The frequency and immunodominance of islet-specific CD8+ T-cell responses change after type 1 diabetes diagnosis and treatment.

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The Frequency and Immunodominance of Islet-specific CD8$^+$ T-cell Responses Change after Type 1 Diabetes Diagnosis and Treatment

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Running title: CD8$^+$ T-cell responses change after type 1 diabetes onset

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Abbreviations used: aAb, autoantibody; IA-2, insulinoma-associated protein 2; IAA, insulin autoantibodies; IFN-γ, interferon-γ; IGRP, islet glucose-6-phosphatase catalytic subunit-related protein; ISL8Spot, Islet-reactive $CD8^+$ T-cell IFN-γ ELISpot; PPI, preproinsulin; SFC, spot-forming cells; T1D, type 1 diabetes.

Abstract

OBJECTIVE–Islet-reactive $CD8^+$ T-cells play a key role in the pathogenesis of type 1 diabetes (T1D) in the NOD mouse. The predominant T-cell specificities change over time, but whether similar shifts also occur after clinical diagnosis and insulin treatment in T1D patients is unknown.

RESEARCH DESIGN AND METHODS–We took advantage of a recently validated islet-specific $CD8^+$ T-cell IFN-γ enzyme-linked immunospot (ISL8Spot) assay to follow responses against preproinsulin (PPI), GAD, IA-2 and islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) epitopes in 15 HLA-$A2^+$ adult T1D patients close to diagnosis and at a second time point 7-16 months after.

RESULTS–$CD8^+$ T-cell reactivities were less frequent at follow-up, as 28.6% of responses tested positive at T1D diagnosis vs. 13.2% after a median of 11 months (P=0.003). While GAD and IA-2 autoantibody (aAb) titers were unchanged in 75% of cases, the fraction of patients responding to PPI and/or GAD epitopes by ISL8Spot decreased from 60-67% to 20% (P<0.02). The previously subdominant IA-$2_{206-214}$ and $IGRP_{265-273}$ peptides were newly targeted, thus becoming the immunodominant epitopes.

CONCLUSIONS–Shifts both in frequency and in immunodominance of $CD8^+$ T-cell responses occur rapidly, as compared to slower changes in aAb titers. These different kinetics may suggest complementary clinical applications for T cell and aAb measurements.

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$CD8^+$ T-cells have recently emerged as crucial actors in the pathogenesis of type 1 diabetes (T1D) in the NOD mouse (1,2). Three different $CD8^+$ pathogenic clones specific for proinsulin $(PI)_{B15-23}$ (3), islet-specific glucose-6-phosphatase catalytic subunit-related protein $(IGRP)_{206-214}$ (4) and dystrophia myotonica kinase $(DMK)_{138-146}$ (5) have been described. These specificities are highly immunodominant in NOD mice, as T-cells recognizing them are abundantly present in the early insulitis infiltrates (6,7). Moreover, the rise in circulating $IGRP_{206-214}$-specific $CD8^+$ T-cells can predict impending T1D onset in NOD mice (8).

More recent data suggest that there could be a hierarchy of spreading from one reactivity to another, as NOD mice made tolerant to PI are protected from diabetes and do not develop IGRP-specific $CD8^+$ responses, while IGRP-tolerant mice still become diabetic and mount PI-specific responses (9). The initiating role of PI is further supported by the fact that PI knockout mice reconstituted with a hormonally active PI transgene carrying a single tyrosine to alanine mutation at position B16 are completely protected from T1D (10,11). This substitution affects both the PI_{B15-23} epitope and an overlapping immunodominant PI_{B9-23} $CD4^+$ epitope (12,13).

Mirroring these mouse data, $CD8^+$ T-cells have recently received increasing attention also for human T1D (14-19), as their detection could provide new autoimmune markers of β-cell aggression. Development of such markers is important to complement autoantibody (aAb) readouts. Indeed, aAbs can separate T1D from healthy subjects with high sensitivity and specificity, making them indispensable tools for diagnostic classification and T1D prediction. However, the utility of aAbs for

immune monitoring purposes is limited by the fact that they do not reflect tolerance restoration following therapeutic manipulation (20,21).

To be clinically applicable, T-cell assays should be developed that can be easily transferred into routine laboratory testing. The first validation step for such assays is to test their diagnostic sensitivity and specificity in separating T1D from healthy subjects. For $CD4^+$ T-cells, the cellular immunoblotting technique of Brook-Worrell et al. (22) was the assay showing the best performance (23). In many other instances, β-cell-specific $CD4^+$ T-cells were detected in all individuals irrespective of disease status (24,25), although probably characterized by different phenotypes (26-28).

For $CD8^+$ T-cells, we have recently proposed an islet-specific $CD8^+$ T-cell IFN-γ ELISpot (ISL8Spot) assay (18,29). The ISL8Spot employs HLA-A2-restricted β-cell epitopes derived from pre-PI (PPI) (30), GAD, insulinoma-associated protein 2 (IA-2) (17) and IGRP (31), which have been mostly identified by proteasome digestion and DNA immunization strategies (32). Using a single IFN-γ secretion readout on unfractionated PBMCs, this assay detects and quantifies β-cell-reactive $CD8^+$ T-cells directly ex vivo (18). Beta-cell epitope-specific $CD8^+$ T-cells were thus found at an average frequency of 0.008%, ranging from 0.0008 to 0.08% of total PBMCs. The presence vs. absence of these cells allowed discrimination of T1D vs. healthy subjects with high sensitivity and specificity (18).

