

# Simultaneous Detection of Circulating Autoreactive CD8<sup>+</sup> T-Cells Specific for Different Islet Cell–Associated Epitopes Using Combinatorial MHC Multimers

Jurjen H. Velthuis,<sup>1,2</sup> Wendy W. Unger,<sup>1</sup> Joana R.F. Abreu,<sup>1</sup> Gaby Duinkerken,<sup>1,2</sup> Kees Franken,<sup>1</sup> Mark Peakman,<sup>3</sup> Arnold H. Bakker,<sup>4</sup> Sine Reker-Hadrup,<sup>4</sup> Bart Keymeulen,<sup>2,5</sup> Jan Wouter Drijfhout,<sup>5</sup> Ton N. Schumacher,<sup>4</sup> and Bart O. Roep<sup>1,2</sup>

**OBJECTIVE**—Type 1 diabetes results from selective T-cell-mediated destruction of the insulin-producing  $\beta$ -cells in the pancreas. In this process, islet epitope-specific CD8<sup>+</sup> T-cells play a pivotal role. Thus, monitoring of multiple islet-specific CD8<sup>+</sup> T-cells may prove to be valuable for measuring disease activity, progression, and intervention. Yet, conventional detection techniques (ELISPOT and HLA tetramers) require many cells and are relatively insensitive.

**RESEARCH DESIGN AND METHODS**—Here, we used a combinatorial quantum dot major histocompatibility complex multimer technique to simultaneously monitor the presence of HLA-A2 restricted insulin B<sub>10–18</sub>, prepro-insulin (PPI)<sub>15–24</sub>, islet antigen (IA)-2<sub>797–805</sub>, GAD65<sub>114–123</sub>, islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP)<sub>265–273</sub>, and prepro islet amyloid polypeptide (ppIAPP)<sub>5–13</sub>-specific CD8<sup>+</sup> T-cells in recent-onset diabetic patients, their siblings, healthy control subjects, and islet cell transplantation recipients.

**RESULTS**—Using this kit, islet autoreactive CD8<sup>+</sup> T-cells recognizing insulin B<sub>10–18</sub>, IA-2<sub>797–805</sub>, and IGRP<sub>265–273</sub> were shown to be frequently detectable in recent-onset diabetic patients but rarely in healthy control subjects; PPI<sub>15–24</sub> proved to be the most sensitive epitope. Applying the “Diab-Q-kit” to samples of islet cell transplantation recipients allowed detection of changes of autoreactive T-cell frequencies against multiple islet cell–derived epitopes that were associated with disease activity and correlated with clinical outcome.

**CONCLUSIONS**—A kit was developed that allows simultaneous detection of CD8<sup>+</sup> T-cells reactive to multiple HLA-A2–restricted  $\beta$ -cell epitopes requiring limited amounts of blood, without a need for in vitro culture, that is applicable on stored blood samples. *Diabetes* 59:1721–1730, 2010

From the <sup>1</sup>Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, the Netherlands; the <sup>2</sup>JDRF Center for Beta Cell Therapy in Diabetes Brussels, Belgium; the <sup>3</sup>Department of Immunobiology, King’s College School of Medicine, Guy’s Hospital, London, U.K.; the <sup>4</sup>Division of Immunology, The Netherlands Cancer Institute, Amsterdam, the Netherlands; and the <sup>5</sup>Diabetes Research Center, Brussels Free University-VUB, Brussels, Belgium.

Corresponding author: Bart O. Roep, boroep@lumc.nl.

Received 7 October 2009 and accepted 15 March 2010. Published ahead of print at <http://diabetes.diabetesjournals.org> on 31 March 2010. DOI: 10.2337/db09-1486.

J.H.V. is currently affiliated with Swan Diagnostics, Department of Cell Biology, Erasmus MC, Rotterdam, the Netherlands.

W.W.U. is currently affiliated with Department of Cell Biology and Immunology, VU University Medical Center, Amsterdam, the Netherlands.

A.H.B. is currently affiliated with University of California, Berkeley, California. S.R.H. is currently affiliated with Center for Cancer Immunotherapy, Department of Hematology, Herlev University Hospital, Herlev, Denmark.

© 2010 by the American Diabetes Association. Readers may use this article as long as the work is properly cited, the use is educational and not for profit, and the work is not altered. See <http://creativecommons.org/licenses/by-nc-nd/3.0/> for details.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**T**ype 1 diabetes results from a selective T-cell-mediated destruction of the insulin-producing  $\beta$ -cells in the pancreas. It is becoming increasingly clear that islet epitope-specific CD8<sup>+</sup> T-cells play a pivotal role in the destruction process and constitute a significant portion of insulinitis (1,2). In accordance, nonobese diabetic mice lacking the expression of major histocompatibility complex (MHC) class I are resistant to autoimmune diabetes (3,4), whereas HLA-A2 transgenic nonobese diabetic mice develop accelerated disease (5). Additionally, transfer of CD8<sup>+</sup> T-cell clones resulted in transfer of type 1 diabetes (6,7). Thus, detection and monitoring of specific CD8<sup>+</sup> T-cells may provide a valuable tool to assess the disease activity.

Islet cell transplantation has considerable potential as a cure for type 1 diabetes (8). Several groups have reported short-term success, using different islet isolation and immunosuppressive regimens (9–12), but long-term insulin independence is rare (13). The rationale behind transplantation of islet cells is replenishment of destroyed cells. Yet, as the insulin-producing cells were destroyed by an autoimmune response, islet cell transplantation could also result in reactivation of the autoimmune response. Recently, we have shown that proliferation of CD4<sup>+</sup> T-cells specific for GAD and IA-2 in patients who underwent islet cell transplantation is associated with clinical outcome (14). Yet, ultimately, the destruction of  $\beta$ -cells is likely to be caused by CD8 T-cells.

The epitopes recognized by the diabetes-specific human autoreactive CD8<sup>+</sup> T-cells are primarily derived from  $\beta$ -cell antigens, most importantly (pre-)(pro-)insulin. Previously, we showed that the presence of CD8<sup>+</sup> T-cells reactive to the naturally processed insulin-peptide B<sub>10–18</sub> in HLA-A2 correlated with islet cell destruction (15). Recently, another important epitope that was uncovered as the signal peptide of pro-insulin was shown to contain a glucose-regulated CD8<sup>+</sup> T-cell epitope (prepro-insulin [PPI]<sub>15–24</sub>) (16), but many other epitopes derived from insulin and a range of other  $\beta$ -cell–derived antigens, such as GAD65 (17), islet antigen (IA)-2 (18), islet-specific glucose-6-phosphatase catalytic subunit related protein (IGRP) (19,20), and prepro islet amyloid polypeptide (ppIAPP) (21), have been reported (rev. in 22). Ideally, monitoring for the presence of CD8<sup>+</sup> T-cells reactive to all of the above-mentioned epitopes simultaneously would be desired, posing considerable constraints on blood volumes accessible for monitoring of islet autoimmunity with conventional immune assays.

Currently, monitoring of CD8<sup>+</sup> T-cells reactive to  $\beta$ -cell-derived antigens requires staining of a large number of, usually fresh, cells with HLA tetramers loaded with a single peptide, or in vitro culture for functional immune assays (proliferation, cytokine production [ELISPOT]). Monitoring multiple epitope-specific CD8<sup>+</sup> T-cell populations by conventional tetramer technology is generally impossible because of the scarcity of material. Furthermore, detection of islet autoreactive T-cells is hampered by their low precursor frequencies in circulation (23,24), low T-cell receptor (TCR) avidity (15), potentially low binding affinity of peptide epitopes to HLA (25), a wide range of candidate islet epitopes (22), and the existence of regulatory T-cells (26,27).

Therefore, we used the recently described combinatorial quantum dot (Qdot) technique (28) to simultaneously detect CD8<sup>+</sup> T-cells specific for six different  $\beta$ -cell-derived antigens, a naturally occurring HLA-A2 derived peptide, and a mix of viral epitopes in HLA-A2 multimers. Using peripheral blood cells from recent-onset type 1 diabetic patients, their siblings, and control subjects, we validated this technique and established the specificity of these stainings. Subsequently, we monitored the presence of reactive CD8<sup>+</sup> T-cells before and at several time points after clinical islet cell transplantation. Altogether, we developed a high-throughput and relatively sensitive and specific Diab-Q-kit, allowing simultaneous detection of autoreactive CD8<sup>+</sup> T-cells to multiple islet epitopes, which is applicable to small volumes of stored blood samples to allow screening in multicenter immune intervention trials.

