Simultaneous detection of circulating autoreactive CD8+ T-cells specific for different islet cell-associated epitopes using combinatorial MHC-multimers

Running title: Detection of circulating autoreactive T-cells

Jurjen H. Velthuis1,5,6, Wendy W. Unger1,6, Joana R. F. Abreu1, Gaby Duinkerken1,5, Kees Franken1, Mark Peakman4, Arnold H. Bakker3,6, Sine Reker-Hadrup3,6, Bart Keymeulen2,5, Jan Wouter Drijfhout2, Ton N. Schumacher3 and Bart O. Roep1,5.

1 Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, NL;
2 Diabetes Research Center, Brussels Free University-VUB, Brussels, Belgium;
3 Division of Immunology, The Netherlands Cancer Institute, Amsterdam, The Netherlands.
4 Department of Immunobiology, King's College School of Medicine, Guy's Hospital, London, U.K.
5 JDRF Center for Beta Cell Therapy in Diabetes.
6 Present addresses: Swan Diagnostics, Dept of Cell Biology, Erasmus MC, Rotterdam, The Netherlands (JHV); Department of Cell Biology and Immunology, VUmc, Amsterdam, The Netherlands (WWU); University of California, Berkeley, USA (AHB); Center for Cancer Immunotherapy, Department of Hematology, Herlev University Hospital, Herlev, Denmark (SRH)

Correspondence:
Prof. Bart O. Roep
Email: boroep@lumc.nl

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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+ T-cells play a pivotal role. Thus, monitoring of multiple islet-specific CD8+ T-cells may prove valuable for measuring disease activity, progression and intervention. Yet, conventional detection techniques (ELISPOT, HLA tetramers) require many cells and are relatively insensitive.

Research Design and Methods - Here, we employed a combinatorial quantum dot MHC multimer technique to simultaneously monitor the presence of HLA-A2 restricted insulin B\textsubscript{10-18}, pre-pro-insulin (PPI)\textsubscript{15-24}, IA-2\textsubscript{797-805}, GAD65\textsubscript{114-123}, IGRP\textsubscript{265-273} and ppIAPP\textsubscript{5-13} specific CD8+ T-cells in recent onset diabetes patients, their siblings, healthy controls and islet cell transplantation recipients.

Results - Using this kit, islet autoreactive CD8+ T-cells recognizing insulin B\textsubscript{10-18}, IA-2\textsubscript{797-805} and IGRP\textsubscript{265-273} were shown to be frequently detectable in recent onset patients, but rarely in healthy controls; PPI\textsubscript{15-24} proved 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 was associated with disease activity and correlated with clinical outcome.

Conclusions - A kit was developed that allows simultaneous detection of CD8+ 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.
Type 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\(^+\) T-cells play a pivotal role in the destruction process and constitute a significant portion of insulitis (1;2). In accordance, NOD mice lacking the expression of MHC class I are resistant to autoimmune diabetes (3;4), whereas HLA-A2 transgenic NOD mice develop an accelerated disease (5). Additionally, transfer of CD8\(^+\) T-cell clones resulted in transfer of type 1 diabetes (6;7). Thus detection and monitoring of specific CD8\(^+\) 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 destructed cells. Yet, as the insulin producing cells were destructed by an auto-immune response, islet cell transplantation could also result in reactivation of the auto-immune response. Recently, we have shown that proliferation of CD4\(^+\) T-cells specific for GAD and IA-2 in patients who underwent islet cell transplantation 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 auto-reactive CD8\(^+\) T-cells are primarily derived from beta-cell antigens, most importantly (pre-)(pro-)insulin. Previously, we showed that the presence of CD8\(^+\) T-cells reactive to the naturally processed insulin–peptide B\(_{10-18}\) in HLA-A2 correlated with islet cell destruction (15). Recently, another important epitope was uncovered as the signal peptide of pro-insulin was shown to contain a glucose-regulated CD8\(^+\) T-cell epitope (PPI\(_{15-24}\)) (16), but many other epitopes derived from insulin and a range of other beta cell derived antigens, such as glutamic acid decarboxylase (GAD)-65 (17), islet antigen (IA)-2 (18), islet-specific glucose-6-phosphatase catalytic subunit related protein (IGRP) (19;20) and pre-pro islet amyloid polypeptide (ppIAPP) (21) have been reported [for review see (22)]. Ideally, monitoring for the presence of CD8\(^+\) T-cells reactive to all 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\(^+\) 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\(^+\) T-cell populations by conventional tetramer technology is generally impossible due to the scarcity of material. Furthermore, detection of islet autoreactive T-cells is hampered by their low precursor frequencies in circulation (23;24), low 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 employed the recently described combinatorial Qdot technique (28) to simultaneously detect CD8\(^+\) 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 controls, we validated this technique and established the specificity of these staining. Subsequently, we
monitored the presence of reactive CD8+ 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+ T-cells to multiple islet epitopes, which is applicable to small volumes of stored blood samples to allow screening in multicenter immune intervention trials.

