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Date:

2012-01-01

Citation:

Coppieters, K. T., Dotta, F., Amirian, N., Campbell, P. D., Kay, T. W. H., Atkinson, M. A., Roep, B. O. & von Herrath, M. G. (2012). Demonstration of islet-autoreactive CD8 T cells in insulitic lesions from recent onset and long-term type 1 diabetes patients. *Journal of Experimental Medicine*, 209 (1), pp.51-60. <https://doi.org/10.1084/jem.20111187>.

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# Demonstration of islet-autoreactive CD8 T cells in insulitic lesions from recent onset and long-term type 1 diabetes patients

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**A direct association of islet-autoreactive T cells with  $\beta$  cell destruction in human pancreatic islets from type 1 diabetes (T1D) patients has never been demonstrated, and little is known about disease progression after diagnosis. Frozen pancreas samples were obtained from 45 cadaveric T1D donors with disease durations ranging from 1 wk to >50 yr, 14 nondiabetic controls, 5 nondiabetics with islet autoantibodies, 2 cases of gestational diabetes, and 6 T2D patients. Sections were systematically analyzed for the presence of insulin-sufficient  $\beta$  cells, CD8<sup>+</sup> insulitic lesions, and HLA class I hyperexpression. Finally, consecutive sections from HLA-A2-expressing individuals were probed for CD8 T cell reactivity against six defined islet autoantigens associated with T1D by in situ tetramer staining. Both single and multiple CD8 T cell autoreactivities were detected within individual islets in a subset of patients up to 8 yr after clinical diagnosis. Pathological features such as HLA class I hyperexpression and insulitis were specific for T1D and persisted in a small portion of the patients with longstanding disease. Insulitic lesions consistently presented in a multifocal pattern with varying degrees of infiltration and  $\beta$  cell loss across affected organs. Our observations provide the first direct proof for islet autoreactivity within human islets and underscore the heterogeneous and chronic disease course.**

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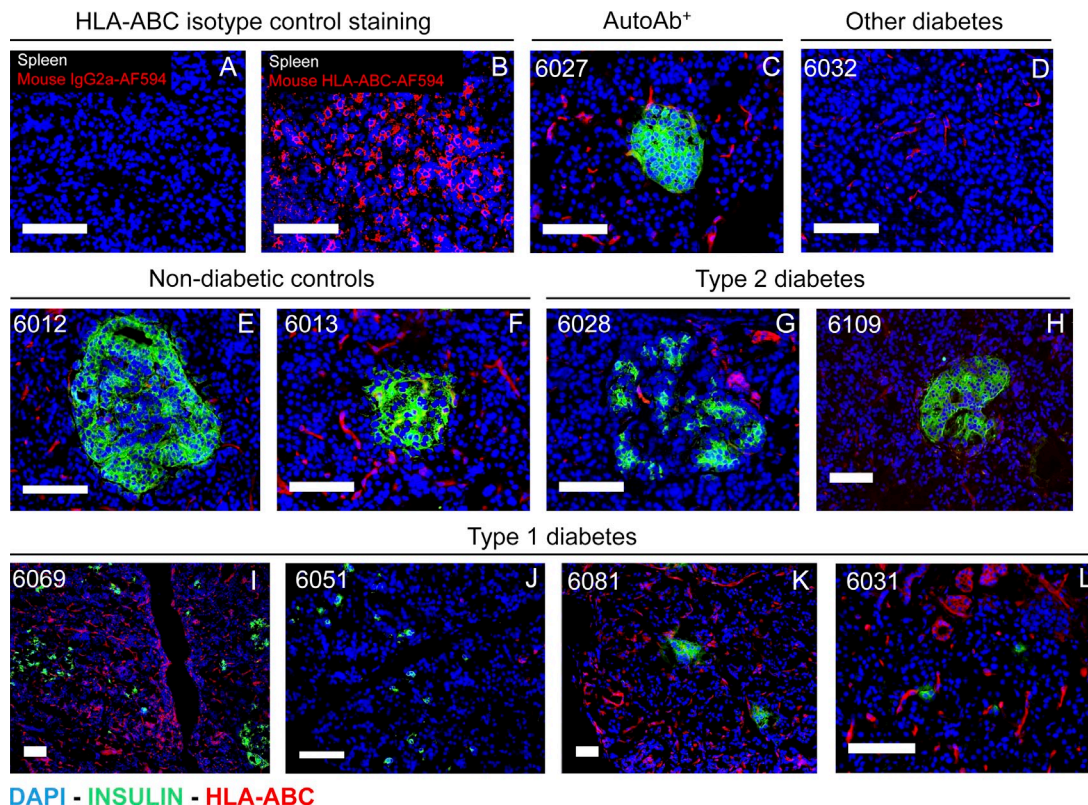
Abbreviations used: NOD, non-obese diabetic; nPOD, Network for Pancreatic Organ Donors with Diabetes; PPI, preproinsulin; T1D, type 1 diabetes.

Type 1 diabetes (T1D) development is associated with inflammation of the pancreatic islets and the presence of leukocyte infiltrates (insulitis) has long served as a hallmark in differentiating the disease from other forms of diabetes (Gepts, 1965). The histological composition of islet infiltrates includes B cells, macrophages, and NK cells but is typically dominated by CD8 T cells (Coppieters and von Herrath, 2009). From a quantitative perspective, islet infiltration was long thought to be substantial, but recent studies suggest occurrence of subtle infiltrates, often with only a few immune cells per given islet (Willcox et al., 2009). T cell infiltration into the islets, including consequential  $\beta$  cell loss, has been proposed to display a

markedly focal distribution, affecting a minority of islets at a given time (Foulis et al., 1986). A recent study in autoantibody-positive organ donors without T1D found evidence of insulitis in only 10% of islets or less (In't Veld et al., 2007). Collectively, these data indicate that the autoimmune response which eventually culminates in overt clinical T1D is distinct from that in animal models.

