitive assay's lower detection limit is set to 1.17 pmol/L, with inter- and intra-assay coefficients of variation at 5.5 and 3.8% at 37 pmol/L. Interleukin-35 (IL-35) concentrations were determined by an ELISA (BioLegend, San Diego, CA). All other cytokines were analyzed using a mesoscale multiplex kit (Meso Scale Diagnostics, Rockville, MD).

Flow Cytometry

Freshly isolated PBMCs were prepared by using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO). The purity of the live cells was >90% in each sample. PBMCs were stained with antibodies for surface markers (Supplementary Table 1). The cells were thereafter fixed and permeabilized with Fixation Permeabilization Buffer (eBioscience, San Diego, CA) for intracellular markers (Supplementary Table 1). The samples were run on LSR II Fortesa (BD, Franklin Lakes, NJ) using DivaDacker software (BD), and one million events were counted for analysis. The fluorescence minus one, isotype, and single-stained controls were used for gating strategies as described previously (7,8). The Flow Cytometry Standard files were analyzed on FlowLogic software (Inivai Technologies, Mentone, Australia). For cytokine staining, we did not stimulate the cells and blocked the cytokine secretion by using cytokine secretion blockers, since they can cause artifacts upon Foxp3 staining (8,9).

Statistics

Where the data were assessed on a continuous scale, the two-tailed Student *t* test was used for comparing the means of two independent samples, and a one-way ANOVA with Tukey post hoc test was used for pairwise comparisons among the means of three or more groups. Where the data were assessed as ranks relative to other participants in the groups, the two-tailed Mann-Whitney test was applied to compare the rank means of two groups, and the Kruskal-Wallis test with the Bonferroni post hoc test was used for pairwise comparisons among the rank means of three or more groups. *P* values <0.05 were considered significant. All values are given as means ± SEM.

RESULTS

Approximately 40% of the investigated patients with type 1 diabetes and ≥10 years duration of disease had measurable fasting C-peptide concentrations (Table 1). The mean age of C-peptide–positive patients was 43.0 years compared with a mean of 37.6 years for C-peptide–negative patients, but the difference was not statistically significant (P = 0.077). The mean age at disease onset was not significantly older for C-peptide–positive patients (15.5 vs. 13.2 years, P = 0.075), and the disease duration did not differ significantly between the two groups. Further, we observed no statistical difference in indicators of metabolic control or insulin needs between C-peptide–positive and –negative patients. The presence of GAD and/or IA2 antibodies did not differ between the two groups. However, we observed the DR4 and DQ3 risk allele to be more frequent in C-peptide–positive patients (Table 1). The levels of IL-35 were found to be significantly higher (twofold, P = 0.0004) in the C-peptide–positive patients compared with the C-peptide–negative patients (Table 1). The levels of IL-15 were decreased