The natural history of $CD8^+$ T-cell responses after T1D clinical onset and start of insulin treatment has not been explored. We know from the NOD mouse that the $PI_{B15\text{-}23}$-specific fraction is highly immunodominant already at 5 wk (i.e., in the pre-

diabetic period), but rapidly disappears, becoming negligible by 20 wk (i.e., after T1D onset). In parallel, the $IGRP_{206-214}$-specific fraction increases in number, while undergoing an avidity maturation process which selects the clonotypes of higher avidity (6,8). Whether similar shifts in epitope targeting also apply to human T1D is unknown. We therefore used the ISL8Spot assay to measure $CD8^+$ T-cell responses against different β-cell epitopes at T1D clinical onset and after some months of follow-up. Our results reveal a fast waning of most ISL8Spot responses, which contrasts with the steady titers of GAD and IA-2 aAbs. Few new T-cell reactivities appear, which focus on previously subdominant IA-2 and IGRP epitopes.

Research design and methods

Peptides. The HLA-A2-restricted β-cell epitopes used (Tab. 1) were previously described (17,18,31). A viral peptide pool of Flu MP_{58-66} (GILGFVFTL), Epstein-Barr virus $BMLF_{280-288}$ (GLCTLVAML) and cytomegalovirus $pp65_{495-503}$ (NLVPMVATV) and phytohemagglutinin (PHA, 1 µg/ml; Sigma, Lyon, France) were used as positive controls. HIV gag_{77-85} (SLYNTVATL) and DMSO diluent were included as negative controls. All peptides were used at 10 µM and were >80% pure (Schafer-N, Copenhagen, Denmark). Responses against these 9- to 10-amino acid-long epitopes originate from $CD8^+$ T-cells, as previously shown (18).

Study subjects. $HLA\text{-}A2^+$ new-onset adult (>16 yr-old) T1D patients with acute onset of symptoms requiring permanent insulin treatment from the time of diagnosis (33) were recruited from the Diabetes Registry of the Province of Turin, Italy and from the GOFEDI Network, France (see Appendix for the detailed list of participating centers). All patients had metabolically controlled disease and were free of recent (<2 weeks) infectious or inflammatory conditions at the time of blood draw. Parallel recruitment of Caucasian healthy control subjects took place at the same Institutions. Available subjects wishing to continue the study were subsequently drawn and tested a second time under identical conditions. All subjects gave informed consent and the study was approved by the relevant Ethics Committees.

Blood processing. All blood samples were shipped overnight at room temperature. Rapid HLA-A2 screening was performed with the BB7.2 mAb, followed by subtyping using the Olerup SSP HLA*02 kit (GenoVision/Qiagen, Vienna, Austria) to verify the presence of the HLA-A*0201 allele (>95% of $HLA\text{-}A2^+$ individuals).

PBMCs were isolated by density gradient centrifugation using lymphocyte separation medium (PAA, Les Mureaux, France), and immediately used or stored frozen (10% DMSO in pooled human male AB serum). The second testing was performed under the same condition (i.e., fresh or frozen PBMCs) of the first testing.

Islet Abs. Serum GAD, IA-2 and insulin (a)Abs were measured by radioligand binding assays, following protocols previously evaluated within the Diabetes Antibody Standardization Program (DASP; Lab no. 137) (34). Sensitivities in the 2005 DASP were 84% for anti-GAD, 76% for anti-IA-2 and 28% for anti-insulin (IAA), where the specificity was set at 95%. Changes >33% between two aAb titer determinations were considered significant.

ISL8Spot assay. Ninety-six well PVDF plates (Millipore, Saint-Quentin-en-Yvelines, France) were coated overnight with an anti-IFN-γ Ab (U-CyTech, Utrecht, The Netherlands). Plates were subsequently blocked with RPMI + 10% human serum (PAA) and peptides added (10 µM final concentration) in triplicate wells along with recombinant human IL-2 (0.5 U/ml; R&D Systems, Lille, France), as previously reported (18). PBMCs were seeded at $3x10^5$ cells/well and cultured for 20-24 h. Following PBMC removal, IFN-γ secretion was visualized with a biotin-conjugated anti-IFN-γ Ab (U-CyTech), alkaline phosphatase-conjugated ExtrAvidin and Sigmafast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) tablets (both from Sigma). Spots were counted using an AID reader (Strassberg, Germany) and means of triplicate wells calculated. All ISL8Spot readouts are expressed as spot-forming cells (SFC)/10^6 PBMCs.

The cut-off for a positive response was set at 3SD above the average basal reactivity (i.e., reactivity against HIV gag_{77-85} and DMSO diluent alone). This was chosen as the cut-off allowing for the best diagnostic sensitivity (i.e., highest number of positive responses to β-cell epitopes in T1D patients) and specificity (i.e., lowest number of positive responses in healthy controls), as determined by receiver-operator characteristics analysis (18).

T-cell clone dilution experiments. A Flu MP_{58-66}-specific HLA-A2-restricted T-cell clone was serially diluted into PBMCs from 3 different HLA-$A2^+$ donors showing no response to the MP_{58-66} peptide and displaying different levels of basal reactivity in the absence of stimuli. These cell mixtures were challenged in the presence of 10 μM Flu MP_{58-66} peptide using the same ELISpot format as above.