Because previous studies largely focused on patients with 1 yr of clinical disease or less, data

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**Figure 1. Histopathological survey of diabetic donors and controls for insulin (green) and MHC class I (red) expression.** (A and B) Positive control staining for HLA-ABC on human spleen section and parallel isotype-matched negative control staining. (C) Nondiabetic individual with autoantibodies against ZnT8. (D) Case of undefined diabetes. (E and F) Nondiabetic donors. (G and H) Type 2 diabetics. (I and L) Longstanding T1D cases without HLA class I hyperexpression. All case numbers correspond to [Table S1](#). Bars, 100  $\mu$ m.

beyond that duration of time are scarce but suggest that some longstanding patients exhibited signs of persistent islet autoimmunity such as insulinitis (Foulis et al., 1986; Meier et al., 2005) and MHC class I hyperexpression (Foulis et al., 1987). In a selective cohort of T1D patients with disease duration of >50 yr, many cases maintained a residual pool of functional  $\beta$  cells at the time they deceased (Keenan et al., 2010). From a therapeutic perspective, validation of this suggestion would be of crucial importance, as it is currently uncertain whether intervention could be feasible late after diagnosis.

Immune assays on circulating T cells are emerging as vital tools in the longitudinal assessment of islet autoimmunity and the efficacy of experimental treatment (Roep and Peakman, 2010). The assumption that peripheral blood offers a representative source of diabetogenic T cell specificities is pivotal to the validity of T cell assays. Animal studies have indicated that T1D-related T cell specificities found in the peripheral blood correspond to some degree with pancreatic infiltrates (Trudeau et al., 2003; Wong et al., 2007; Enée et al., 2008). However, support for a similar concordance in humans is currently lacking. In fact, the presence of autoantigen-specific CD8 T cells at the level of the target organ (i.e., the human pancreatic islet) as ultimate proof of the autoimmune nature of T1D has never been demonstrated so far.

As diabetic pancreata are difficult tissues to obtain, we have taken advantage of a series of rare cases and combined these with tissues from the Network for Pancreatic Organ donors with Diabetes (nPOD), which procures freshly frozen pancreas samples from T1D patients and antibody-positive prediabetic individuals in a coordinated effort across the United States and beyond ([www.jdrfnpod.org](http://www.jdrfnpod.org)). Our studies provide definitive proof of presence of islet-reactive CD8 T cells in insulitic lesions from patients diagnosed with T1D.

## RESULTS

### Systematic screening for insulin production, MHC class I hyperexpression, and CD8<sup>+</sup> infiltration in T1D pancreata and controls

Pancreas sections from all 72 available samples were systematically assayed for the presence and distribution pattern of insulin-sufficient islets, CD8<sup>+</sup> insulinitis, and HLA class I hyperexpression (see [Table 2](#) and [Table S1](#)).

Specificity of the antibody against a common epitope from the HLA-ABC gene products was ascertained on human spleen sections ([Fig. 1, A and B](#)) and nondiabetic pancreata (negative control, [Fig. 1, E and F](#)). Furthermore, none of the islet autoantibody-positive samples ([Fig. 1 C](#)), T2D cases ([Fig. 1, G and H](#)), or undefined/gestational diabetics ([Fig. 1 D](#)) showed any MHC class I expression on their islet cells. From parallel CD8 staining

**Table 1.** Demographic information

ID	Duration	Auto-Ab	Age	Gender	Ethnicity	C peptide	BMI	HLA typing
			yr			ng/ml		
<b>Recent onset cases</b>								
Patient A	<1 wk	mIAA <sup>+</sup> IA2ic <sup>+</sup>	9	F	Caucasian	undetectable	N.D.	A2, 25 B8 DR3,4
Patient B	1 wk	mIAA <sup>+</sup> GAD65 <sup>+</sup> IA2ic <sup>+</sup>	5	M	Caucasian	undetectable	N.D.	A2, 25 B8, 60 DR3, 4
Patient C	10 wk	GAD65 <sup>+</sup>	24	M	Caucasian	1.61	22.9	A3,29 B7,44 DR7,13
Patient D	6 mo	IA2ic <sup>+</sup> GAD65 <sup>+</sup>	14	F	Caucasian	0.65	20.1	A2, 25 B8 DR3, 3
Patient E	9 mo	GAD65 <sup>+</sup> IA2ic <sup>+</sup>	26	F	Caucasian	1.12	24.8	A2,2 B18,44 DR3,4
<b>Longstanding cases</b>								
Patient 1	1 yr	GAD65 <sup>+</sup> IA2ic <sup>+</sup>	17	F	Caucasian	0.9	21.2	A2,25 B8 DR3,4
Patient 2	1 yr	ICA512 <sup>+</sup> mIAA <sup>+</sup> IA2ic <sup>+</sup>	12	M	African American	0.18	20.3	A*0201, 6801 B*0602, 1502 DRB1*0901, 1602 DQA1*0102, 0301 DQAB1*0303, 0502
Patient 3	3 yr	GAD65 <sup>+</sup>	19	M	Caucasian	0.35	24.5	A2,2 B18,44 DR3,3
Patient 4	3 yr	GAD65 <sup>+</sup>	19	M	Caucasian	1.2	ND	A*0101, 0201, B*0801, 5101 DR*0301, 0404
Patient 5	7 yr	ICA215 <sup>+</sup> mIAA <sup>+</sup> IA-2ic <sup>+</sup>	22	F	Caucasian	<0.05	21.6	A*0201, 0205 B*3801, 5801 DRB1*1001, 1601 DQA1*0101, 0102 DQAB1*0501, 0502
Patient 6	8 yr	ICA512 <sup>+</sup> ZnT8 <sup>+</sup> IA-2ic <sup>+</sup>	18	F	Caucasian	<0.05	25.2	A*0201, 0301 B*1501, 3901 DRB1*0101, 0401 DQA1*0101, 0301 DQAB1*0302, 0501

ICA512, islet cell autoantigen 512; ZnT8, zinc transporter 8; IA-2ic, intracytoplasmic domain of the tyrosine phosphatase IA-2; mIAA, micro assay for insulin autoantibodies; GAD65, Glutamic acid decarboxylase 65; ND, not determined

results, we also conclude that substantial exocrine infiltration does not necessarily correlate with MHC class I up-regulation on islet cells (e.g., case 6101 and case 6028; Table S1). This observation suggests that MHC class I up-regulation, although it is a strictly T1D-associated phenomenon, is not merely a consequence of inflammatory local conditions.