Table 1—Descriptive data for C-peptide–positive and –negative patients

Parameter	C-peptide positive	C-peptide negative	*P* values
Patients (*n*, %)	46 (41)	67 (59)	
Male sex (*n*, %)	28 (61)	35 (52)[a]	0.71
Age (years)	43.0 ± 2.5	37.6 ± 1.8[b]	0.077
Age at onset (years)	15.5 ± 1.8	13.2 ± 1.0[c]	0.075
Disease duration (years)	28.0 ± 2.5	24.5 ± 1.5[c]	0.54
BMI (kg/m^2)	25.3 ± 0.5	25.0 ± 0.5[c]	0.62
C-peptide (pmol/L)	28.6 ± 15.0	0 ± 0[c]	<0.0001
IL-35 (ng/mL)	20.3 ± 2.5	9.7 ± 1.8[c]	0.0004*
IFN-γ (pg/mL)	8.8 ± 0.9	9.8 ± 1.4[c]	0.43
IL-10 (pg/mL)	0.38 ± 0.05	0.41 ± 0.05[c]	0.19
IL-12 (pg/mL)	122 ± 12	119 ± 8[c]	0.84
IL-15 (pg/mL)	2.3 ± 0.17	2.60 ± 0.08[c]	0.018*
IL-17 (pg/mL)	1.4 ± 0.16	1.5 ± 0.2[c]	0.89
IL-1β (pg/mL)	0.06 ± 0.02	0.02 ± 0.008[c]	0.078
IL-2 (pg/mL)	0.1 ± 0.01	0.5 ± 0.3[c]	0.47
IL-4 (pg/mL)	0.02 ± 0.004	0.02 ± 0.003[c]	0.32
IL-5 (pg/mL)	0.3 ± 0.03	0.4 ± 0.04[c]	0.51
IL-6 (pg/mL)	0.6 ± 0.05	0.8 ± 0.1[c]	0.53
TNF-α (pg/mL)	2.5 ± 0.1	2.4 ± 0.1[c]	0.92
DR3†, *n*/*n*	7/14	7/14[a]	1.0
DR4†, *n*/*n*	14/14	8/14[a]	0.016
DQ3†, *n*/*n*	14/14	8/14[a]	0.016
Creatinine (μmol/L)	79.8 ± 4.1	76.7 ± 2.9[c]	0.46
Glucose (mmol/L)	11.2 ± 0.7	10.5 ± 0.6[b]	0.45
HbA_{1c} (mmol/mol, %)	64.3 ± 1.9 (8.0 ± 0.2)	61.5 ± 1.4 (7.8 ± 0.2)[c]	0.21
GAD (IE/mL)	62 ± 14	64 ± 12[c]	0.75
Number of GAD positive (*n*, %)	26 (57)	37 (55)[a]	1.0
IA2 (kE/L)	48 ± 14	23 ± 7[c]	0.25
Number of IA2 positive (*n*, %)	21 (46)	20 (30)[a]	0.12
Total insulin doses (units/24 h)	50.7 ± 3.6	46.3 ± 2.2[c]	0.71
Insulin doses adjusted for bodyweight (units/kg/24 h)	0.66 ± 0.04	0.62 ± 0.027[c]	0.70

All blood samples were collected after overnight fasting. All data are given as means ± SEM unless otherwise indicated. *P* values <0.05 were considered statistically significant. For the cytokine data (12 different parameters), the Kruskal-Wallis test of Bonferroni was applied to correct for multiple testing. †Analyzed for 14 patients in each group. [a]Fisher exact test. [b]Two-tailed Student *t* test. [c]Two-tailed nonparametric Student *t* test (Mann-Whitney) based on ranks. *Bonferroni corrected *P* value for IL-35 = 0.0019 and for IL-15 >0.99. IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α.

in C-peptide–positive patients, although this did not remain statistically significant when controlling the type 1 error by correcting for multiple cytokine analyses (Table 1). All other cytokines measured were similar in both groups (Table 1). C-peptide concentrations were stable over time in most patients (Supplementary Fig. 1).

Since IL-35 is produced by regulatory T cells (Tregs), $CD8^+Foxp3^+$ cells, regulatory B cells (Bregs), and tolerogenic antigen-presenting cells (APCs) (10–15), we determined the proportion of IL-35^+ cells among these cell types. The proportion of IL-35^+ Tregs was decreased in C-peptide–negative patients when compared with both healthy control subjects and C-peptide–positive patients (Fig. 1*A* and Supplementary Fig. 2), although not the median fluorescent intensities (MFIs) for subunits of IL-35 (Ebi3 and IL-12p35; data not shown). We did not observe any differences in the proportions of Tregs in total (Fig. 1*B*), or of Tregs, thymic-derived Tregs (tTregs), or peripherally induced Tregs (pTregs) among $CD4^+$ T cells (data not shown) in C-peptide–negative patients when compared with healthy control subjects and C-peptide–positive patients. $CD8^+Foxp3^+$ among $CD8^+$ cells were decreased in the C-peptide–negative patients compared with C-peptide–positive patients (Fig. 1*C*), whereas the total proportion of $CD8^+Foxp3^+$ did not differ between the groups (Fig. 1*D*).

The proportion of IL-35^+ Bregs was decreased in C-peptide–negative patients compared with healthy control subjects (Fig. 2*A* and Supplementary Fig. 3), whereas the total proportion of Bregs among $CD19^+$ cells was similar between the groups (Fig. 2*B* and Supplementary Fig. 3). The proportion of IL-35^+ APCs was similar in all groups (data not shown). The MFIs of both Ebi3 and IL-12p35 among Bregs and tolerogenic APCs were also similar in all groups (data not shown).