Statistical analysis. Values are expressed as mean ±SD or median (range), according to their distribution. Comparisons between proportions were made with the χ^2 test and Fisher's exact test, when appropriate. Comparisons of means between two groups were carried out with the Student's *t* test for normal distributed variances or with the Mann-Whitney *U* test for non-normal variances. $P<0.05$ was considered to be of statistical significance.

Results

Assay validation

The reproducibility of our ELISpot technique has been previously reported (18). Variability was found to be of 14.1% intra-assay, 4.2% at the analytical inter-assay level (i.e., using thawed PBMC samples frozen on the same occasion) and 9.2% at the pre-analytical and analytical level (i.e., using separate blood draws from the same donor). One further problem that is encountered in a longitudinal setting is that the basal reactivity level (i.e., spontaneous IFN-γ production in the absence of stimuli) can be different not only among different subjects, but also within the same individual when analyzed at different time points. To address whether these variations could affect the detection sensitivity of the technique, we took HLA-$A2^+$ healthy donors displaying different IFN-γ background levels that demonstrated no response to the Flu MP_{58-66} epitope, mixed their PBMCs with serially diluted numbers of a Flu MP_{58-66}-specific clone, and tested these mixtures in ELISpot against the MP_{58-66} peptide. As shown in Figure 1, background levels more than 10-fold different (7.3, 21.7 and 85.5 SFC/10^6 PBMCs, respectively) did not significantly affect the detection sensitivity of the system (i.e., the basal-subtracted net signal) over a wide range of frequencies. Moreover, all counts were above the basal + 3SD positive cut-off value calculated from the respective backgrounds.

Most $CD8^+$ T-cell responses are short-lived after T1D onset

We considered β-cell epitopes that we previously defined as targets of $CD8^+$ T-cells in 19-50% of adult new-onset T1D patients, but not of age-matched healthy controls (17,18). These epitopes were derived from four different β-cell Ags, namely PPI, GAD, IA-2 and IGRP. A complete list is presented in Table 1.

Fifteen new-onset T1D patients were available for follow-up within a median of 11 months (range 7-16; Tab. 2). Their mean age at diagnosis was of 27.6±8.4 years and their median T1D duration at the time of the first blood draw was of 13 days (range 2-180). Fifteen age-matched healthy controls (mean age 28.8±5.9) were also tested twice, within a median follow-up period of 14 months (range 12-26).

Results expressed as number of SFC/10^6 PBMCs, are summarized in Figure 2. All healthy control subjects were negative at follow-up, including two individuals (i.e., H08 and H09) previously found to be weakly positive for 1-2 epitopes. The number of T1D patients positive for T-cell responses dropped from 80% (12/15) at diagnosis to 53% (8/15) during follow-up (P=0.12). Moreover, while positive patients at the time of diagnosis recognized multiple epitopes (median reactivities/patient = 2, range 0-6), there were mostly single reactivities that persisted during follow-up (median 1, range 0-3; P=0.05). Overall, the number of positive responses out of the total tested decreased from 28.6% (34/119) to 13.2% (16/121; P=0.003).

Those patients with detectable reactivities could be divided in two categories: on one side, patients already positive at diagnosis (i.e., patients P04, P09, P10, P11, P14 and P15), in whom at least one of the original epitopes was still recognized, while new ones sometimes emerged (i.e., P04, P11, P14). On the other side, patients whose responses were all negative at diagnosis and who developed scattered reactivities at follow-up (1-2 epitopes recognized; patients P06 and P08). $CD8^+$ T-cell responses against a pool of viral epitopes were included as internal controls. They decreased or disappeared only in 2 patients (P08 and P11), remaining unchanged in all other instances (13/15; 87%).

$CD8^+$ T-cell responses shift towards IA-$2_{206-214}$ and $IGRP_{265-273}$ epitopes after T1D onset

A simplified graphical representation of these data is provided in Figure 3, where only $CD8^+$ T-cell responses testing positive either at diagnosis or follow-up are represented. Such responses are displayed as absent (<3SD above the mean basal reactivity), low (3-4SD), intermediate (4-5SD) or high (>5SD), following the quantitative ranking reported in Figure 2.

Of the total positive T-cell responses to islet epitopes observed at diagnosis, the majority (26/42; 62%) dropped to non-detectable levels, while few of them (8/42; 19%) remained detectable at follow-up. Only in 5 patients (i.e., P04, P06, P08, P11 and P14) did new epitopes become targeted (8/42; 19%). Except for patient P04 who recognized PI_{B18-27}, in all other instances (7/8; 87.5%) the newly targeted epitopes were IA-$2_{206-214}$ and $IGRP_{265-273}$. These two epitopes were recognized together in 3 of 4 patients.