CD8<sup>+</sup> cell accumulation and MHC class I hyperexpression within pancreatic islets was found exclusively within a subset of T1D subjects. Although 10 T1D donors showed evidence of concurrent  $\beta$  cell preservation, CD8<sup>+</sup> infiltration,

and MHC class I hyperexpression (see Table 2 and next section), six longstanding cases showed residual  $\beta$  cells only (Fig. 1, I–L). Of note, only two (nPOD 6081 and 6038; Table S1) out of five (the others are nPOD 6051, 6031, and 6065; C-peptide data from 6069 are not available) of these longstanding, insulin-positive donors without infiltration or MHC class I hyperexpression had detectable C-peptide levels. We thus present further evidence of the persistence of insulin-producing  $\beta$  cells, even in patients that have no detectable levels of C-peptide and with up to 56 yr of disease.

**Table 2.** Histopathological features

ID	Duration	Insulin <sup>+</sup> islets	CD8 <sup>+</sup> cells/islet (islets counted)	Islet HLA class I (pattern)	Tetramer reactivity <sup>a</sup> (pattern)
%					
<b>Recent onset cases</b>					
Patient A	<1 wk	Isolated cells only	8.8 (160)	Positive	Insulin, IGRP (monospecific)
Patient B	1 wk	None	8.4 (125)	Positive	Insulin, IGRP (monospecific)
Patient C	10 wk	Partial loss	<1 (128)	Positive	HLA-mismatched control (no staining)
Patient D	6 mo	25	3.5 (116)	Positive	Insulin
Patient E	9 mo	50	4.1 (150)	Positive	Insulin
<b>Longstanding cases</b>					
Patient 1	1 yr	<50	3.2 (140)	Positive	IGRP
Patient 2	1 yr	10	4.3 (40)	Positive	IGRP, IA-2, insulin (mono-/multispecific)
Patient 3	3 yr	15	1.5 (70)	Positive	No staining
Patient 4	3 yr	39	6.5 (20)	Positive	IGRP, GAD65, pplAPP, insulin (multispecific)
Patient 5	7 yr	70	9.4 (30)	Positive (tail region only)	IGRP, GAD65, PPI, insulin, IA-2 (mono-/multispecific)
Patient 6	8 yr	70	3.5 (20)	Positive (lobular)	No staining

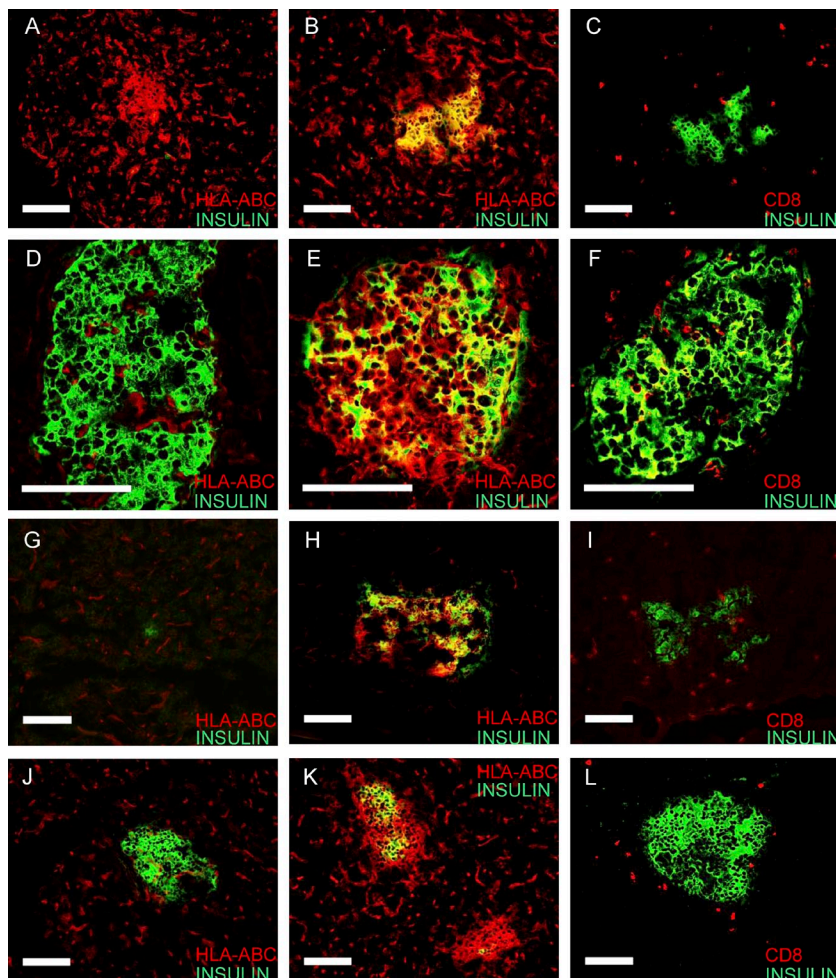
<sup>a</sup>patients A, B, C, D, E, 1, and 3 were stained for reactivity against IGRP, insulin, and PPI in Siena. Patients 2, 4, 5, and 6 were stained for reactivity against IGRP, insulin, PPI, IA-2, GAD65, and pplAPP in La Jolla.

### Multifocal islet autoimmunity, detectable in recent-onset disease, persists in a subset of longstanding T1D patients

All 11 HLA-A0201 haplotyped T1D patients with clear pathological MHC class 1 up-regulation and CD8<sup>+</sup> infiltration were selected (Table 1) and subjected to detailed histological analysis within multiple pancreatic regions (Table 2). Patients were arbitrarily categorized as recent-onset (<1-yr duration) or longstanding (≥1-yr duration). Acuteness and severity of clinical presentation is usually inversely correlated with age at diagnosis (Pipeleers et al., 2008). Indeed, the two youngest subjects (9 and 5 yr old) showed little to no remaining insulin-containing islets within a week after diagnosis. Despite the almost complete loss of functional β cells in these individuals, ubiquitous CD8<sup>+</sup> insulitic lesions and HLA class I hyperexpression on islet cells was found shortly after clinical diagnosis. The other three recent-onset cases showed partial and lobular loss of insulin secretion, low to moderate levels of islet inflammation, and pronounced hyperexpression of HLA class I (Foulis et al., 1986).