In both type 1 diabetes animal models and in patients, T-helper 17 (Th17) cells play an important role in the reduction of β-cell mass (16,17). Interestingly, the proportion of IL-$17a^+$ cells among Tregs was increased in C-peptide–negative patients (Fig. 3*A*), although MFIs of CD25 in Tregs were similar in all the groups (Fig. 3*B*). The proportions of IL-$17a^+$ cells among

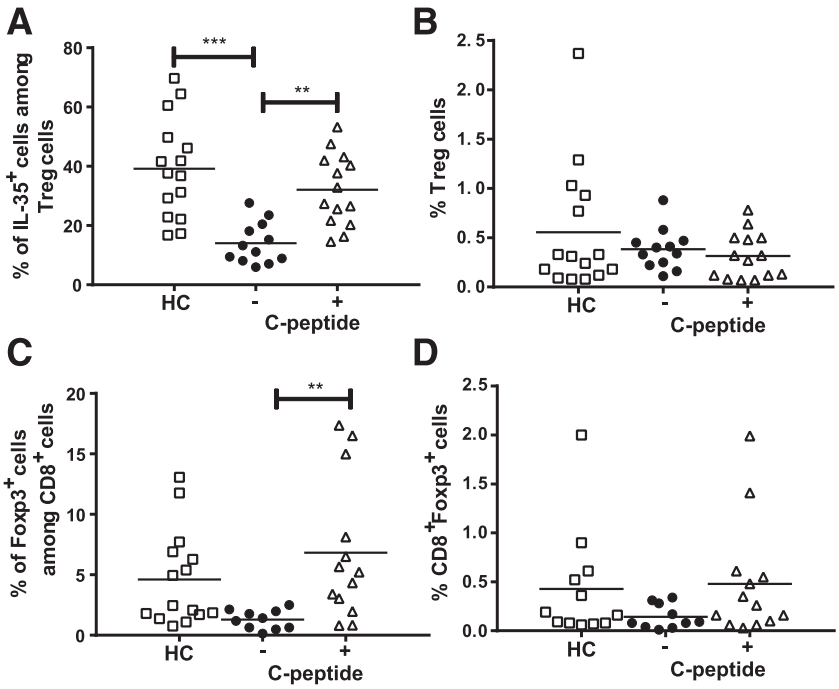

Figure 1—IL-35–producing Tregs are increased in patients with type 1 diabetes with remaining C-peptide. The proportions of IL-35$^+$ cells among CD4$^+$CD25$^+$CD127$^-$Foxp3$^+$ Tregs (*A*) and CD4$^+$CD25$^+$CD127$^-$Foxp3$^+$ Tregs (*B*). *C*: The proportion of Foxp3$^+$ among CD8$^+$ T cells. *D*: The proportion of CD8$^+$Foxp3$^+$ cells. The proportions of cells were determined by using a flow cytometer. Representative gating strategies are shown in Supplementary Fig. 2. One-way ANOVA with Tukey post hoc test was used for comparisons; n = 12–15/group. $^{**}P < 0.01$; $^{***}P < 0.001$. C-peptide−, patients with type 1 diabetes with no measurable C-peptide in plasma; C-peptide+, patients with type 1 diabetes with remaining C-peptide in plasma; HC, healthy control subjects.

the CD4$^+$ and CD8$^+$ T cells were also lower in the C-peptide–positive patients (Fig. 3*C* and *D* and Supplementary Figs. 4 and 5).

IL-15 regulates the homeostasis of CD4$^+$, CD8$^+$, natural killer (NK), and natural killer T (NKT) cells and promotes the development of CD8$^+$ diabetogenic cells (18). Therefore, we analyzed the proportion of CD3$^+$CD56$^-$, CD4$^+$CD25$^-$CD8$^+$, CD56^{low}(NK), CD56^{high}(NK), and CD3$^+$CD56$^+$(NKT) cells but observed no differences between C-peptide–negative and –positive patients (data not shown). The proportions of plasmacytoid dendritic cells and APCs were also similar in C-peptide–positive patients and –negative patients (data not shown).