While the number of patients responding to epitopes derived from PPI and GAD significantly decreased (60% to 20% for PPI; 67% to 20% for GAD; $P<0.02$; Fig. 4A), IA-$2_{206-214}$ became the major immunodominant epitope detected at follow-up. This was true both in terms of IA-$2_{206-214}$-responding patients (5/14, 36%; Fig. 4B) and of frequency of IA-$2_{206-214}$ among all targeted epitopes (5/16, 31%; Fig. 5). Second in order came $IGRP_{265-273}$, which was recognized by 27% (3/11) of patients (Fig. 4B), thus constituting 19% (3/16) of all reactivities detected (Fig. 5). Another 20% of patients and 19% of reactivities were covered at follow-up by $GAD_{114-123}$ and PI_{B18-27}, which were the two immunodominant epitopes at diagnosis (53% and 36% prevalence and 23% and 14% relative frequency, respectively). Thus, 4 epitopes (i.e., the newly immunodominant IA-$2_{206-214}$ and $IGRP_{265-273}$, along with $GAD_{114-123}$ and

PI_{B18-27}) accounted for 88% of all reactivities detected at follow-up. Three epitopes (PI_{B10-18}, $GAD_{536-545}$ and $IGRP_{228-236}$) were instead no longer recognized. This focusing of T-cell responses towards more selected epitopes contrasted with the situation at diagnosis (Fig. 4B and Fig. 5) where, besides $GAD_{114-123}$ and PI_{B18-27} emerging as the immunodominant specificities, the other 7 epitopes were evenly ranked (13-29% prevalence, 6-12% of total reactivities).

aAb titers remain stable in face of fading $CD8^+$ T-cell responses.

A sharp dichotomy was observed when comparing the progression of aAb titers and T-cell responses (Tab. 2). While the fraction of positive ISL8Spot responses decreased from 28.6% to 13.2% (P=0.003), that of positive GAD or IA-2 aAb responses did not change (63.3% vs. 60.7%; P=0.83). Even the titers of GAD and IA-2 aAbs remained nearly identical in the majority of cases (21/28; 75.0%). A decrease in titers was registered only in 17.9% (5/28) of cases, while both GAD and IA-2 further rose only in one patient.

The picture was different when we compared (a)Ab and T-cell responses against PPI. In line with their adult onset T1D, all but two (13/15; 86.7%) patients were negative for IAA at diagnosis (Tab. 2). Following initiation of insulin therapy, insulin Ab titers became positive in most patients, with the exception of P03 and P10. P10 was also one of the three patients for whom PI-specific T-cell responses were still detectable at follow-up.

Discussion

We here describe the progression of β-cell-directed $CD8^+$ T-cell responses after T1D clinical onset and initiation of insulin therapy. As the aim of this study was the longitudinal evaluation of T1D patients rather than their comparison with healthy controls, the fact that these two groups were matched only with respect to age does not bias our conclusions. In this longitudinal setting, it is instead essential to distinguish between changes in T-cell responses due to the variability of the ISL8Spot technique from those reflecting true biological fluctuations. In this respect, the 9.2% inter-assay variability registered when analyzing separate blood draws from the same individual (18) is reassuring. Although some fluctuations in the ELISpot background noise are unavoidable, most of these fluctuations were shown to have a minimal impact on the final basal-subtracted signal obtained (18). Moreover, detection of a serially diluted clone over different basal backgrounds was also similar. Three main conclusions can thus be drawn: *i*) most $CD8^+$ T-cell responses are short-lived after clinical onset; *ii*) some new reactivities appear, which preferentially target the previously subdominant IA-$2_{206-214}$ and $IGRP_{265-273}$ epitopes; *iii*) GAD and IA-2 aAb titers display less fluctuations.

There are several hypotheses which, without being mutually exclusive, could explain the rapid disappearance of most β-cell specific IFN-γ-producing $CD8^+$ T-cells after T1D diagnosis. The simplest explanation is that these T-cells are no longer detected just because they are no longer present at sufficient frequencies. They may have been deleted or suppressed as part of the natural blunting of (auto)immune responses. Although some β-cells are thought to survive for a long time after T1D onset, the increasing paucity of islet Ags may doom most of these T-cells to die for lack of Ag

stimulation. These cells may also become undetectable because of a change in their recirculation or homing behavior. Alternatively, β-cell-specific T-cells may have stopped secreting IFN-γ, changing their phenotype to a different one. There are indeed examples in the setting of both chronic virus infections (35) and of neoplastic diseases (36), where activated virus- or tumor-specific $CD8^+$ T-cells persist, but lose their effector function (i.e., cytotoxicity and cytokine production).

A third explanation is that the epitope specificity of the $CD8^+$ T-cell population may have shifted towards different targets. Although it is possible that other unknown specificities become prevalent, an epitope focusing phenomenon was observed. Indeed, only six of the nine original epitopes were still targeted at follow-up. Furthermore, a shift in the epitopes preferentially targeted by $CD8^+$ T-cells was observed, as two previously subdominant IA-2 and IGRP epitopes became targeted by 27-36% of patients, while the previously immunodominant responses against PI and GAD epitopes became less prominent. IA-$2_{206-214}$ and $IGRP_{265-273}$ (but not $IGRP_{228-236}$) were also the two epitopes newly targeted in the vast majority (87.5%) of cases. These results are relevant in light of the recent debate as to whether there is a hierarchy of epitope spreading in the T1D pathogenic process. Data in the NOD mouse suggest that PI may stand at the source of this spreading (10,11), while IGRP would lie downstream in the cascade (9). Our human data is consistent with this model. Indeed, PI- and GAD-specific responses decreased, while IA-$2_{206-214}$-specific and – to a lesser extent – $IGRP_{265-273}$-specific T-cells became immunodominant. While the decreasing PI-specific responses and the increasing of $IGRP_{265-273}$ reactivity are reminiscent of NOD mouse data (6), the finding of a similar phenomenon for GAD and IA-2 is unparalleled. This is not surprising, since GAD and IA-2 are