## RESEARCH DESIGN AND METHODS

**Recent-onset diabetic patients.** Samples from recent-onset type 1 diabetic patients were retrieved from the Kolibri type 1 diabetes cohort, which includes material from 350 patients with juvenile-onset type 1 diabetes (median age 8.7 years [range 1–17 years]). The cohort was collected consecutively after diagnosis by pediatricians in the southwestern part of the Netherlands between 1995 and 1999. The diagnosis was made according to International Society of Pediatric and Adolescent Diabetes and World Health Organization criteria. All patients were HLA-A2 positive. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Isopaque density gradient centrifugation. PBMCs were frozen in a solution of 20% human pooled serum and 10% DMSO ( $5\text{--}10 \times 10^6$  cells per vial) and kept in liquid nitrogen until use. Blood samples of islet transplant recipients were stored in liquid nitrogen for 12–36 months.

**Islet cell transplanted patients.** Seven patients were transplanted with islet cell grafts after signing informed consent and under appropriate ethical approval as reported previously (29). None of the patients presented alloantibodies against HLA alloantigen that was expressed on the donor cells before transplantation. Graft recipients were long-term type 1 diabetic patients without any earlier transplantation, with plasma C-peptide  $<0.09$  ng/ml, large variation in blood glucose levels (coefficient of variation  $\geq 25\%$ ), A1C concentration  $>7\%$ , and one or more chronic diabetes lesions. Exclusion criteria were body weight  $>90$  kg, active smoking, pregnancy, disturbed liver function tests, history of hepatic disease, presence of HLA antibodies, or negative Epstein-Barr virus serostatus.

Donor organs were procured from multiple heart-beating donors through the Eurotransplant Foundation (Leiden, the Netherlands) and processed at the Beta Cell Bank in Brussels, Belgium, to  $\beta$ -cell-enriched fractions that were cultured for 2–20 days (median 6 days). Immunosuppressive induction therapy consisted of antithymocyte globulin (ATG) (Fresenius HemoCare, Redmond, WA) with a single infusion of 9 mg/kg and subsequently with 3 mg/kg for 6 days except when the T-cell count was  $<50/\text{mm}^3$ . Maintenance immunosuppression consisted of tacrolimus (Prograf; Fujisawa/Pharma Logistics; dose according to trough level: 8–10 ng/ml in the first 3 months post-transplantation, 6–8 ng/ml thereafter) and mycophenolate mofetil (2,000 mg/day; Roche, Basel, Switzerland). The HLA typing of the patients is depicted in the supplementary table (available in an online appendix at <http://diabetes.diabetesjournals.org/cgi/content/full/db09-1486/DC1>). Islet cell recipients were age-matched with the healthy control subjects.

TABLE 1  
Combinations of Qdot-labeled HLA-A2 multimers

| Origin  | Position/protein | Sequence    | Signal         |
|---------|------------------|-------------|----------------|
| CMV     | pp65             | NLVPMTVATV  | Qdot 585 + 655 |
| EBV     | LMP2             | CLGGLTMTV   | Qdot 585 + 655 |
| Measles | H250             | SMYRVFEVGV  | Qdot 585 + 655 |
| HLA-A2  | 140–149          | YAYDGKDYIA  | Qdot 585 + 605 |
| Insulin | B 10–18          | HLVEALYLV   | Qdot 605 + 655 |
| PPI     | 15–24            | ALWGPDPAAA  | Qdot 705 + 655 |
| GAD65   | 114–123          | VMNiLLQYVYV | Qdot 800 + 655 |
| IA-2    | 797–805          | MVWESGCTV   | Qdot 705 + 605 |
| IGRP    | 265–273          | VLFGGLGFAI  | Qdot 800 + 605 |
| ppIAPP  | 5–13             | KLQVFLIVL   | Qdot 705 + 800 |

CVM, cytomegalovirus; EBV, Epstein-Barr virus.

**Qdot-labeled HLA-A2-peptide multimers.** Multimeric HLA-A2-peptide complexes were prepared essentially as previously described (30). Briefly, recombinant HLA-A2 and human  $\beta_2$ -microglobulin were solubilized in urea and injected together with each synthetic peptide into a refolding buffer consisting of 100 mmol/l Tris (pH 8.0), 400 mmol/l arginine, 2 mmol/l EDTA, 5 mmol/l reduced glutathione, and 0.5 mmol/l oxidized glutathione. Refolded complexes were biotinylated by incubation for 2 h at 30°C with BirA enzyme (Avidity, Denver, CO). The biotinylated complexes were purified by gel filtration on a Superdex 75 column (Amersham Pharmacia Biotech, Piscataway, NJ). Multimeric HLA-peptide complexes were produced by addition of streptavidin-conjugated Qdots (28) (Invitrogen, Breda, the Netherlands) to achieve a 1:20 streptavidin-Qdot/biotinylated HLA class I ratio. Qdots used are Qdot-585, -605, -655, -705, and -800. Samples from HLA-A2-positive subjects were stained with a mixture containing six diabetes-associated epitopes, a HLA-A2 epitope expressed in HLA-A2, and a mix of viral antigens (Table 1).

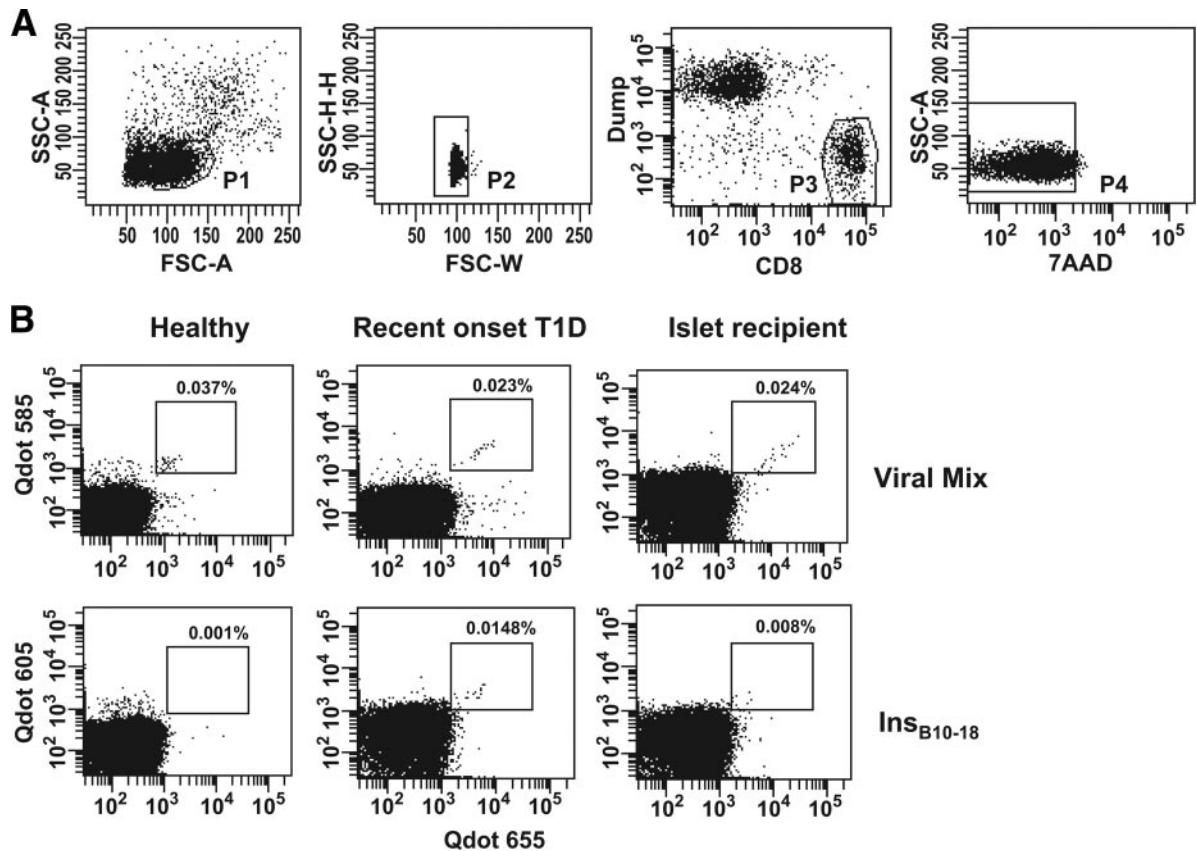
**Cell staining with Qdot-labeled multimeric complexes.** PBMCs ( $2 \times 10^6$ ) were stained simultaneously with all Qdot-labeled multimers (0.1  $\mu\text{g}$  of each specific multimer) in 60  $\mu\text{l}$  of PBS supplemented with 2% BSA and incubated for 15 min at 37°C (Table 1). Subsequently, 10  $\mu\text{l}$  antigen-presenting cell (APC)-labeled anti-CD8 (stock 1:10) and 10  $\mu\text{l}$  fluorescein isothiocyanate-labeled anti-CD4, -CD14, -CD16, -CD19, and -CD40 antibodies (Becton Dickinson, Franklin Lakes, NJ) were added for 30 min at 4°C. After being washed twice, cells were resuspended in PBS/2% BSA containing 7-aminocincomycin D (7-AAD; eBioscience, San Diego, CA) to exclude dead cells, and samples were analyzed using the LSR II (Becton Dickinson).