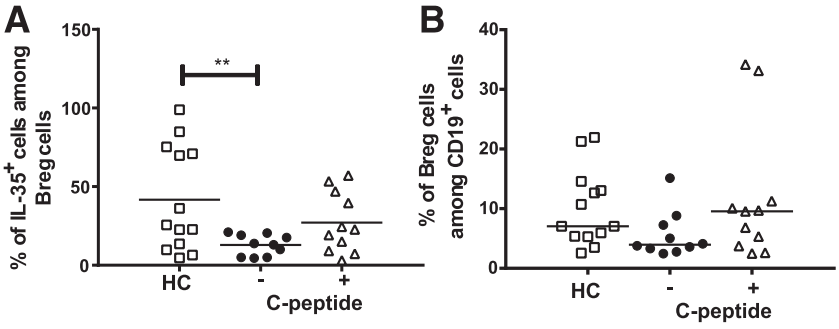

Figure 2—IL-35–producing Bregs are decreased in patients with type 1 diabetes with no measurable remaining C-peptide. *A*: The proportion of IL-35$^+$ cells among CD19$^+$CD24$^+$CD40$^+$CD38$^+$ Bregs. *B*: The proportion of CD19$^+$CD24$^+$CD38$^+$CD40$^+$ among CD19$^+$ B cells. Representative gating strategies are shown in Supplementary Fig. 3. One-way ANOVA with Tukey post hoc test was used for comparisons; n = 10–13/group. $^{**}P < 0.001$. C-peptide−, patients with type 1 diabetes with no measurable C-peptide in plasma; C-peptide+, patients with type 1 diabetes with remaining C-peptide in plasma; HC, healthy control subjects.

CONCLUSIONS

To date, there are no immunological studies to explain why some patients with type 1 diabetes have spared β-cells for decades. We hypothesized that the remaining C-peptide concentrations in patients with type 1 diabetes may be due to a beneficial shift in the immunological profile. Several of the investigated pro- and anti-inflammatory cytokines have previously been suggested to be important players in affecting disease development in both animal models of type 1 diabetes and in patients with type 1 diabetes. Noteworthy, the IL-35 concentrations in blood plasma were observed to be twice as high in the patients with remaining C-peptide. Approximately 40% of patients with type 1 diabetes and ≥10 years disease duration had measurable fasting C-peptide concentrations, which is similar to the results of a previous study (2) using the same technique for the measurements. The presence of even low concentrations of circulating C-peptide indicates residual β-cells spared by the immune system. Since samples were collected under fasting conditions and C-peptide production therefore was not maximally stimulated during controlled conditions, we cannot exclude that some patients were erroneously classified as C-peptide–negative patients. However, it should be noted that the mean blood glucose concentrations at sampling were >10 mmol/L in both groups, implying a moderate degree of glucose stimulation to C-peptide secretion. The current study was the first to perform follow-up C-peptide measurements ≥12 months after first measurements, and we found the levels to be stable over time in most patients.

We and others have previously reported that systemic treatment with IL-35 (8) or its overexpression in β-cells (19) could prevent development of disease in animal models of type 1 diabetes. Systemic IL-35 treatment even reversed manifest diabetes in both multiple low-dose streptozotocin–treated mice and in spontaneously diabetic NOD mice (8). We have also reported on decreased plasma levels of IL-35 in new-onset and long-standing type 1 diabetes compared with healthy controls (8).

The observed combination of lower proportion of IL-35$^+$ Tregs, IL-35$^+$ Bregs, and IL-35–producing CD8$^+$Foxp3$^+$ cells in