thought to be important target Ags in human T1D (15,17,26,37-39), but not in the NOD mouse (40-43). Consideration of additional epitopes poorly recognized at T1D onset (17,18) may reveal further specificities becoming immunodominant at later time points. It is also interesting that, similarly to the overlapping PI_{B9-23} and PI_{B15-23} mouse epitopes, the immunodominant human $CD8^+$ T-cell epitopes at diagnosis (i.e., $GAD_{114-123}$) and at follow-up (i.e., IA-$2_{206-214}$) overlap with the DR4-restricted epitopes $GAD_{115-127}$ (44,45) and IA-$2_{206-221}$ (46).

The observed shift in epitope specificity could be due either to the emergence of novel clonotypes or to an avidity maturation of pre-existing ones. Indeed, if IA-$2_{206-214}$-specific $CD8^+$ T-cells undergo avidity maturation, this would lower the ISL8Spot detection threshold and thus increase their measured frequencies. Alternatively, the measured frequencies could increase due to higher precursor numbers following *de novo* generation and/or expansion. The study of epitope-specific $CD8^+$ clones obtained at different time points will clarify this issue.

Another important question is whether the observed changes in $CD8^+$ T-cell responses are part of the T1D natural history, or rather reflect an immunological effect induced by insulin therapy. Indeed, daily insulin treatment in these patients may have modulated T-cell responses. Consistent with this hypothesis, PI-specific responses were frequently undetected at follow-up (12 of 15 patients; 80%), a trend which contrasted with the opposite behavior of insulin Ab responses, which rose in most (10/12; 83%) instances. The increase in insulin Ab titers could also contribute to this shift in T-cell responses, as Abs could change the efficiency of presentation of PI-derived T-cell epitopes (47,48). Besides the possibility of a direct effect of PI as an Ag on its cognate T-cells, an indirect tolerogenic effect could also be mediated by the

hormonal activity of PI. Insulin treatment can indeed reduce the functional pressure on the β-cell, which may translate in reduced β-cell apoptosis and/or reduced β-cell Ag release.

The slower changes observed for aAb titers as compared to T-cell responses have important implications. Autoreactive memory B-cells are long-lived, and can keep replenishing the plasma cell compartment without further need for Ag-specific stimulation (49). It is not completely clear whether the same rules apply to $CD8^+$ T-cell memory (50). The efficient B-cell homeostasis may make it possible for the aAb titers to be maintained longer than T-cell responses. The faster dynamics of $CD8^+$ T-cell responses could make their measurement more suitable to promptly reflect the autoimmune modifications of the disease. In this scenario, aAb and T-cell measurements could find complementary clinical applications. We hypothesize that the slow changes of aAb titers may make them more suitable for predicting the "ifs" of T1D (i.e., 5-year probability to develop disease in at-risk subjects). Conversely, the faster kinetics of T-cell responses may be less useful to predict long-term odds, but better suited to reflect more proximal events, i.e., the "whens" of T1D once disease onset, remission or relapse are approaching.

Another important application for T-cell assays will be for the etiologic classification of borderline cases. One such example is patient P03, whose diagnosis of T1D could be questioned, having ketoacidosis and insulin-dependency at diagnosis but being seronegative and genetically protected ($DR15^+$ with no HLA Class II susceptibility allele). Nonetheless, his robust $CD8^+$ T-cell responses at diagnosis hint an autoimmune component for his diabetes.

The disappearance of most $CD8^+$ T-cell responses following T1D onset and initiation of insulin therapy has also important implications for the application of T-cell assays in the clinics. There is quite some parallelism between human and NOD mouse data, as in both cases T-cell responses significantly wane after clinical onset (8). If this human/mouse parallel holds true also for the pre-diabetic period, we may find the zenith of T-cell responses before diabetes onset. Longitudinal follow-up of at-risk subjects will shed further light on the time course of these events.

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Appendix

The following colleagues and Institutions contributed to the study with patient recruitment:

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Tables

Table 1. The HLA-A2-restricted β-cell epitope panel used in the ISL8Spot assay.

Name	Sequence
PPI_{2-10}	ALWMRLLPL
PI_{B10-18} (PPI_{34-42})	HLVEALYLV
PI_{B18-27} (PPI_{42-51})	VCGERGFFYT
PI_{A12-20} ($PPI_{101-109}$)	SLYQLENYC
$GAD65_{114-123}$	VMNILLQYVV
$GAD65_{536-545}$	RMMEYGTTMV
$IA\text{-}2_{206-214}$	VIVMLTPLV
$IGRP_{228-236}$	LNIDLLWSV
$IGRP_{265-273}$	VLFGLGFAI

For PPI, aa numbering is given both with respect to the PI sequence alone (aa B1-A21) and to the complete PPI sequence (aa 1-110).

Table 2. Characteristics of HLA-A2^{+} new-onset T1D patients and age-matched healthy controls.