**Statistical analysis.** Statistical analysis on recent-onset diabetic patients versus their siblings was performed using a Wilcoxon matched-pairs test. Differences between recent-onset diabetic patients and control subjects were tested using the unpaired *t* test with Welch correction (for HLA-A2 peptide and PPI<sub>15–24</sub>; passed normality test) or the Mann-Whitney test (all other epitopes). Changes in epitope reactivity of islet cell transplant recipients over time were tested using the Friedman test followed by a Dunn multiple comparisons test. All statistical analyses were performed using GraphPad Prism software.

## RESULTS

**Simultaneous monitoring of multiple epitopes.** Recently, the use of multidimensional encoded MHC multimers was reported as a powerful tool to allow the parallel detection of multiple antigen-specific T-cell populations within a single sample (28). This technology opens the possibility of designing kits of defined peptide-MHC multimers that may be used to report on disease state or vaccine response. To test this concept, a Qdot-based combinatorial approach was developed to simultaneously monitor multiple islet epitopes associated with the development of type 1 diabetes. HLA-A2 molecules were loaded with identified diabetes peptides, and MHC multimers were created by labeling with Qdots such that T-cells specific for each of these epitopes are defined by binding of MHC multimers with a unique combination of two Qdots (Table 1).

During flow cytometric analysis, single-cell lymphocytes were gated that stained positive for CD8 (on average



**FIG. 1.** Flow cytometric analysis of epitope-specific CD8<sup>+</sup> T-cells using the combinatorial Qdot approach. **A:** Gating strategy: viable CD8<sup>+</sup> single T-cells were analyzed by gating lymphocytes on the basis of FSC-A and SSC-A. Subsequently, single cells were gated (FSC-W and FSC-H) and CD8-APC-positive but dump-channel fluorescein isothiocyanate (CD4 + CD14 + CD16 + CD20 + CD40)-negative cells were gated, of which the 7-AAD-positive cells were gated out. **B:** Qdot staining: within the viable CD8<sup>+</sup> single T-cells, the cells recognizing the epitopes in the viral mix (Qdot 585 + 655) and insulin B<sub>10-18</sub> (Qdot 605 + 655) are shown as a typical example for a healthy control, a recent-onset type 1 diabetic patient (T1D), and a pretransplantation islet cell recipient.

60,000 CD8 T-cells were gated per blood sample) but negative for the “dump”-channel (combination of CD4, CD14, CD16, CD19, and CD40) and negative for the exclusion (viability) dye 7-AAD (Fig. 1A). Staining of PBMCs of healthy control subjects (Fig. 1B, typical example shown) with a mixture of three virus-derived epitopes resulted in a clearly distinguishable population characterized by a positive signal for both fluorescent signals used to encode the peptide-MHC multimers. A clear population of CD8<sup>+</sup> T-cells reactive to insulin B<sub>10-18</sub> was seen in the sample of the recent-onset diabetic patient. These insulin B<sub>10-18</sub>-reactive CD8 T-cells were also found in an islet cell transplantation recipient, in which virus-specific CD8<sup>+</sup> T-cells also were seen. No CD8<sup>+</sup> T-cells reactive to insulin B<sub>10-18</sub> were found in the healthy control subjects (Fig. 1), whereas few virus-specific CD8<sup>+</sup> T-cells were detectable in samples of recent-onset diabetic patients (Fig. 1). In terms of reproducibility, the coefficient of variation between experiments was 9.5% across Qdot multimers. For the separate epitopes, the variation varied (HLA-A2 peptide, 10.8%; virus mix, 34.9%; InsB, 15.9%; IA-2, 0.0%; IGRP, 0.0%; PPI, 6.3%; GAD65, 4.5%; and pplAPP, 6.9%) (supplementary Figs. 1–3, available in the online appendix).

**Selectivity of the examined epitopes.** To determine whether the simultaneously measured islet epitopes were sensitively and specifically detected in the circulation of recent-onset type 1 diabetic patients, we determined the frequency of CD8<sup>+</sup> T-cells specific for all currently known

epitopes in recent-onset diabetic patients, their siblings (when available), and healthy control subjects.

Unfortunately, of the 20 recent-onset diabetic patients studied, material from just 5 HLA-A2-expressing siblings was available, corresponding to 3 recent-onset diabetic patients, allowing a direct comparison of the presence of CD8<sup>+</sup> T-cells specific for the islet epitopes (Fig. 2, left panels). Generally, the frequency of  $\beta$ -cell antigen-reactive CD8<sup>+</sup> T-cells was higher in recent-onset diabetic patients than in their siblings, but because of low numbers, no statistically significant differences were observed. Also, we analyzed the frequency of CD8<sup>+</sup> T-cells in the circulation of all examined recent-onset diabetic patients ( $n = 20$ ) and matched control blood donors ( $n = 15$ ). Clearly, the frequencies of CD8<sup>+</sup> T-cells recognizing the islet cell-derived epitopes were all significantly higher in recent-onset diabetic patients than in control subjects. Conversely, a higher frequency of virus-reactive CD8<sup>+</sup> T-cells was seen in the control subjects. These data point to higher frequencies of islet-reactive CD8<sup>+</sup> T-cells within the circulation of recent-onset diabetic patients.

Next, we determined the sensitivity and specificity of T-cell responses to each epitope, defining a frequency of one cell reactive in 10,000 CD8<sup>+</sup> T-cells (i.e., 0.010%) as a cutoff, with the exception of the IGRP<sub>265-273</sub>, where a frequency of 1 in 20,000 (i.e., 0.005%) was used as a cutoff. Three epitopes were found to be 100% specific because no relevant frequencies were seen in the control subjects:



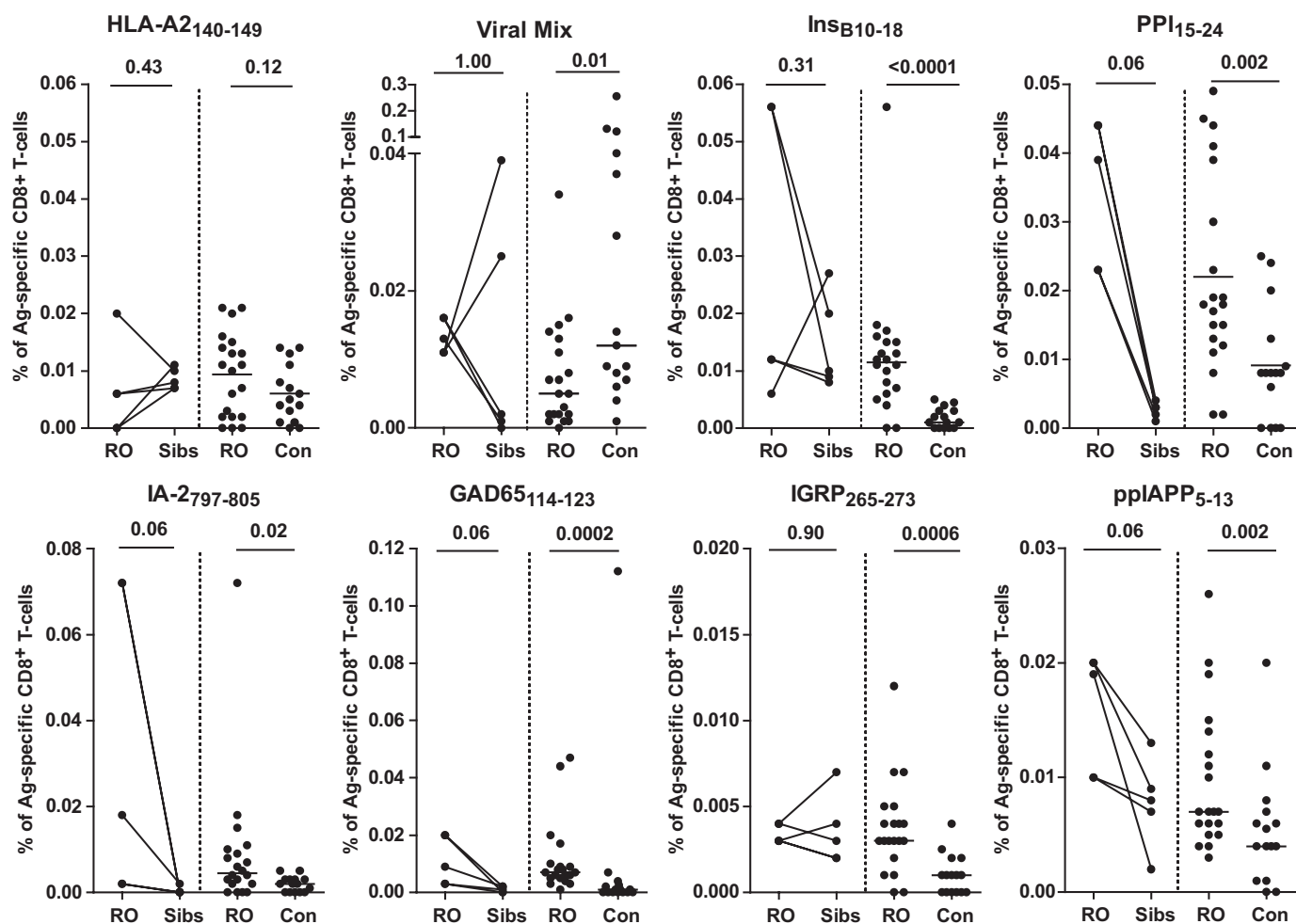


FIG. 2. Frequencies of epitope-specific CD8<sup>+</sup> T-cells in recent-onset diabetic patients, their siblings, and healthy control subjects. The frequencies of CD8<sup>+</sup> T-cells recognizing the epitopes HLA-A2<sub>140-149</sub>, the viral mix, insulin B<sub>10-18</sub>, PPI<sub>15-24</sub>, GAD65<sub>114-123</sub>, IA-2<sub>797-805</sub>, IGRP<sub>265-273</sub>, and pplAPP<sub>5-13</sub> in HLA-A2 as determined by flow cytometry are depicted. First, the frequency detected in recent-onset diabetic patient material (RO, *n* = 3) and that of their siblings (Sibs, *n* = 5) was compared (*left panels*). Statistical analysis was performed using the Wilcoxon matched-pairs test. The frequencies detected in all RO (*n* = 20) and control subjects (Con, *n* = 15) were compared (*right panels*). Statistical analysis was performed using the unpaired *t* test with Welch correction (for HLA-A2 peptide and PPI<sub>15-24</sub>) or the Mann-Whitney test (all other epitopes).

insulin B<sub>10-18</sub> showed a sensitivity of 65% and was the most specific and considerably sensitive epitope detected; IA-2<sub>797-805</sub> and IGRP<sub>265-273</sub> provided a specificity of 100% but a sensitivity of 25% (Table 2). The epitope with the highest sensitivity was PPI<sub>15-24</sub>, as epitope-specific CD8<sup>+</sup> T-cell reactivity was detectable in 17 of 20 recent-onset diabetic patients (85%). Yet, 4 of 15 control subjects also

exhibit CD8<sup>+</sup> T-cells against this epitope, affecting the specificity (73%). Qdot stainings of HLA-A2-negative patients or control subjects with HLA-A2 multimers were always below the detection limit (*n* = 27), regardless of the peptide epitopes tested, supporting the specificity of these reagents. Overall, our data indicate that we can discretely monitor the specific presence of multiple

TABLE 2  
Selectivity of the epitopes tested

| Epitope                          | Cutoff       | Control subjects | Recent-onset type 1 diabetic patients | Sensitivity | Specificity |
|----------------------------------|--------------|------------------|---------------------------------------|-------------|-------------|
| <i>n</i>                         |              | 15               | 20                                    |             |             |
| Insulin B <sub>10-18</sub>       | >1 in 10,000 | 0                | 13                                    | 65          | 100         |
| Pre-pro-insulin <sub>15-24</sub> | >1 in 10,000 | 4                | 17                                    | 85          | 73          |
| IA-2 <sub>797-805</sub>          | >1 in 10,000 | 0                | 5                                     | 25          | 100         |
| GAD65 <sub>114-123</sub>         | >1 in 10,000 | 1                | 5                                     | 25          | 93          |
| IGRP <sub>265-273</sub>          | >1 in 20,000 | 0                | 5                                     | 25          | 100         |
| pplAPP <sub>5-13</sub>           | >1 in 10,000 | 2                | 8                                     | 40          | 87          |

Data are *n* or percent for sensitivity and specificity.

epitope reactive CD8<sup>+</sup> T-cells simultaneously using the newly developed Diab-Q-kit.

**Epitope-specific CD8<sup>+</sup> T-cells in islet cell transplantation.** Next, the Diab-Q-kit was used to monitor the presence of HLA-A2 epitope-specific CD8<sup>+</sup> T-cells in seven islet cell transplant recipients, at four different time points: before transplantation, 6 weeks thereafter (reconstitution of the T-cell compartment after ATG-induction treatment), 26 weeks after transplantation, and 52 weeks after transplantation. Examination of the frequency of CD8<sup>+</sup> T-cells recognizing endogenously processed HLA-A2 peptide presented in the HLA-A2 molecule showed reactivity in two recipients at two different time points (Fig. 3). Viral reactivity was seen in two of seven HLA-A2-positive islet cell transplantation recipients before transplantation (Fig. 3, viral mix), but clearly the induction therapy with ATG strongly reduced their frequency in circulation. One year after transplantation, virus-specific T-cells had reappeared, albeit to a lower level than before transplantation.

Despite disease durations up to several decades, islet autoreactive CD8<sup>+</sup> T-cells were still detectable in the majority of type 1 diabetic patients at the time of islet transplantation (0 weeks; Fig. 4). No particular islet epitope or pattern of reactivity dominated. After reconstitution of the T-cell compartment under maintenance immunosuppression after ATG induction therapy (4 weeks; Fig. 4), the cumulative numbers of autoreactive CD8<sup>+</sup> T-cells waned in a minority of recipients. In four of seven patients, islet autoreactivity increased after islet transplantation, one patient displayed stable frequencies of circulating islet reactive T-cells, and, in two case subjects, a stable decline was observed.

The dynamics of T-cells specific for the (pre)-(pro-) insulin epitopes (insulin B<sub>10-18</sub> and PPI<sub>15-24</sub>) were most pronounced (Fig. 3): considerable frequencies of these cells were detected before islet cell infusion and (re-)emerged at later time points thereafter. In contrast, T-cell frequencies against GAD65<sub>114-123</sub> and IGRP<sub>265-273</sub> were infrequently seen, with only a single increase late after islet implantation in patients 6 and 7, respectively (Fig. 3). These two case subjects also displayed the most epitope spreading at that time point (Fig. 4). Of note, patient βct #7 cytomegalovirus-converted after transplantation.

**Clinical outcome.** Subsequently, the patterns of autoreactive CD8<sup>+</sup> T-cell frequencies were correlated with clinical outcome. All immune parameters were defined and interpreted without prior knowledge of clinical outcome. Because reactivity to single epitopes was sometimes observed in healthy control subjects (Fig. 2), only the presence of two or more epitope-specific autoreactive CD8<sup>+</sup> T-cells at any time after transplantation was interpreted as being detrimental; to predict the clinical outcome based on the full autoimmune spectrum, the presence of CD8<sup>+</sup> T-cells reactive to the recently uncovered PPI epitopes PPI<sub>76-84</sub> and PPI<sub>79-88</sub> in HLA-A3 as well as PPI<sub>4-13</sub> in HLA-B7 were also considered (W.W.U., J.H.V. et al., unpublished observations) as indicated in Table 3. Consequently, the data obtained with the novel Diab-Q-kit predicted that six of seven recipients studied would not reach insulin independence.