MATERIAL AND METHODS
Recent onset diabetes patients. Samples from recent onset type 1 diabetes (T1D) patients were retrieved from the Kolibri T1D cohort, which includes material from 350 patients with juvenile-onset T1D (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. PBMCs were isolated by Ficoll-isopaque density gradient centrifugation. PBMCs were frozen in a solution of 20% human pooled serum and 10% DMSO (5-10.106 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 prior to transplantation. Graft recipients were long-term type 1 diabetes patients without any earlier transplantation, with plasma C-peptide <0.09 ng/ml, large variation in blood glucose levels (Coefficient of variation [CV] ≥25%), HbA1c 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 EBV 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 to beta cell enriched fractions that were cultured for 2–20 days (median 6 days). Immunosuppressive induction therapy consisted of anti-thymocyte globulin (ATG, Fresenius, Fresenius Hemocare, WA, USA) with a single infusion of 9 mg/kg and subsequently with 3 mg/kg for 6 days except when T-lymphocyte count was under 50/mm3. Maintenance immunosuppression consisted of tacrolimus (Prograft, Fujisawa/Pharma Logistics, dose according to trough level: 8–10 ng/ml in the first three months post transplantation, 6–8 ng/ml thereafter) and mycophenolate mofetil (MMF, Roche, 2000 mg/day). The HLA typing of the patients is depicted in the supplementary table in the online appendix available at http://diabetes.diabetesjournals.org. Islet recipients were age-matched with the healthy control subjects.

Quantum-Dot-labeled HLA A2 - peptide multimers. Multimeric HLA-A2-peptide complexes were prepared essentially as previously described (30). Briefly, recombinant HLA-A2 and human β2M were solubilized in urea and injected together with each synthetic peptide into a refolding buffer consisting of 100 mM Tris (pH 8.0), 400 mM arginine, 2 mM EDTA, 5 mM reduced glutathione, and 0.5 mM 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 quantum-dots (Qdots; 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 subject were stained with a mixture containing 6 diabetes associated epitopes, a HLA-A2 epitope expressed in HLA-A2 and a mix of viral antigens (Table I).

**Cell Staining with Qdot-labeled multimeric complexes.** PBMC (2 × 10^6) were stained simultaneously with all Qdot-labeled multimers (0.1 µg of each specific multimer) in 60 µl of PBS supplemented with 2% BSA and incubated for 15 min at 37°C (see table I). Subsequently, 10 µl APC-labeled anti-CD8 (stock 1:10) and 10 µl FITC-labeled anti-CD4; -CD14; -CD16; -CD19 and –CD40 antibodies (Becton Dickinson) were added for 30 min at 4°C. After washing twice, cells were resuspended in PBS/2% BSA containing 7-AAD (eBioscience, San Diego, CA, USA) to exclude dead cells and samples were analyzed using the LSRII (Becton Dickinson).

**Statistical analysis.** Statistical analysis on recent onset diabetes patients versus their siblings was performed using Wilcoxon matched pairs test. Differences between recent onset diabetes patients and controls were tested using the unpaired t-test with Welch correction (for HLA-A2 peptide and PPI15-24, 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 the Dunn’s 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 to design kits of defined peptide-MHC multimers that may be used to report on disease state or vaccine response. To test this concept, a quantum-dots (Qdots) based combinatorial approach was developed to simultaneously monitor multiple islet epitopes associated with the development of type I diabetes. Hereto, 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 I).