Case no.	Age at 1st draw (yr)	Sex	T1D duration at diagnosis (days)	Follow-up time (months)	HLA DRB1	GAD Ab (AU) 1st	GAD Ab (AU) 2nd	IA-2 Ab (AU) 1st	IA-2 Ab (AU) 2nd	Insulin Ab (AU) 1st	Insulin Ab (AU) 2nd
T1D patients (n=15)											
P01	38	M	180	13	04-16	**2,817**	**2,384**	13	21	**2.54**	**4.43**
P02	37	M	60	11	07-08	**2,898**	**1,118**	30	16	0.24	**0.89**
P03	43	M	5	11	11-15	60	61	11	18	0.30	0.45
P04	21	F	24	16	03-16	**2,894**	**2,044**	**598**	**244**	0.40	**1.36**
P05	23	M	16	7	04-13	58	-	18	-	0.31	**21.7**
P06	33	M	19	14	-	**2,114**	**5,102**	**505**	**1,204**	0.44	**1.99**
P07	16	F	6	7	01-01	**1,441**	**1,413**	**578**	**315**	0.29	**2.30**
P08	18	M	13	14	03-15	**883**	**722**	11	35	0.35	**9.30**
P09	29	M	26	12	03-04	**2,548**	**2,572**	**1,570**	**1,334**	0.35	**2.10**
P10	27	M	70	14	03-13	**3,292**	**3,105**	30	22	**0.77**	0.43
P11	24	F	4	7	03-13	**1,602**	88	**2,876**	**3,688**	0.33	**11.4**
P12	40	M	5	8	04-07	**298**	**273**	20	19	0.62	**3.47**
P13	18	F	2	8	01-04	**2,352**	**2,105**	**5,542**	**5,255**	0.40	**3.84**
P14	22	M	11	8	01-03	**190**	125	21	14	0.33	**3.25**
P15	25	M	6	7	04-11	114	87	**3,638**	**1,872**	0.58	**3.05**
Healthy subjects (n=15)											
H01	25	F		24	13-14	-	40	-	19	-	0.23
H02	26	F		16	09-11	-	37	-	11	-	0.20
H03	30	F		26	01-16	-	41	-	15	-	0.37
H04	32	M		15	11-16	-	60	-	10	-	0.22
H05	33	F		15	04-14	-	63	-	9	-	0.23
H06	27	M		14	07-15	-	25	-	16	-	0.26
H07	26	F		22	08-11	-	43	-	13	-	0.47
H08	25	F		22	11-15	-	48	-	14	-	0.36
H09	23	F		12	03-11	-	41	-	26	-	**0.76**
H10	27	M		14	01-11	-	66	-	28	-	0.20
H11	26	F		14	01-07	-	43	-	27	-	0.28
H12	26	F		14	13-15	-	70	-	14	-	0.15
H13	25	M		14	-	-	76	-	20	-	0.28
H14	48	F		14	04-11	-	70	-	13	-	0.13
H15	33	M		13	07-13	-	60	-	14	-	0.49

The 97.5th percentile cut-off values for Ab titers are as follows: GAD Abs: 180 arbitrary units (AU). IA-2 Ab: 80 AU. IAA: 0.7 AU. Positive Ab titers are displayed in bold, while changes in titers >33% are shaded in grey. –, not determined.

Figure Legends

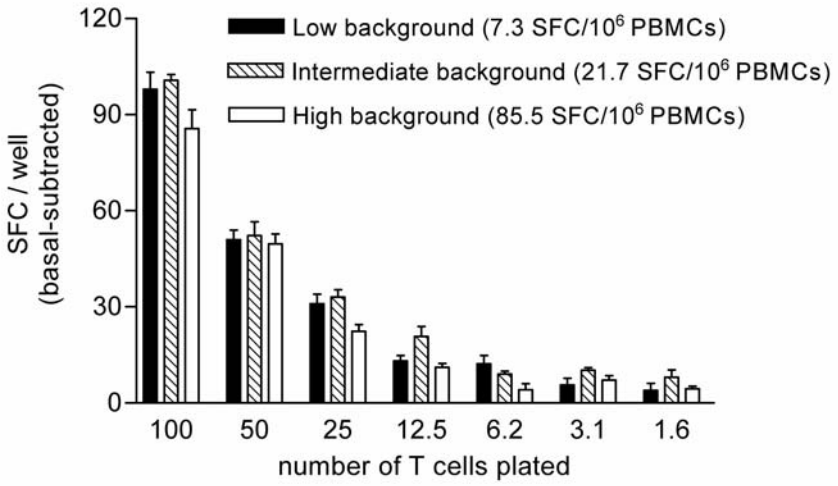
Figure 1. ELISpot detection of epitope-specific signals over different background noise levels. Flu MP_{58-66}-specific T-cells were serially diluted into PBMCs displaying different background noise values, as indicated. The number of MP_{58-66}-specific T cells plated is shown on the X axis, while the Y axis displays the SFC counted per well after basal background subtraction. All counts were above the basal + 3SD cutoff value for a positive response, as calculated from the respective background noise levels. A representative experiment out of two performed is shown.