These histopathological features could persist long after clinical diagnosis, their quantitative distribution being similar to that typically observed in recent-onset T1D pancreata (Table 2; Fig. 2). Patient 2 (1-yr disease duration) had residual insulin-positive β cells in the tail region only (Fig. 2, A–C). HLA class I hyperexpression was found on all islets, regardless of their insulin content and islet composition (Fig. 2, A and B). Insulin-deficient islets showed no significant

CD8<sup>+</sup> cellular infiltrate, whereas small insulin-expressing islets did (Fig. 2 C). Patient 4 (3-yr duration) had a substantive amount of large insulin-expressing islets (Fig. 2, D–F), most of which highly expressed HLA class I (Fig. 2 E) and were infiltrated by CD8<sup>+</sup> cells (Fig. 2 F), with only few exceptions (Fig. 2 D). Patient 5 (7-yr duration) had a limited number of insulin<sup>+</sup> islet cell clusters outside the tail region, all of which expressed HLA class I similar to nondiabetic controls (Fig. 2 G). The tail region, however, contained considerable numbers of insulin<sup>+</sup> islets, which all showed high expression of HLA class I (Fig. 2 H), and variable numbers of CD8<sup>+</sup> cells ranging from none to >50 (Fig. 2 I). Patient 6 (8 yr of clinical disease) showed evidence of insulin-positive β cells within the majority of blocks examined (Fig. 2, J–L). The degree of HLA class I up-regulation on islet cells was highly variable between lobules, with some regions lacking hyperexpression (Fig. 2 J), whereas increased levels were found on most insulin-expressing islets in other areas (Fig. 2 K). CD8<sup>+</sup> cells were found in association with insulin<sup>+</sup> islets (Fig. 2 L). Importantly, our multi-region histological analysis exposed a lack of correspondence between C-peptide values, a measure of β cell function, and insulin-positive β cell mass in longstanding patients 5 and 6 and, to a lesser extent, recent-onset patient A. This discrepancy is easily overlooked when analyzing only a single region of the pancreas, which can be completely devoid of insulin, whereas other regions do in fact show insulin expression ([www.jdrfnpod.org](http://www.jdrfnpod.org)).



**Figure 2. Histological analysis of longstanding cases shows protracted pathology.** Frozen pancreas sections from patient 2 (A–C), patient 4 (D–F), patient 5 (G–I), and patient 6 (J–L) were stained for insulin, CD8, and with antibodies that recognize a common epitope from the HLA-A, -B, and -C gene products. Bars, 100  $\mu$ m.

two longstanding cases showed no CD8 T cell reactivity against any of the epitopes tested, in spite of insulinitis. In conclusion, sensitive in situ detection suggests stochastic infiltration and expansion of CD8 T cells with single  $\beta$  cell specificity, whereas insulinitic lesions long after diagnosis contain lesions with diverse and multiple T cell autoreactivities.

#### Immunofluorescent detection

To colocalize CD8 detection with insulin within the same section, immunofluorescent techniques are required. To amplify the fluorescent signal originating from in situ tetramer binding, an approach was adopted from McGavern et al. (2002) that we previously applied (Christen et al., 2004). In accordance with our immunohistochemical data, islet-autoreactive T cells were undetectable in any of the inflamed islets in Patient 6 (unpublished data). Likewise, none of the imaged CD8<sup>+</sup> cells colocalized with tetramer fluorescence beyond background in Patient 4 (unpublished data). The apparent contrast with the results obtained in this pa-

tient by immunohistochemistry may result from different pancreatic regions being sectioned and stained. Alternatively, the TCRs targeted may be of lower avidity that can only be detected by the more sensitive method.

Across eight consecutive pancreatic sections from Patient 5, containing numerous inflamed and insulin-positive islets, fluorescent signal was observed in association with tetramers of five different islet-autoreactive specificities (Fig. 4). Of note, the frequency of positivity on a per-islet basis was highly variable ranging from a single T cell reactive against IGRP to virtual monospecificity associated with IA-2 or preproinsulin (PPI; Fig. 4 A). The presence of multiple specificities of autoreactive T cells within a consecutively cut islet was confirmed with reactivity against GAD65 (not depicted), IA-2, and insulin but predominantly PPI (Fig. 4 B). In Patient 2, CD8<sup>+</sup> islet infiltrates specific for IGRP, insulin, and IA-2 were detected (unpublished data). An insulin-positive islet could be captured in all eight consecutive sections that showed signal for two tetramers, i.e., IGRP and insulin (unpublished data). Consistent with the magnitude of the signal as detected by flow cytometry, and despite the amplification strategy used in this paper, fluorescence associated with in situ tetramer staining was generally dim. Collectively, these

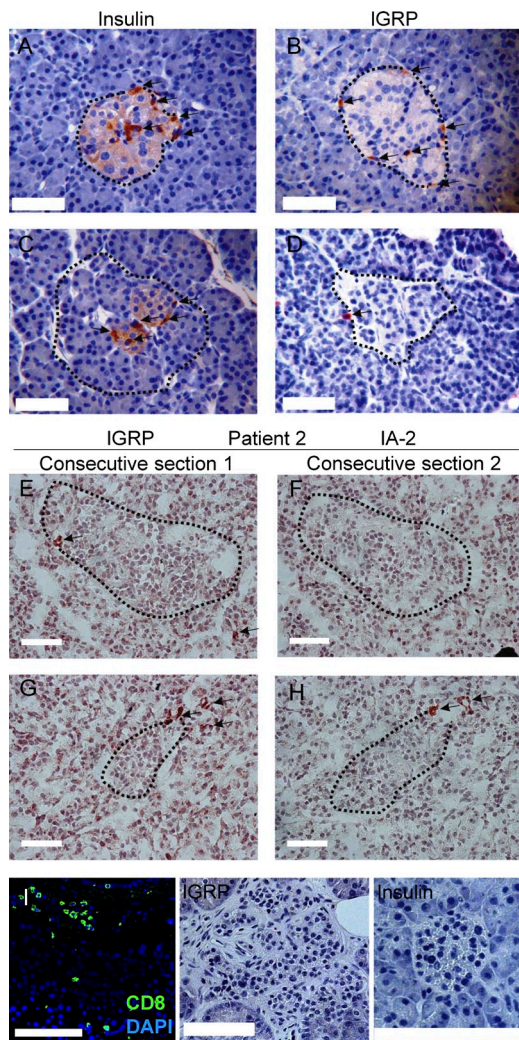
#### Detection of islet-reactive CD8 T cells in pancreatic islets from T1D patients with varying clinical disease duration