Previously, we reported that proliferation of CD4<sup>+</sup> T-cells to whole IA-2 and GAD65 before transplantation predicted transplantation outcome (14). For comparison, the prediction of clinical outcome based on this proliferation assay was also taken into consideration (Table 3). Prediction based on proliferation of islet-specific CD4

T-cells pointed toward four patients not reaching insulin independence. For three patients, there was agreement in the prediction by both immunological end points.

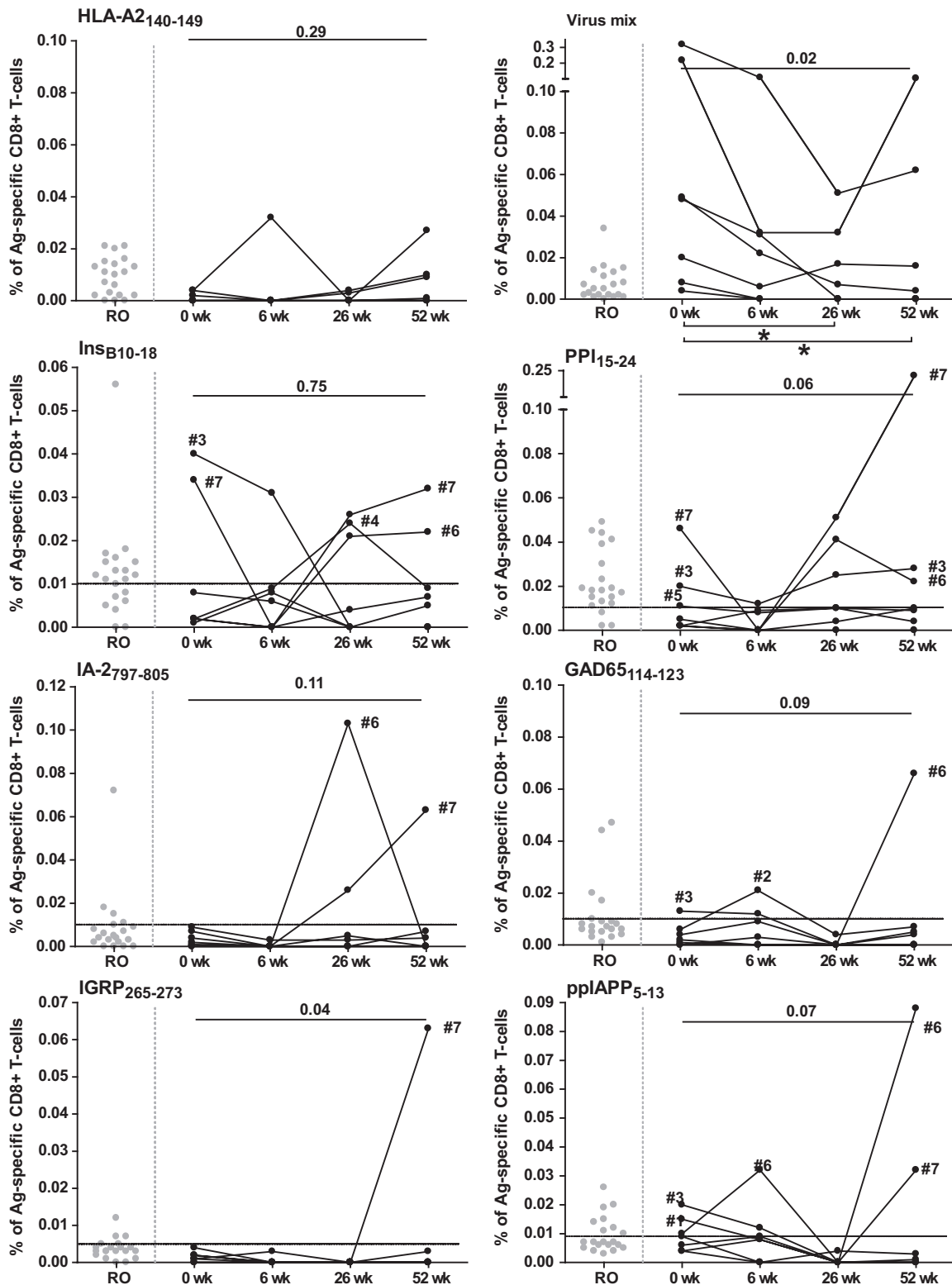
The Diab-Q-kit predicted that six recipients would remain insulin requiring, of which four actually required exogenous insulin injection. Thus, this method showed a prediction accuracy of 66%. The prediction of clinical outcome based on proliferation (14) predicted insulin requirement in four case subjects, of which three were correct (accuracy of 75%). However, when both methods agreed in their prediction, all transplant recipients remained insulin requiring after transplantation, and thus the combined method provided an accuracy of 100%, underlining the importance of monitoring CD8<sup>+</sup> T-cell frequencies.

## DISCUSSION

Our study is the first to implement simultaneous detection of multiple islet cell-specific CD8<sup>+</sup> T-cell responses, by development of the Diab-Q-kit. To this purpose, multidimensional encoded MHC multimers were used. Recently, their use was extensively validated and shown to be a powerful tool to parallel detect antigen-specific T-cells (28). Here, we determined the sensitivity and specificity of previously reported HLA-A2 restricted epitopes (15–18,20) in recent-onset type 1 diabetic patients, their siblings, control blood donors, and islet cell transplant recipients. Insulin B<sub>10-18</sub> was found to be 100% specific, as no CD8<sup>+</sup> T-cell frequencies of >1 in 10,000 cells were seen in PBMCs from healthy control subjects. Although this also holds true for IGRP<sub>265-273</sub> and IA-2<sub>797-805</sub>, CD8<sup>+</sup> T-cells recognizing insulin B<sub>10-18</sub> showed the highest sensitivity of these epitopes. The relevance of insulin B<sub>10-18</sub> in type 1 diabetes is underlined by previously published observations that PBMCs from a type 1 diabetic patient produced interferon-γ in response to this peptide (31), that expression of insulin B<sub>10-18</sub> renders target cells sensitive to killing by CTL lines (32), and, most importantly, that the presence of insulin B<sub>10-18</sub>-specific CD8<sup>+</sup> T-cells correlates with destruction of β-cells (15). As the transplantation of isolated islet cells can result in reactivation of CD8<sup>+</sup> T-cell-mediated autoreactivity toward islet cell-specific epitopes, peripheral blood from islet cell transplantation recipients can be used to monitor the factors important in β-cell destruction. Also in this cohort, the presence of insulin B<sub>10-18</sub>-reactive CD8<sup>+</sup> T-cells after transplantation correlated with a poor clinical outcome, with the exception of the cytomegalovirus-converted recipient.

Recently, we reported PPI<sub>15-24</sub> as a naturally produced and presented HLA-A2 epitope (16). Cytotoxic CD8<sup>+</sup> T-cells against this peptide could be cloned that killed β-cells in vitro in a glucose concentration-dependent fashion, linking β-cell immunogenicity with its functional activity (16). This study underscores the relevance of CD8 islet autoreactivity in the pathogenesis of type 1 diabetes, and it indicates that β-cells are actively involved in their own demise. Interestingly, this PPI epitope provided the highest sensitivity (85%) combined with a specificity of 73%. All but one HLA-A2-positive islet cell recipient exhibited increased frequencies against PPI<sub>15-24</sub> at a time point after transplantation. Of these patients, half did not reach insulin independence after transplantation.