Figure 2. Summary of HLA-$A2^+$ T1D patients (n = 15) and healthy control subjects (n = 15) assayed for β-cell reactivities by ISL8Spot close to diagnosis (0 mo) and at follow-up (7-14 mo, as indicated). All values, including basal + n SD cutoffs, are expressed as SFC/10^6 PBMCs and are basal subtracted. Unsubtracted basal values (reactivities to DMSO and gag_{77-85}) are shown in the last row of each column. Reactivities are ranked as low (between 3 and 4 SD, in yellow), intermediate (between 4 and 5 SD, in orange), and high (more than 5 SD, in red). ++++, off-scale ELISpot reading.

Figure 3. Longitudinal follow-up of ISL8Spot responses against β-cell epitopes in T1D patients. Patients previously tested by ISL8Spot close to diagnosis (Fig. 2; 0 mo here) were re-assayed after 7-14 mo follow-up, as indicated. Reactivities testing positive at either time point are depicted and ranked as absent (<3SD above basal), low (3-4SD), medium (4-5SD) and high (>5SD) as in Fig. 2. Responses against a pool of viral epitopes are included as positive controls.

Figure 4. (A) Cumulative prevalence of $CD8^+$ T-cell responses against epitopes derived from PPI, GAD, IA-2 and IGRP in T1D patients at diagnosis and at follow-up. *P<0.02. (B) Prevalence of single epitope specificities in T1D patients at diagnosis and at follow-up. **P<0.05.

Figure 5. Relative distribution of epitope specificities. The percent prevalence of each epitope out of all epitopes recognized at diagnosis (n=34) and at follow-up (n=16) is shown.



	T1D PATIENTS (n=15)																													
	P01		P02		P03		P04		P05		P06		P07		P08		P09		P10		P11		P12		P13		P14		P15	
	0 mo	13 mo	0 mo	11 mo	0 mo	11 mo	0 mo	16 mo	0 mo	7 mo	0 mo	14 mo	0 mo	7 mo	0 mo	14 mo	0 mo	12 mo	0 mo	14 mo	0 mo	7 mo	0 mo	8 mo	0 mo	8 mo	0 mo	8 mo	0 mo	7 mo
PPI 2-10	27.2	4.5	0	0	5.5	0.5	4.5	0.5	0	10.6	0	0	16.7	9.0	16.8	7.2	7.1	0	697.1	148.9	19.4	13.8	54.0	0	0	0	0	58.3	15.0	6.7
PI B10-18 (PPI 34-42)	18.3	6.7	43.4	0	12.2	0	2.2	6.1	0	0	0	1.1	35.5	0	0	1.7	0		26.1		0	1.5	45.1	6.7	1.7	0	2.0	0	16.1	0
PI B18-27 (PPI 42-51)	29.3	5.6	0	0	24.4	0	0	31.6	0	0	2.8	14.4	4.4	6.9	0	1.7			832.7	147.8	7.8	0.4	58.5	1.2	27.3	1.6	0	0	25.0	23.3
PI A12-20 (PPI 101-109)	78.3	0	7.3	0	7.8	1.7	10.0	29.4	0	0	2.6	12.2	20.0	0	21.3	0	0				0	12.6	75.1	0	0	0	0	0	12.8	7.8
GAD 114-123	34.4	13.3	35.0	0	4.4	18.3	10.0	0	11.1	13.9	0	0	0	9.0	9.1	9.5	20.4	143.3	820.5	87.8	7.8	31.5	0	0	7.3	0	117.6	167.2	37.2	0
GAD 536-545	48.9	1.1	15.0	0	16.7	3.9	1.1	18.3	3.3	0	0	37.7	27.8	5.2		6.1						12.6	32.9	2.3	1.2	0	23.1	32.8	15.0	3.3
IA-2 206-214	56.7	7.8	0	0	17.8	7.2	1.1	0	0	23.9	0	112.2	1.1	0.7	19.1	72.8			720.5	222.2	2.8	47.1	86.2	4.5	0	0	47.6	180.5	7.2	8.9
IGRP 228-236	81.7	12.2	0	0	26.9	2.8	1.1	6.1	36.1	0	0.4	12.2		0	12.4	3.9						16.0	52.9	2.3	91.7	0	7.6	119.4	0	6.7
IGRP 265-273	11.1	2.2	6.1	0			3.3	6.1	41.1	0	0			8.5	13.5	17.2						29.3	126.8	0	18.4	0	5.4	295.0	0	3.3
Viral mix control	53.3	30.6	532.8	588.9	160.0	86.1	343.3	147.2	223.3	325.0	837.0	910.4	287.8	114.0	180.2	17.2	458.2	476.6	1053.8	978.8	32.2	11.5	606.2	548.9	319.5	447.7	500.9	673.8	284.4	276.1
PHA	++++	148.9	++++	++++	++++	++++	712.2	++++	638.8	259.4	++++	++++	211.1	324.0	++++	561.6	1557.8		++++		733.4	72.6	212.9	++++	165.0	++++	++++	++++	++++	807.7
Basal+3SD	26.5	18.3	19.1	86.9	16.3	20.4	5.2	27.9	25.8	57.8	15.1	53.5	16.3	36.5	55.0	14.7	4.4	5.7	66.2	110.6	5.8	26.5	76.7	10.3	150.1	31.1	48.9	135.3	21.1	13.7
Basal+4SD	35.3	24.3	25.5	115.8	21.8	27.2	6.9	37.2	34.4	77.0	20.1	71.4	21.8	48.7	73.4	19.6	5.9	7.7	88.3	147.6	7.7	35.3	102.3	13.8	200.1	41.5	65.2	180.3	28.2	18.2
Basal+5SD	44.0	30.4	32.0	144.8	27.3	34.0	8.6	46.4	43.0	96.3	25.3	89.2	27.2	60.8	91.7	24.5	7.3	9.5	110.5	184.5	9.5	44.2	127.9	17.2	250.2	51.8	81.5	225.4	35.2	22.8
Basal	20.0	11.1	18.3	24.4	8.9	3.9	1.1	92.8	42.2	147.2	8.5	137.8	8.9	50.4	68.7	6.1	0.7	5.6	85.0	221.1	2.2	21.8	90.4	4.4	120.5	37.3	72.4	226.1	43.9	18.9