During flow cytometric analysis, single cell lymphocytes were gated that stained positive for CD8 (on average 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 PBMC of healthy controls (Figure 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 (fig 1B left). A clear population of CD8^+ T-cells reactive to insulin B_{10-18} was seen in the sample of the recent onset patient (fig 1C, right). These insulin B_{10-18} reactive CD8 T-cells were also found in an islet cell transplantation recipient (fig 1D, right), in which also virus specific CD8^+ T-cells were seen (fig 1D, left). No CD8^+ T-cells reactive to insulin B_{10-18} were found in the healthy controls (fig 1B right), whereas few virus specific CD8^+ T-cells were detectable in samples of recent onset patients (fig 1C, left). In terms of reproducibility, the co-efficient of variation between experiments was 9.5% across Qdot multimers. For the
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- 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%; ppIAPP: 6.9%) (Supplementary Figures 1, 2 and 3).

Selectivity of the examined epitopes. To determine if the simultaneously measured islet epitopes were sensitively and specifically detected in the circulation of recent onset type 1 diabetes patients, we determined the frequency of CD8+ T-cells specific for all currently known epitopes in recent onset patients, their siblings (when available) and healthy controls.

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

Next, we determined the sensitivity and specificity of T-cell responses to each epitope, defining a frequency of 1 cell reactive in 10,000 CD8+ T-cells (i.e. 0.010%) as cut-off, with the exception of the IGRP265-273 where a frequency of 1 in 20,000 (i.e. 0.005%) was used as a cut-off. Three epitopes were found to be 100% specific as no relevant frequencies were seen in the controls: insulin B10-18 showed a sensitivity of 65%, and was the most specific and considerably sensitive epitope detected; IA-2 797-805 and IGRP265-273 provided a specificity of 100%, but a sensitivity of 25% (Table II). The epitope with the highest sensitivity was PPI15-24, as epitope specific CD8+ T-cell reactivity was detectable in 17 out of 20 recent patients (85%). Yet, 4 out of the 15 controls also exhibit CD8+ 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 detection limit (n=27), regardless of the peptides epitopes tested, supporting the specificity of these reagents.

Overall, our data indicate that we can discretely monitor the specific presence of multiple epitope reactive CD8+ T-cells simultaneously using the newly developed ‘Diab-Q-kit’.

Epitope specific CD8+ T-cells in islet cell transplantation. Next, the ‘Diab-Q-kit’ was employed to monitor the presence of HLA-A2 epitope specific CD8+ T-cells in seven islet cell transplant recipients, at four different time-points: prior to transplantation, 6 weeks thereafter (reconstitution of the T-cell compartment after ATG-induction treatment), 26 weeks and 52 weeks after transplantation. Examination of the frequency of CD8+ T-cells recognizing endogenously processed HLA-A2 peptide presented in the HLA-A2 molecule showed reactivity in two recipients at two different time points (figure 3). Viral reactivity was seen in two out of the seven HLA-A2 positive islet cell transplantation recipients prior to transplantation (figure 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. In spite of disease durations up to several decades, islet autoreactive CD8+ T-cells were
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still detectable in the majority of type 1 diabetes patients at the time of islet transplantation (figure 4; 0 weeks). No particular islet epitope or pattern of reactivity dominated. After reconstitution of the T-cell compartment under maintenance immunosuppression following ATG induction therapy (fig 4; 6 weeks), the cumulative numbers of autoreactive CD8+ T-cells weaned in a minority of recipients. In four out of seven patients, islet autoreactivity increased after islet transplantation, one patient displayed stable frequencies of circulating islet reactive T-cells and in two case a stable declined was observed.

The dynamics of T-cells specific for the (pre-) (pro-)insulin epitopes (insulin B10-18 and PPI15-24 were most pronounced (figure 3): considerable frequencies of these cells were detected prior to islet cell infusion and (re-)emerged at later time-points thereafter. In contrast, T-cell frequencies against GAD65114-123 and IGRP265-273 were infrequently seen, with only a single increase late after islet implantation in patients #6 and #7, respectively (figure 3). These two cases also displayed most epitope spreading at that time point (figure 4). Of note, βct #7 CMV-converted after transplantation.

Clinical outcome. Subsequently, the patterns of autoreactive CD8+ 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 controls (figure 2), only the presence of two or more epitope specific auto-reactive CD8+ 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+ T-cells reactive to the recently uncovered PPI-epitopes PPI76-84 and PPI79-88 in HLA-A3 as well as PPI4-13 in HLA-B7 were also considered (Unger W, Velthuis J, et al. submitted) as indicated in table III. Consequently, the data obtained with the novel ‘Diab-Q-kit’ predicted that six of the seven recipients studied would not reach insulin independence.