We next investigated whether islet antigen-specific CD8 T cells could be found within insulinitic lesions of individuals suffering recent-onset or longstanding T1D. Protocol optimization was performed using NRP-V7 tetramer on frozen pancreas sections from nonobese diabetic (NOD) mice previously screened for CD8 T cell reactivity against IGRP islet antigen in the blood (not depicted; see Material and methods for details). We included two types of negative controls: concurrent staining of all pancreatic tissues using tetramers loaded with irrelevant peptide and inclusion of an HLA-mismatched tissue.

#### Immunohistochemical detection

Single CD8 islet-autoreactive T cell specificities could be detected per islet in insulinitic lesions from recent-onset patients, whereas anatomically nearby islets could express single autoreactivity against a distinct  $\beta$  cell autoantigen (Fig. 3, A–C). In some longstanding patients, however, these islet infiltrates were characterized by the simultaneous presence of multiple CD8 T cell reactivities (Fig. 3, G–H).

Overall, reactivity against IGRP was most ubiquitous within lesions of all patients examined (Fig. 3 and not depicted). Finally,



**Figure 3. Immunohistochemical detection of islet antigen-reactive CD8 T cells within insulinitic lesions of T1D patients.** (A–C) Typical tetramer staining as observed on pancreas sections from recent-onset patients. (A and B) Reactivity against insulin and IGRP in two distinct islets from patient B. (C) insulin reactivity in patient A. (D) IGRP reactivity in long-standing patient 1. (E–H) In long-standing patient 2, tetramers loaded with IGRP and IA-2 yielded distinct signal. (E–H) Analysis of the same islet within consecutive sections showed that both these specificities could be detected within a single islet (G and H), whereas others contained only a single specificity (E and F). (I) Negative controls included HLA-mismatched pancreas tissue from patient C, which contained CD8<sup>+</sup> insulinitis but did not show tetramer signal. Arrows indicate tetramer-positive cells. Dashed lines are islet contours. Bars, 100 μm.

fluorescent staining data confirm that in situ tetramer staining signal colocalizes with the CD8 marker in proximity to islets and thus identify the presence of islet-reactive CD8 T cells within insulinitic lesions.

### Overview

The graphical overview in Fig. 5, based on all samples analyzed in this study, illustrates that the pathological signs studied in this paper are entirely absent in islet antibody-positive individuals,

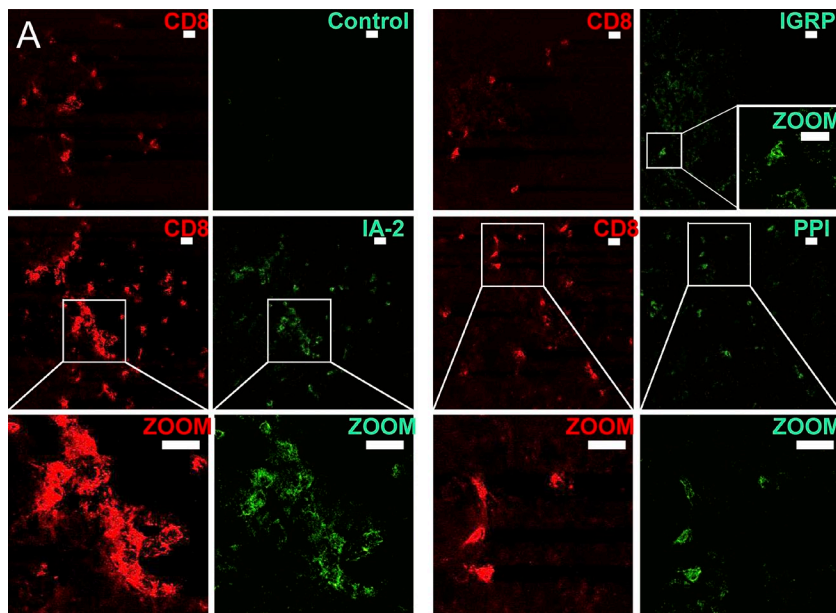
and that these subjects possess normal C-peptide levels and insulin-sufficient islets. Importantly, only detection of multiple autoantibodies is closely associated with disease risk (Zhang and Eisenbarth, 2011), but even in an individual with three autoantibodies no abnormalities were detected. Inflammation, CD8 T cell autoreactivity, and MHC class I hyperexpression peaks in the patient subset with duration <1 yr, whereas the majority of these individuals maintain at least some degree of C-peptide and insulin production. Beyond 1 yr of disease duration, all these parameters concurrently decline. Although infiltration and MHC class I hyperexpression become undetectable 10 yr after diagnosis, a fraction of these cases still shows evidence of C-peptide and insulin production. These findings indicate that although significant retention of CD8<sup>+</sup> islet infiltration requires the presence of insulin antigen, a minor population of β cells may escape autoimmune destruction. It is also clear from this overview that the selected set of longstanding cases with protracted disease represents only a subset of the entire cohort, as in most pancreata no signs of inflammation or MHC class I up-regulation were detected.

### DISCUSSION

We report the histopathology of the largest contemporary cohort of T1D cases and compared this with 27 nondiabetic and diabetic controls. Exploiting the unprecedented quality and availability of frozen pancreas tissue, the specificity of the autoimmune response within pancreatic islets from a series of T1D patients with disease duration ranging from 1 wk to 8 yr could be investigated.