The HLA-A2-restricted islet epitopes IA-2<sub>797-805</sub>, GAD65<sub>114-123</sub>, IGRP<sub>265-273</sub>, and ppiAPP<sub>5-13</sub> also exhibited a highly specific staining, with only incidental reactivity in



**FIG. 3.** Frequencies of epitope-specific CD8<sup>+</sup> T-cells in islet cell recipients over time. The frequencies of CD8<sup>+</sup> T-cells recognizing the epitopes HLA-A2<sub>140-149</sub>; the viral mix, insulinB<sub>10-18</sub>, PPI<sub>15-24</sub>, GAD65<sub>114-123</sub>, IA-2<sub>797-805</sub>, IGRP<sub>265-273</sub>, and ppiAPP<sub>5-13</sub> in HLA-A2 as determined by flow cytometry are depicted. The frequencies of CD8<sup>+</sup> T-cells were measured at four different time points: before transplantation, 6 weeks thereafter (reconstitution of the T-cell compartment after ATG induction treatment), 26 weeks after transplantation, and 52 weeks after transplantation. Changes in epitope reactivity of islet cell transplant recipients over time were tested using the Friedman test followed by a Dunn multiple comparisons test with \**P* < 0.05 values considered statistically significant. Data points of recent-onset diabetic patients are depicted to allow easy comparison of “recent-onset reactivity” and “islet cell transplant reactivity.”

healthy control subjects. In contrast to previously reported results (18), we did not observe any CD8<sup>+</sup> T-cells reactive to IA-2<sub>797-805</sub> in healthy control subjects. How-

ever, we performed a direct assessment of the CD8<sup>+</sup> T-cell frequency in PBMCs, rather than functional assays, whereas Takahashi et al. (18) cultured CD8<sup>+</sup> cells for 14

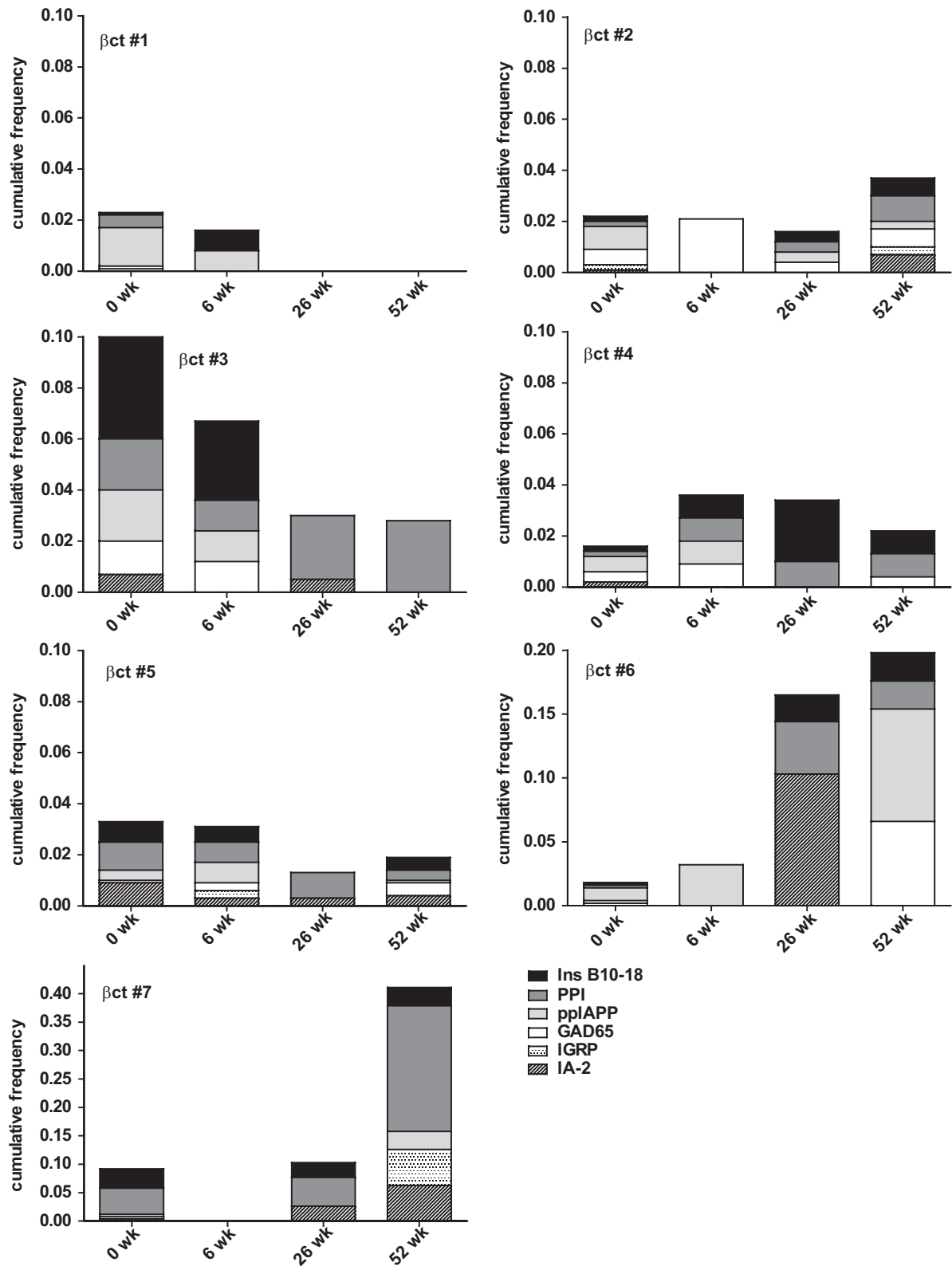


FIG. 4. Cumulative frequencies of epitope-specific CD8<sup>+</sup> T-cells in islet cell recipients at different time points. The cumulative frequencies of CD8<sup>+</sup> T-cells recognizing the epitopes insulin B<sub>10-18</sub>, PPI<sub>15-24</sub>, GAD65<sub>114-123</sub>, IA-2<sub>797-805</sub>, IGRP<sub>265-273</sub>, and pplAPP<sub>5-13</sub> in HLA-A2 as determined by flow cytometry are depicted for each islet cell recipient individually. Note the different axis for patients beta ct #6 and beta ct #7.

days with autologous APC expressing the IA-2 peptide and a cocktail of cytokines. Their observations may therefore be influenced by in vitro phenomena.

Reactivity to IA-2<sub>797-805</sub>, GAD65<sub>114-123</sub>, and IGRP<sub>265-273</sub> were almost exclusively seen in combination with reactivi-

ties to insulin B<sub>10-18</sub> and/or PPI<sub>15-24</sub>, suggesting that PPI epitopes may comprise the primary epitopes after reactivation of autoimmunity upon islet cell transplantation, whereas reactivity to the other may result from epitope spreading.

TABLE 3  
Clinical outcome

| Patient No. | CD8 T-cell reactivity against islet epitopes after transplantation |                                  |                        |   | Prediction |               | Clinical outcome# |        |                   |
|-------------|--|----------------------------------|------------------------|---|------------|---------------|-------------------|--------|-------------------|
|             | 0 weeks  | 6 weeks                          | 26 weeks               | 52 weeks                                | CD8        | CD4           | Insulin needs     | CV (%) | Time to Ins Indep |
|             |  |                                  |                        |   | Diab-Q-kit | Proliferation |                   |        |                   |
| βct #1      |  | Tx1<br>A2 A3 B7                  | Tx2<br>A2 A3           |   |            |               |                   |        |                   |
| HLA-A2      | ppIAPP   |                                  |                        |   | Ins Req    |               | Ins Req           | 26.5   | NA                |
| HLA-A3      | PPI <sub>76-84</sub> *   | PPI <sub>76-84</sub> *           | PPI <sub>76-84</sub> * |   |            |               |                   |        |                   |
| HLA-B7      | PPI <sub>79-88</sub> *   |                                  | PPI <sub>79-88</sub> * | PPI <sub>79-88</sub> *                  |            |               |                   |        |                   |
|             | PPI <sub>4-13</sub> *  |                                  |                        | PPI <sub>4-13</sub> *                   |            |               |                   |        |                   |
| βct #2      |  |                                  | Tx1<br>B7              |   |            |               |                   |        |                   |
| HLA-A2      |  |                                  |                        | PPI                                     | Ins Req    | Ins Req       | Ins Req           | 20.0   | NA                |
| HLA-B7      |  | GAD65<br>PPI <sub>4-13</sub> *   | PPI <sub>4-13</sub> *  |   |            |               |                   |        |                   |
| βct #3      |  | Tx1<br>A2                        | Tx2<br>A2              |   |            |               |                   |        |                   |
| HLA-A2      | B10-18<br>PPI<br>GAD65<br>ppIAPP                                   | B10-18<br>PPI<br>GAD65<br>ppIAPP | PPI                    | PPI                                     | Ins Req    | Ins Req       | Ins Req           | 50.2   | NA                |
| βct #4      |  | Tx1<br>A2                        | Tx2<br>A2              |   |            |               |                   |        |                   |
| HLA-A2      |  |                                  | B10-18<br>PPI          |   | Ins Req    |               | Ins Indep         | 32.1   | 19.6 weeks        |
| βct #5      |  | Tx1<br>A2 A3                     | Tx2<br>A2              |   |            |               |                   |        |                   |
| HLA-A2      | PPI  |                                  | PPI                    |   |            | Ins Req       | Ins Indep         | 22.2   | 15.0 weeks        |
| HLA-A3      |  |                                  |                        |   |            |               |                   |        |                   |
| HLA-B7      |  | PPI <sub>4-13</sub> *            |                        |   |            |               |                   |        |                   |
| βct #6      |  | Tx1                              | Tx2<br>A2              |   |            |               |                   |        |                   |
| HLA-A2      |  |                                  | B10-18<br>PPI<br>IA-2  | B10-18<br>PPI                           | Ins Req    | Ins Req       | Ins Req           | 24.1   | NA                |
|             | ppIAPP   | ppIAPP                           |                        | GAD65<br>ppIAPP                         |            |               |                   |        |                   |
| βct #7†     |  | Tx1<br>A2                        | Tx2<br>A2              |   |            |               |                   |        |                   |
| HLA-A2      | B10-18<br>PPI  |                                  | B10-18<br>PPI<br>IA-2  | B10-18<br>PPI<br>IA-2<br>IGRP<br>ppIAPP | Ins Req    |               | Ins Indep         | 25.4   | 26.9 weeks        |