	HEALTHY CONTROLS (n=15)																													
	H01		H02		H03		H04		H05		H06		H07		H08		H09		H10		H11		H12		H13		H14		H15	
	0 mo	24 mo	0 mo	16 mo	0 mo	26 mo	0 mo	15 mo	0 mo	15 mo	0 mo	14 mo	0 mo	22 mo	0 mo	22 mo	*0 mo*	12 mo	0 mo	14 mo	0 mo	14 mo	0 mo	14 mo	0 mo	14 mo	0 mo	14 mo	0 mo	13 mo
PPI 2-10	0	30.6	0	5.6	19.5	8.8	0	0	0	0	8.9	0	0	3.9	7.7	9.4	5.6	17.8	0	3.9	2.7	0	0	0	0	37.3	1.2	0	0	0
PI B10-18 (PPI 34-42)	0	0	0	0	0	0	5.9	0	5.9	0	7.8	0	0	2.8	0	0	12.2	0	0	0.5	2.7	0	0	0	0.5	43.9	0	0	0	0
PI B18-27 (PPI 42-51)	0	0	0	0	0	6.6	4.8	0	4.8	0	5.6	16.1	0.4	15.0	0	16.1		0	0	1.6	0	9.4	0	0	0.5	20.6	2.3	7.7	5.0	35.0
PI A12-20 (PPI 101-109)	0	6.2	0	1.1	0	0	0	0	0	0	1.1	0	0	1.7	10.0	18.3	31.1	19.4	18.3	0.5	1.6	0	2.8	0	0.5	0	31.1	0	0.6	0
GAD 114-123	1.1	64.5	0	0	4.0	0	11.5	1.6	11.5	1.6	6.7	0	0	15.0		19.4	12.2	1.7	20.6	3.9	2.7	0	12.8	18.3	0	0	37.8	0	0	0
GAD 536-545	0	0	8.5	3.3	0	0	4.8	0	4.8	0	6.7	0	0	12.8		0		1.1	0	2.7	1.6	0	0	0	0	0	0	0	2.8	0
IA-2 206-214	0	15.0	0	25.6	0	0	0.4	0	5.5	20.0	7.8	0	0	5.0	161.1	61.6		26.7	0	1.6	0.5	1.7	6.1	0	0	2.8	21.1	0	0	0
IGRP 228-236	0	0	0	1.1	0	4.4	0	0	0	0	4.5	0	42.7	0	117.7	0		2.0	0	0	2.7	0	0	0	0.5	1.7	6.7	6.6	0	0
IGRP 265-273	3.3	58.4	34.0	24.4	0	0	20.4	0	0	0		0	0	7.3		36.1		3.3	0	0.5	2.7	0	0	0	0	9.5	52.3	13.3	0.6	35.0
Viral mix control	13.3	39.5	466.2	642.2	806.1	516.0	238.1	136.6	91.1	216.6	364.4	371.1	3.8	135.0	326.6	165.0	202.2	178.9	345.0	100.0	502.7	508.3	353.9	268.3	106.1	130.6	534.4	568.8	260.6	138.3
PHA	677.7	478.1	++++	806.6	++++	701.0	++++	873.2	724.3	++++	606.6	++++	++++	363.9	++++	205.5	1340.0	++++	++++	208.3	674.9	++++	819.4	898.3	397.2	++++	++++	778.8	407.2	518.3
Basal+3SD	127.9	66.5	44.8	43.4	32.7	60.5	57.1	34.1	8.2	50.2	26.3	33.7	44.4	16.0	114.7	84.9	19.0	29.6	102.4	5.5	4.1	30.8	13.3	50.5	51.1	51.1	73.1	27.3	40.7	97.7
Basal+4SD	170.5	88.6	59.8	57.8	43.6	80.7	76.1	45.4	10.9	66.9	35.1	44.9	59.2	21.4	152.9	113.3	22.7	39.4	136.6	7.3	5.4	41.1	17.7	67.3	68.2	68.2	97.5	36.4	54.3	130.3
Basal+5SD	213.3	110.8	74.8	72.3	54.5	100.9	95.2	56.8	13.6	83.7	43.9	56.2	74.0	26.7	191.3	141.6	28.4	49.2	170.7	9.1	6.8	51.4	22.2	84.2	85.2	85.2	121.9	45.5	67.8	162.9
Basal	36.7	60.5	30.4	20.0	32.7	55.6	59.6	16.7	5.6	20.0	3.3	27.2	34.0	9.4	66.7	102.8	7.8	13.3	57.2	1.7	0.6	25.0	6.1	18.3	89.4	89.4	54.4	57.8	19.4	225.0