Detailed histopathological analysis of the subjects with longstanding disease revealed several features that are typically associated with new manifestation of T1D, such as lobular hyperexpression of MHC class I and variable patterns of β cell loss and insulinitis across lobules. These features appear to persist only in a relatively small subset of cases long after diagnosis, and it could be speculated that overall T1D pathology may, under certain conditions, adopt a more protracted course. Some theories suggest that autoimmunity and β cell decay in T1D occur at variable tempo in distinct patients resulting in different rates of progression to clinical hyperglycemia (van Belle et al., 2011). If this assumption holds, it is plausible that such differences continue to exist after diagnosis and may account for our present observations. Furthermore, our work confirms earlier data by Foulis et al. (1987) in the sense that HLA class I hyperexpression is a phenomenon restricted to T1D. We find, however, that hyperexpression does not require insulin positivity or inflammation on the individual islet level; in other words, we found significant numbers of pseudo-atrophic, MHC class I<sup>high</sup> islets (Fig. 2 A).

We also present further evidence of the persistence of insulin-producing β cells (Gianani et al., 2010; Keenan et al., 2010), even in patients that have no detectable levels of C peptide and with up to 56 yr of disease. Our studies provide compelling evidence of substantial β cell mass at diagnosis of T1D that may last for many years in a subset of patients. Extensive β cell preservation, in spite of loss in β cell function,

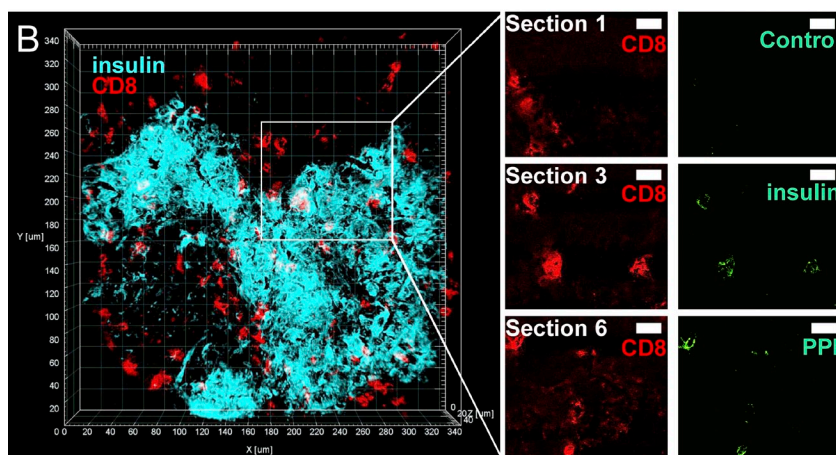


**Figure 4. Immunofluorescent detection of islet-reactive CD8 T cells in patient 5.** (A) Representative examples of positive signal from distinct CD8<sup>+</sup> inflamed islets within eight consecutive pancreas sections. Background threshold was set on multiple islets from the section stained with irrelevant control tetramer and tetramer-associated signal was then collected using these instrument settings. (B) Reconstructed 3D image from a distinct pancreatic region, representing a single islet captured within consecutively cut sections (not by z-stacking, see Materials and methods). Magnified region shows tetramer positivity within distinct sections of the stack. Staining with pplAPP in this patient's pancreatic tissue exhibited high background, as indicated by its apparent lack of colocalization with CD8, and was therefore not interpreted. Bars, 20 μm.

β cell replacement therapy (Voltarelli et al., 2007; Couri et al., 2009).

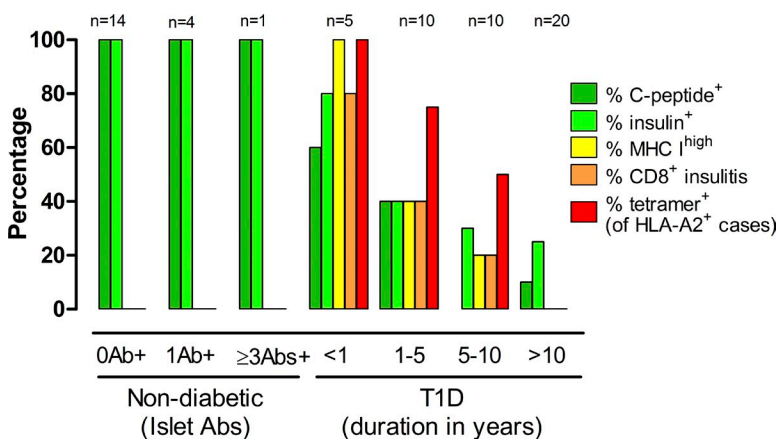
The patterns of infiltration and β cell loss in fact bear resemblance to the multifocal loss of melanocytes as observed in vitiligo, an analogy coined by Eisenbarth (2010). Both conditions share other characteristics such as chronicity, autoantibodies, occurrence of inflammatory lesions, and HLA-associated susceptibility (Passeron and Ortonne, 2005). We propose that understanding the mechanisms selectively targeting a subset of anatomically close cells at the time will provide insights in the etiologies of both autoimmune diseases.

Our in situ tetramer experiments directly indicate that, at least in some patients, CD8 T cells found within insulinitic lesions recognize islet antigens and that



may explain the unexpected, lasting remission, rapid recovery of C-peptide production, and insulin dependency observed in recent-onset type 1 diabetic patients after nonmyeloablative therapy and autologous bone marrow transplantation, without

T1D in these patients is likely caused by an autoimmune response. Early after diagnosis, no islets were observed that contained more than a single specificity of islet-autoreactive CD8 T cells, whereas in case of longstanding disease, inflamed islets usually contained multiple islet-reactive specificities. In some occasions, however, the majority of CD8<sup>+</sup> cells associated with a particular islet within a focal plane consisted of a single specificity, possibly of clonal origin. T cells



**Figure 5. Graphical overview of results from systematic staining and in situ tetramer assay.** The graph represents combined data from Table S1, Table 1, and Table 2, according to presence of autoantibodies and disease duration. The number of samples within each category is shown at the top. Tetramer staining was performed only on HLA-0201-positive samples with insulinitis. A total of 14 nondiabetic controls (Table S1), 5 auto-antibody positive subjects (Table S1), and 34 (Table S1) + 11 (Table I) Type 1 diabetics were included in the graph.