Overview of all detected CD8-reactive epitopes at four different time points. For each recipient, the first and second (if applicable) transplantation is listed including the presence of the relevant HLA restriction in the graft received. In the resulting prediction of clinical outcome, the presence of CD8 T-cells against novel HLA-A3- and HLA-B7-restricted epitopes also was considered. Clinical outcome is defined as insulin independence (Ins Indep) or still requiring insulin (Ins Req), the coefficient of variation in fasting glycemia in the first 6 months (CV), and the time to reach insulin independence. NA, not applicable. #All experiments were performed blinded from clinical outcome. \*Non-HLA-A2-restricted T-cell responses (W.W.U., J.H.V. et al., unpublished observations). †This patient cytomegalovirus-converted upon transplantation.

Intriguingly, in two patients without islet epitope reactive CD8<sup>+</sup> T-cells after the transplantation induction therapy (i.e., at 6 weeks), CD8<sup>+</sup> T-cells specific for insulin

B<sub>10-18</sub> and PPI<sub>15-24</sub> were among the first CD8<sup>+</sup> T-cells to occur. Their occurrence after ATG treatment may have resulted from homeostatic proliferation (33–35), indicat-



ing that these CD8<sup>+</sup> T-cells, although undetectable in the peripheral blood after ATG induction, remain as a memory population in lymphatic organs. Because in one of these patients these cells were undetectable because transplantation, this may indicate that these cells persist many years after destruction of the islet cells. From these data, we speculate that homeostatic proliferation of autoreactive T-cells, including CD8-expressing cells, may be detrimental to islet cell transplantation, similar as homeostatic proliferation of alloreactive cells can be to solid organ transplantation (36,37). We cannot conclude on any order of reactivity among islet autoantigens, because there may be technical explanations for differences in precursor frequencies of the corresponding islet autoreactive CD8 T-cells, such as avidity of the Qdots for the TCR or affinity of the peptide epitope to HLA-A2, that contribute to differences in the detection limit.

Virus-specific CD8 T-cells showed frequencies in some control subjects that were higher than those in recent-onset diabetic subjects, an inverse pattern compared with frequencies of autoreactive T-cells in patient and healthy subjects. Yet, the difference was moderate and was lost completely if frequencies of virus-specific T-cells of patients before islet transplantation were combined with those of patients with newly diagnosed diabetes. We have no clear explanation for this trend, other than patients being slightly younger than control subjects. Yet, this finding suggests that the increases in antigen-specific CD8 T-cells in type 1 diabetes are not a general phenomenon reflecting hyperimmune reactivity per se but seem more specific for islet autoreactive T-cells.

Even though the gate settings will be largely similar among individuals and among experiments, analyzing (auto)antigen T-cell specificities is subject to subtle differences in background stainings between individual subjects that require adjusting the gate settings accordingly. This may partly result from the need to compensate the light channels on each day that blood samples are analyzed on the FACS LSR II. Importantly, the minor differences in background staining did not distinguish patients from healthy subjects. We recommend that a longitudinal series of blood samples from a given subject be analyzed on the same day to minimize interassay variation, as we pursued for the analyses of blood samples of islet transplant recipients. We contend that the availability of a second dimension of staining in our combinatorial approach (each epitope being represented by two different colors) facilitates setting the gates and to a great extent copes with the difficulties of distinguishing background from positive staining of low-avidity TCR.

It will be interesting to use our new technology to assess functional and phenotypic differences in islet autoreactive T-cells between type 1 diabetic patients and other subjects (siblings, healthy unrelated subjects, and patients with other diseases). Preliminary results suggest that CD8 T-cells recognizing the PPI<sub>15-24</sub> epitope in an islet cell-transplanted patient are largely of a memory phenotype (supplementary Fig. 2).

In conclusion, our novel, highly sensitive detection system allows for direct assessment of circulating autoreactive CD8<sup>+</sup> T-cells against an array of islet epitopes simultaneously. Another major advance over the current procedures to determine islet-specific epitopes is the freedom from in vitro culture and expansion that is otherwise required in most studies on novel diabetes-

associated antigens to be able to detect responses (15–18). Testing multiple epitope specificities in the same sample further reduces the blood volumes required for analysis and extends opportunities for testing for additional and novel immune reactivities. Finally, our methods provided applicable and informative data on thawed blood samples that had been stored for up to 15 years, for the first time allowing assessment of cellular islet autoreactivity retrospectively, and enabling use in the context of large cohorts and multicenter immune intervention studies. It is conceivable that other or yet to be discovered epitopes may provide a stronger correlation with clinical outcome. Even though the currently used Qdot-MHC multimer technique allows a highly sensitive, combinatorial assessment of multiple islet epitope-specific CD8<sup>+</sup> T-cell populations in type 1 diabetes to study pathogenesis, prediction, progression, and intervention of the disease, it can be modified and extended to up to 25 different epitopes in the future.

#### ACKNOWLEDGMENTS

These studies were supported by the Juvenile Diabetes Research Foundation, the Dutch Diabetes Research Foundation, and a VICI award to B.O.R. from The Netherlands Organisation for Health Research and Development.

No potential conflicts of interest relevant to this article were reported.

#### REFERENCES

1. In't Veld P, Lievens D, De Grijse J, Ling Z, Van der Auwera B, Pipeleers-Marichal M, Gorus F, Pipeleers D. Screening for insulinitis in adult autoantibody-positive organ donors. *Diabetes* 2007;56:2400–2404
2. Bottazzo GF, Dean BM, McNally JM, MacKay EH, Swift PG, Gamble DR. In situ characterization of autoimmune phenomena and expression of HLA molecules in the pancreas in diabetic insulinitis. *N Engl J Med* 1985;313:353–360
3. Foulis AK, Liddle CN, Farquharson MA, Richmond JA, Weir RS. The histopathology of the pancreas in type 1 (insulin-dependent) diabetes mellitus: a 25-year review of deaths in patients under 20 years of age in the United Kingdom. *Diabetologia* 1986;29:267–274
4. Santamaria P, Utsugi T, Park BJ, Averill N, Kawazu S, Yoon JW. Beta-cell-cytotoxic CD8<sup>+</sup> T cells from nonobese diabetic mice use highly homologous T cell receptor alpha-chain CDR3 sequences. *J Immunol* 1995;154:2494–2503
5. Marron MP, Graser RT, Chapman HD, Serreze DV. Functional evidence for the mediation of diabetogenic T cell responses by HLA-A2.1 MHC class I molecules through transgenic expression in NOD mice. *Proc Natl Acad Sci U S A* 2002;99:13753–13758
6. Graser RT, DiLorenzo TP, Wang F, Christianson GJ, Chapman HD, Roopenian DC, Nathanson SG, Serreze DV. Identification of a CD8 T cell that can independently mediate autoimmune diabetes development in the complete absence of CD4 T cell helper functions. *J Immunol* 2000;164:3913–3918
7. Wong FS, Visintin I, Wen L, Flavell RA, Janeway CA Jr. CD8 T cell clones from young nonobese diabetic (NOD) islets can transfer rapid onset of diabetes in NOD mice in the absence of CD4 cells. *J Exp Med* 1996;183:67–76
8. Naftanel MA, Harlan DM. Pancreatic islet transplantation. *PLoS Med* 2004;1:e58
9. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med* 2000;343:230–238
10. Keymeulen B, Ling Z, Gorus FK, Delvaux G, Bouwens L, Gruppig A, Hendrickx C, Pipeleers-Marichal M, Van Schravendijk C, Salmela K, Pipeleers DG. Implantation of standardized beta-cell grafts in a liver segment of IDDM patients: graft and recipients characteristics in two cases of insulin-independence under maintenance immunosuppression for prior kidney graft. *Diabetologia* 1998;41:452–459
11. Hering BJ, Kandaswamy R, Harmon JV, Ansite JD, Clemmings SM, Sakai T, Paraskevas S, Eckman PM, Sageshima J, Nakano M, Sawada T, Matsumoto I, Zhang HJ, Sutherland DE, Bluestone JA. Transplantation of cultured