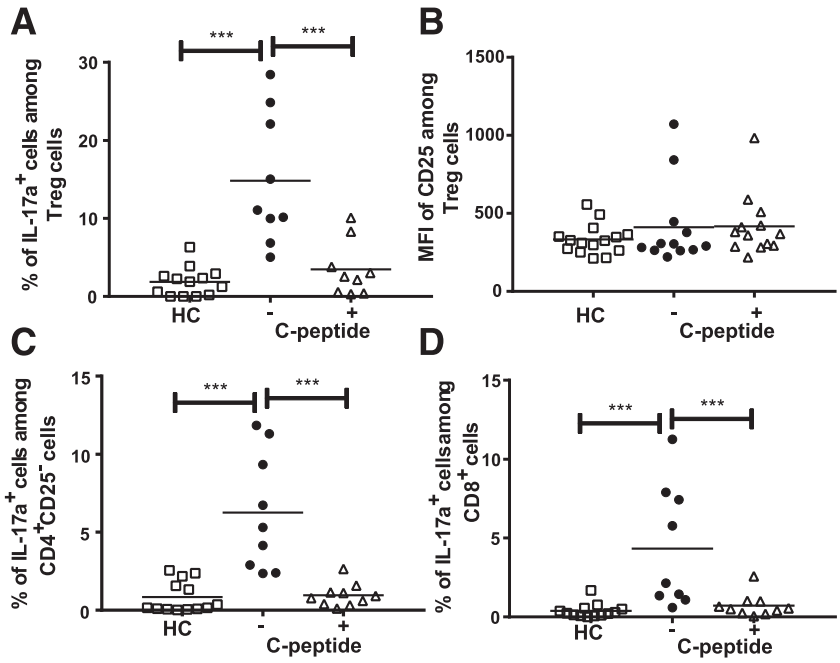

Figure 3—IL-17a$^+$ cells are increased in patients with type 1 diabetes with no measurable C-peptide levels. *A*: The proportions of IL-17a$^+$ cells among CD4$^+$CD25$^+$Foxp3$^+$ Tregs. *B*: MFIs of CD25 in CD4$^+$CD25$^+$CD127$^-$Foxp3$^+$ Tregs. The proportions of IL-17a$^+$ cells among CD4$^+$CD25$^-$ (*C*) and CD8$^+$ (*D*) T cells. The proportions of these cells were determined by using a flow cytometer. Representative gating strategies are shown in Supplementary Figs. 4 and 5. One-way ANOVA with Tukey post hoc test was used for comparisons; n = 9–13/group. ***$P < 0.001$. C-peptide−, patients with type 1 diabetes with no measurable C-peptide in plasma; C-peptide+, patients with type 1 diabetes with remaining C-peptide in plasma; HC, healthy control subjects.

C-peptide–negative patients can explain their lower concentrations of IL-35 in plasma. We did not find any differences in the proportion of tTregs and pTregs in our study. tTregs and pTregs were distinguished by using Helios as a marker for tTregs (7), and it has been reported that Helios$^+$ Tregs are more suppressive of immune responses than Helios$^-$ Tregs (7,20). Furthermore, it has been shown that Helios, together with neuropilin-1, plays a role in the secretion of IL-35 by Tregs (21).

IL-35 has been shown to maintain the suppressive phenotype of Tregs, block the differentiation of Th17 cells, and thereby dampen the ongoing immune destruction of β-cells (8). In line with this, we found that the proportions of IL-17a$^+$ cells among the Tregs, CD4$^+$ T cells, and CD8$^+$ T cells were lower in the C-peptide–positive patients. This could be an important mechanism for the maintenance of β-cell function in these patients (16,17).

Kuczyński et al. (22) have reported on elevated IL-15 concentrations in patients with type 1 diabetes compared with healthy control subjects. In NOD mice, IL-15 expression by pancreatic β-cells leads to the development of insulitis and β-cell destruction, but manifest disease could be reversed by blocking IL-15 signaling (23). The presently observed decreased circulating levels of IL-15 in the C-peptide–positive patients therefore seem to be in agreement with a less active destruction of β-cells.

Since this is an association study, it is impossible to answer whether the immunological differences are causal for protection of residual β-cells. A prospective longitudinal study would be needed to understand whether the more favorable immunological situation in long-standing C-peptide–positive patients with type 1 diabetes was present already at the debut of the disease, or if it had been acquired. Nevertheless, it is tempting to speculate on the latter, since we recently measured plasma IL-35 concentrations, by the same assay presently used, in eight newly diagnosed patients with type 1 diabetes and found them consistently low (<18 ng/mL), with a mean value even lower than that of C-peptide–negative patients in the current study (8). Similar to Keenan et al. (1), we observed the DR4 risk allele to be more frequent in C-peptide–positive patients, and we also found DQ3 to be more common in these patients. The association of this to the immunological findings remains to be determined.

In conclusion, we observed immunological changes, which may explain the long-term survival of functional β-cells in some patients with type 1 diabetes. There was especially a striking difference in circulating levels of IL-35 between patients with type 1 diabetes that remained C-peptide positive and those where no residual β-cell function could be discerned. Based on the data in the current report and our previous work in NOD mice (8), it would be of great interest to further explore the potential of IL-35 as a treatment option in both new-onset and long-standing type 1 diabetes.

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