**Table 3.** Antibodies used for characterization of the sections prior to tetramer staining

Antigen	Primary antibody	Detection antibody
Insulin	Polyclonal guinea pig anti-insulin (Dako; 1/140 dilution, 1-h incubation)	Polyclonal goat anti-guinea pig IgG, highly cross-adsorbed, Alexa Fluor 488 (Invitrogen; 1/1000 dilution, 30-min incubation)
HLA-ABC	Mouse monoclonal (clone W6/32) IgG2a against a monomorphic epitope on the 45 kD polypeptide products of the HLA-A, B and C loci (Dako; 1/100 dilution, 1 h incubation)	Polyclonal goat anti-mouse IgG2a, isotype-specific, Alexa Fluor 594 (Invitrogen; 1/1000 dilution, 30-min incubation)
CD8	Mouse monoclonal (clone HIT8a) IgG1 against the CD8 alpha subunit (BD; 1/100 dilution, 1-h incubation)	F(ab') <sub>2</sub> fragment of goat anti-mouse IgG, Alexa Fluor 594 (Invitrogen; 1/1000 dilution, 30-min incubation)

in the islets of diabetic NOD mice proliferate vigorously (Fousteri et al., 2010), allowing us to envision a scenario of local clonal T cell expansion that would account for the localized areas of monospecificity. We propose that a concept of determinant spreading may explain the evolution from T cell monospecificity to multispecificity within islets as disease progresses (Lehmann et al., 1993). This might involve the progressive release of novel antigens as a consequence of ongoing apoptosis caused by a primary population of diabetogenic T cells. The ensuing secondary generation and influx of a new wave of islet-specific T cells would then contribute to disease progression.

Of note, some of the specificities detected in this paper are currently under scrutiny as candidates for experimental tolerization therapy in recent-onset patients (Peakman and von Herrath, 2010). Our present data suggest that, after diagnosis, these CD8 T cell effectors may mediate terminal  $\beta$  cell loss and could codetermine the disease's tendency to recur even after immune therapies. Tolerization of these CD8 T cell species may thus be a requirement to achieve lasting remission. Our histopathological assessment shows many parallels with the recent-onset cohort that was recently revisited (Willcox et al., 2009), and the infiltrating CD8 T cells may thus bear relevance in the protracted effector phase that on some occasions characterizes the course of T1D after clinical onset. Interventions aimed at preventing lymphocyte migration from one islet or lobule to other regions may ultimately be required to halt disease progression.

In conclusion, we show first proof that autoimmune, islet-reactive CD8 T cell species infiltrated islets from individuals with recent-onset and longstanding T1D. This data directly underscores the functional relevance of T cell reactivities that were previously detected in the circulation and offers a strong rationale for their detection as biomarkers for treatment and intervention.

## MATERIALS AND METHODS

### Patients

6- $\mu$ m sections from freshly frozen pancreas samples were obtained via nPOD (See Table 1 and Table S1 for full details). nPOD case numbers 6046, 6052, and 6070 were here relabeled patients 6, 2, and 5, respectively, to improve readability. Additional samples were from Italy (patients A, B, D, E, 1, and 3) and Melbourne (case SVI021-08, patient 4). Sections from patient C were provided by P. Marchetti (University of Pisa, Pisa, Italy). Samples were from cadaveric donor pancreases and demographic information is disclosed in

Table 1 and Table S1. Cases for tetramer staining were selected from a total of 45 patients diagnosed with T1D based on the presence of CD3<sup>+</sup> insulinitis and HLA-A0201 haplotype. Upon arrival, the first section of a consecutive series was assayed for insulin and CD8<sup>+</sup> insulinitis. After confirmation, the remainder of the consecutive sections was stained with the respective tetramers in a single experimental run per patient. Standard operating procedures for organ processing, C-peptide, and autoantibody determination can be found on <http://www.jdrfpod.org/sops.php>. All experimental procedures were approved by the Human Subjects Committee at the La Jolla Institute for Allergy and Immunology (protocol number DI3-054-1209).

### Tetramer production and quality control

HLA-A0201 tetramers were produced by the Tetramer Core Facility at Leiden University Medical Centre, Leiden, Netherlands (Pinkse et al., 2005). Tetramers were designed carrying the following T1D-associated, HLA-A0201-restricted islet antigens: insulin (Pinkse et al., 2005), IA-2 (tyrosine phosphatase-like protein aa 797–805; Takahashi et al., 2001), IGRP (islet-specific glucose-6-phosphatase catalytic subunit related protein aa 265–273; Unger et al., 2007), PPI (aa 15–23; Skowera et al., 2008), GAD65 (glutamic acid decarboxylase aa 114–123; Panina-Bordignon et al., 1995), and pIAPP (human islet amyloid polypeptide precursor protein [preproIAPP] aa 5–13; Panagiotopoulos et al., 2003). Negative control tetramer was loaded with YAYDGGKDYIA, derived from the HLA class I molecule HLA-A2.

### Immunohistochemistry and immunofluorescence

**Initial screening for insulin, MHC class I, and CD8.** For characterization of the sections before tetramer staining, staining for insulin, CD8, and HLA-ABC (all at room temperature) was performed using the antibodies listed in Table 3. Fixation was with acetone and sections were blocked with goat serum in a standard immunofluorescent staining protocol. An islet was determined as hyperexpressing MHC class I based on a threshold of three unequivocally positive cells, which was the maximum number found in all control islets examined.

### In situ tetramer staining

**Immunohistochemistry.** Human pancreas sections were initially probed for presence of islet-autoreactive CD8 T cells on basis of tetramer positivity by conventional enzymatic immunohistochemistry, as this technique has superior sensitivity over immunofluorescence. Identical procedures were performed in two independent laboratories on the samples available to the respective groups (i.e., patients 2, 4, 5, and 6 in La Jolla and patients A, B, C, D, E, 1, and 3 in Siena). Unfixed, frozen sections were dried and loaded overnight at 4°C with 1  $\mu$ g APC-labeled tetramer diluted in 500  $\mu$ l PBS. After gentle washing, sections were fixed in 0.4% paraformaldehyde solution for 15 min. After washing, endogenous peroxidase activity was blocked by incubation in 0.03% H<sub>2</sub>O<sub>2</sub> and avidin/biotin blocking was performed according to the manufacturer's instructions (Vector Laboratories). Biotinylated mouse anti-allophycocyanin (BioLegend) was added at a 1/500 dilution for 1 h at room temperature. Finally, Avidin D coupled to horseradish peroxidase (Vector Laboratories) was applied at 1/2,000 dilution and NovaRed enzymatic substrate (Vector Laboratories)

was applied for detection (30 min at room temperature). After washing, sections were mounted and analyzed with a microscope (Eclipse 80i; Nikon) using 20 and 40× air objectives.