- islets from two-layer preserved pancreases in type 1 diabetes with anti-CD3 antibody. *Am J Transplant* 2004;4:390–401
12. Ricordi C, Inverardi L, Kenyon NS, Goss J, Bertuzzi F, Alejandro R. Requirements for success in clinical islet transplantation. *Transplantation* 2005;79:1298–1300
  13. Ryan EA, Paty BW, Senior PA, Bigam D, Alfadhli E, Kneteman NM, Lakey JR, Shapiro AM. Five-year follow-up after clinical islet transplantation. *Diabetes* 2005;54:2060–2069
  14. Huurman VA, Hilbrands R, Pinkse GG, Gillard P, Duinkerken G, van de LP, Meer-Prins PM, Versteeg-van der Voort Maarschalk MF, Verbeeck K, Alizadeh BZ, Mathieu C, Gorus FK, Roelen DL, Claas FH, Keymeulen B, Pipeleers DG, Roep BO. Cellular islet autoimmunity associates with clinical outcome of islet cell transplantation. *PLoS One* 2008;3:e2435
  15. Pinkse GG, Tysma OH, Bergen CA, Kester MG, Ossendorp F, van Veelen PA, Keymeulen B, Pipeleers D, Drijfhout JW, Roep BO. Autoreactive CD8 T cells associated with beta cell destruction in type 1 diabetes. *Proc Natl Acad Sci U S A* 2005;102:18425–18430
  16. Skowera A, Ellis RJ, Varela-Calviño R, Arif S, Huang GC, Van-Krinks C, Zaremba A, Rackham C, Allen JS, Tree TI, Zhao M, Dayan CM, Sewell AK, Unger WW, Unger W, Drijfhout JW, Ossendorp F, Roep BO, Peakman M. CTLs are targeted to kill beta cells in patients with type 1 diabetes through recognition of a glucose-regulated preproinsulin epitope. *J Clin Invest* 2008;118:3390–3402
  17. Panina-Bordignon P, Lang R, van Endert PM, Benazzi E, Felix AM, Pastore RM, Spinaz GA, Sinigaglia F. Cytotoxic T cells specific for glutamic acid decarboxylase in autoimmune diabetes. *J Exp Med* 1995;181:1923–1927
  18. Takahashi K, Honeyman MC, Harrison LC. Cytotoxic T cells to an epitope in the islet autoantigen IA-2 are not disease-specific. *Clin Immunol* 2001;99:360–364
  19. Unger WW, Pinkse GG, Mulder-van der Kracht S, van der Slik AR, Kester MG, Ossendorp F, Drijfhout JW, Serreze DV, Roep BO. Human clonal CD8 autoreactivity to an IGRP islet epitope shared between mice and men. *Ann N Y Acad Sci* 2007;1103:192–195
  20. Takaki T, Marron MP, Mathews CE, Guttman ST, Bottino R, Trucco M, DiLorenzo TP, Serreze DV. HLA-A\*0201-restricted T cells from humanized NOD mice recognize autoantigens of potential clinical relevance to type 1 diabetes. *J Immunol* 2006;176:3257–3265
  21. Panagiotopoulos C, Qin H, Tan R, Verchere CB. Identification of a beta-cell-specific HLA class I restricted epitope in type 1 diabetes. *Diabetes* 2003;52:2647–2651
  22. Di Lorenzo TP, Peakman M, Roep BO. Translational mini-review series on type 1 diabetes: systematic analysis of T cell epitopes in autoimmune diabetes. *Clin Exp Immunol* 2007;148:1–16
  23. Seyfert-Margolis V, Gisler TD, Asare AL, Wang RS, Dosch HM, Brooks-Worrell B, Eisenbarth GS, Palmer JP, Greenbaum CJ, Gitelman SE, Nepom GT, Bluestone JA, Herold KC. Analysis of T-cell assays to measure autoimmune responses in subjects with type 1 diabetes: results of a blinded controlled study. *Diabetes* 2006;55:2588–2594
  24. Naik RG, Beckers C, Wentwoord R, Frenken A, Duinkerken G, Brooks-Worrell B, Schloot NC, Palmer JP, Roep BO. Precursor frequencies of T-cells reactive to insulin in recent onset type 1 diabetes mellitus. *J Autoimmun* 2004;23:55–61
  25. Ouyang Q, Standifer NE, Qin H, Gottlieb P, Verchere CB, Nepom GT, Tan R, Panagiotopoulos C. Recognition of HLA class I-restricted beta-cell epitopes in type 1 diabetes. *Diabetes* 2006;55:3068–3074
  26. Lindley S, Dayan CM, Bishop A, Roep BO, Peakman M, Tree TI. Defective suppressor function in CD4<sup>+</sup>CD25<sup>+</sup> T-cells from patients with type 1 diabetes. *Diabetes* 2005;54:92–99
  27. Tree TI, Duinkerken G, Willemen S, de Vries RR, Roep BO. HLA-DQ-regulated T-cell responses to islet cell autoantigens insulin and GAD65. *Diabetes* 2004;53:1692–1699
  28. Hadrup SR, Bakker AH, Shu CJ, Andersen RS, van Veluw J, Hombrink P, Castermans E, Thor Straten P, Blank C, Haanen JB, Heemskerk MH, Schumacher TN. Parallel detection of antigen-specific T-cell responses by multidimensional encoding of MHC multimers. *Nat Methods* 2009;6:520–526
  29. Keymeulen B, Gillard P, Mathieu C, Movahedi B, Maleux G, Delvaux G, Ysebaert D, Roep B, Vandemeulebroucke E, Marichal M, In't Veld P, Bogdani M, Hendrickx C, Gorus F, Ling Z, van Rood J, Pipeleers D. Correlation between beta cell mass and glycemic control in type 1 diabetic recipients of islet cell graft. *Proc Natl Acad Sci U S A* 2006;103:17444–17449
  30. Altman JD, Moss PA, Goulder PJ, Barouch DH, McHeyzer-Williams MG, Bell JI, McMichael AJ, Davis MM. Phenotypic analysis of antigen-specific T lymphocytes. *Science* 1996;274:94–96
  31. Toma A, Haddouk S, Briand JP, Camoin L, Gahery H, Connan F, Dubois-Laforgue D, Caillat-Zucman S, Guillet JG, Carel JC, Muller S, Choppin J, Boitard C. Recognition of a subregion of human proinsulin by class I-restricted T cells in type 1 diabetic patients. *Proc Natl Acad Sci U S A* 2005;102:10581–10586
  32. Hassainya Y, Garcia-Pons F, Kratzer R, Lindo V, Greer F, Lemonnier FA, Niedermann G, van Endert PM. Identification of naturally processed HLA-A2-restricted proinsulin epitopes by reverse immunology. *Diabetes* 2005;54:2053–2059
  33. Monti P, Scirpoli M, Maffi P, Ghidoli N, De Taddeo F, Bertuzzi F, Piemonti L, Falcone M, Secchi A, Bonifacio E. Islet transplantation in patients with autoimmune diabetes induces homeostatic cytokines that expand autoreactive memory T cells. *J Clin Invest* 2008;118:1806–1814
  34. Ernst B, Lee DS, Chang JM, Sprent J, Surh CD. The peptide ligands mediating positive selection in the thymus control T cell survival and homeostatic proliferation in the periphery. *Immunity* 1999;11:173–181
  35. Goldrath AW, Bevan MJ. Low-affinity ligands for the TCR drive proliferation of mature CD8<sup>+</sup> T cells in lymphopenic hosts. *Immunity* 1999;11:183–190
  36. Wu Z, Bensinger SJ, Zhang J, Chen C, Yuan X, Huang X, Markmann JF, Kassae A, Rosengard BR, Hancock WW, Sayegh MH, Turka LA. Homeostatic proliferation is a barrier to transplantation tolerance. *Nat Med* 2004;10:87–92
  37. Brook MO, Wood KJ, Jones ND. The impact of memory T cells on rejection and the induction of tolerance. *Transplantation* 2006;82:1–9