**Immunofluorescence.** Protocol optimization was done on frozen pancreas sections from NOD mice previously screened with NRP-V7 (IGRP 206–214-reactive; Lieberman et al., 2003) tetramer obtained from R. Tang (University of British Columbia Child and Family Research Institute, Vancouver, British Columbia, Canada). The presence of IGRP-reactive CD8 T cells in the blood correlates with their appearance in the pancreas, and positive NOD animals thus serve as an ideal source of tetramer-reactive cells in the pancreas (Trudeau et al., 2003). Negative controls were spleen sections from insulin B:9–23 TCR transgenic mice (gift from G. Eisenbarth, Barbara Davis Center for Childhood Diabetes, University of Colorado, Denver, CO) and positive controls were spleen sections from IGRP 206–214 TCR transgenic NOD8.3 mice (gift from R. Tang). As the NRP-V7 tetramers (1/20 of 0.57 µg/µl stock; overnight at 4°C) were PE labeled, detection was achieved with polyclonal rabbit anti-PE (1/700, 3 h at 4°C; Serotec), followed by highly cross-adsorbed Alexa Fluor 647 goat anti-rabbit (1/500, 3 h at 4°C; Invitrogen) in conjunction with rat anti-CD8 (1/1,000, added overnight at 4°C together with tetramer; BD) and highly cross-adsorbed Alexa Fluor 488 donkey anti-rat (1:1,000, 3 h at 4°C; Invitrogen). All animal procedures were approved by the Animal Care Committee at the La Jolla Institute for Allergy and Immunology. We were able to detect low numbers of relatively low-avidity auto-reactive T cells in the islets by using a high-avidity peptide/MHC class I tetramer. This procedure was thus designed to anticipate accurate detection of rare antigen-specific CD8<sup>+</sup> T cell populations, possibly of low avidity, in human islets.

A modified version of the protocol used by McGavern et al. (2002) was designed to optimize signal-to-noise ratio for fluorescent detection. Because all human tetramers (used here at same dilution and under same conditions as in Immunohistochemistry) were labeled with APC, we aimed to amplify signal intensity by choosing secondary detection reagents labeled to fluorophores within the same spectral range. To this end, we used biotinylated mouse anti-allophycocyanin, which does not quench the fluorescence originating from the APC target it binds. As a third step, streptavidin coupled to Alexa Fluor 647 was applied, which has spectral emission characteristics that closely parallel APC. Analogous to the immunohistochemistry protocol, sections were fixed after overnight incubation with tetramer in PBS. Sections were then stained with anti-CD8 (1/50, 1 h RT; clone HIT8a; BD) and detection with highly cross-adsorbed goat anti-mouse Alexa Fluor 555 (1/1,000, 1 h RT; Invitrogen) was performed. These steps were performed first to avoid cross-reaction with subsequent anti-insulin and particularly anti-APC antibodies. After washing, guinea pig anti-insulin (1/140, 1 h RT; Dako) and biotinylated mouse anti-APC (1/500, 1 h RT; BioLegend) were added simultaneously, followed by a final detection step with highly cross-adsorbed goat anti-guinea pig Alexa Fluor 488 (1/1,000, 1 h RT; Invitrogen) and streptavidin coupled to Alexa 647 (1/1,000 RT; Invitrogen). Sections were washed and mounted with Prolong Gold antifade mounting medium with DAPI (Invitrogen).

Image acquisition was performed with a confocal microscope (MRC-1024; Bio-Rad Laboratories) equipped with an argon-krypton laser for excitation at 488, 568, and 647 nm and attached to an inverted microscope (TE300; Nikon) carrying a 40× 1.3 N.A. oil objective. Initially, the first section of a consecutive series was stained with control tetramer, loaded with YAYDG-KDYIA, derived from HLA0201 class I antigen. The section was imaged and laser power, pinhole size, detector gain, and offset were set at near-background levels, with a focus on CD8<sup>+</sup> infiltrated islets. All parameters were then kept constant during acquisition of tetramer-stained serial sections. After acquisition, images from consecutive sections of the same islet were grouped in stacks with ImageJ (National Institutes of Health) and pixel histogram optimization (brightness/contrast function in ImageJ) was performed simultaneously. Thus, any raise in signal intensity was introduced equally in both controls and test samples. The Brightness and Contrast function allows one to improve the signal-to-background ratio and contrast of an image after acquisition. The image's

histogram shows the number of pixels at each different intensity value found in that image and its lower threshold (black) and upper threshold (saturated) for display can be manually set. Thus, the minimum pixel value was increased to display the image's background noise as black and the maximum value was decreased to display the brightest objects as saturated (full manual: <http://imagej-docu.tudor.lu/>). No z-stacking was performed, and all images represent single focal planes. Images of separate serial sections were aligned using the Stackreg plugin for ImageJ (Thévenaz et al., 1998) and 3D reconstruction was performed using Imaris software (Bitplane). The 3D images presented thus are based on individual images from consecutively cut sections that were aligned in silico and spaced 6 µm apart (i.e., section thickness).

#### Online supplemental material

Table S1 shows insulin, CD8, and HLA Class I expression patterns within pancreatic islets from diabetic individuals and controls. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20111187/DC1>.

We thank Kees Franken for providing customized HLA tetramers.

This research was performed with the support of the nPOD, a collaborative T1D research project sponsored by the Juvenile Diabetes Research Foundation International (JDRF). Organ Procurement Organizations, partnering with nPOD to provide research resources, are listed at [www.jdrfnpod.org/our-partners.php](http://www.jdrfnpod.org/our-partners.php). This work was supported by a National Institutes of Allergy and Infectious Diseases U-prevention center grant to M.G. von Herrath and the Brehm Coalition. F. Dotto is supported by the European Union (Collaborative Project NAIMIT in the Framework Program 7), by the Italian Ministry of Research, by the Italian Ministry of Health, and by the Tuscany Region.

The authors declare that no competing financial interests related to this manuscript exist.

Submitted: 10 June 2011

Accepted: 6 December 2011

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