Previously, we reported that proliferation of CD4+ T-cells to whole IA-2 and GAD65 prior to transplantation, predicted transplantation outcome (14). For comparison, the prediction of clinical outcome based on this proliferation assay was also taken into consideration (table III). Prediction based on proliferation of islet-specific CD4 T-cells pointed towards four patients not reaching insulin independence. For three patients there was agreement in the prediction by both immunological endpoints. The Diab-Q-kit predicted that six recipients would remain insulin requiring, of which four actually required exogeneous insulin injection. Thus, this method showed a prediction accuracy of 66%. The prediction of clinical outcome based on proliferation (14) predicted insulin requirement in 4 cases, of which three were correct (accuracy of 75%). However, when both methods agreed in their prediction all transplant recipients remained insulin requiring following transplantation and thus the combined method provided an accuracy of 100%, underlining the importance of monitoring CD8+ T-cell frequencies.

DISCUSSION

Our study is the first to implement simultaneous detection of multiple islet cell specific CD8+ T-cell responses, by development of the Diab-Q-kit. To this purpose, multidimensional encoded MHC multimers were employed. 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 diabetes patients, their siblings, control blood donors and islet cell transplant recipients. Insulin B10-18 was
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found to be 100% specific, as no CD8+ T-cell frequencies of over 1 in 10,000 cells were seen in PBMC from healthy controls. Although this also holds true for IGRP265-273 and IA-2797-805, CD8+ T-cells recognizing insulin B10-18 showed the highest sensitivity of these epitopes. The relevance of insulin B10-18 in type 1 diabetes is underlined by previously published observations that PBMC from a type 1 diabetic patient produced IFN-γ in response to this peptide (31), that expression of insulin B10-18 renders target cells sensitive to killing by CTL lines (32) and most importantly, that the presence of insulin B10-18 specific CD8+ T-cells correlates with destruction of beta-cells (15). As the transplantation of isolated islet cells can result in reactivation of CD8+ T-cell mediated auto-reactivity towards islet cell specific epitopes, peripheral blood from islet cell transplantation recipients can be used to monitor the factors important in beta cell destruction. Also in this cohort, the presence of insulin B10-18 reactive CD8+ T-cells post-transplantation correlated with a poor clinical outcome, with the exception of the CMV-converted recipient.

Recently, we reported PPI15-24 as being a naturally produced and presented HLA-A2 epitope (16). Cytotoxic CD8+ T-cells against this peptide could be cloned that killed β-cells in vitro in a glucose concentration dependent fashion, linking beta-cell immunogenicity with its functional activity (16). This study underscores the relevance of CD8 islet autoreactivity in the pathogenesis of T1D, while it indicates that beta-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 PPI15-24 at a time-point after transplantation. Of these patients, half did not reach insulin independence after transplantation. Also the HLA-A2 restricted islet epitopes IA-2797-805, GAD65114-123, IGRP265-273 and ppIAPP5-13 exhibited a highly specific staining, with only incidental reactivity in healthy controls. In contrast to previously reported results (18) we did not observe any CD8+ T-cells reactive to IA-2797-805 in healthy controls. However, we performed a direct assessment of the CD8+ T-cell frequency in PBMC, rather than functional assays, whereas Takahashi et al. (18) cultured CD8+ cells for 14 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-2797-805, GAD65114-123 and IGRP265-273 were almost exclusively seen in combination with reactivities to insulin B10-18 and/or PPI15-24, suggesting that preproinsulin epitopes may comprise the primary epitopes following reactivation of auto-immunity upon islet cell transplantation, whereas reactivity to the other may result from epitope spreading. Intriguingly, in two patients without islet epitope reactive CD8+ T-cells after the transplantation induction therapy (i.e. at 6 weeks), CD8+ T-cells specific for insulin B10-18 and PPI15-24 were among the first CD8+ T-cells to occur. Their occurrence after ATG treatment may have resulted from homeostatic proliferation (33-35) indicating that these CD8+ T-cells although undetectable in the peripheral blood after ATG induction, remain as a memory population in lymphatic organs. As in one of these patients these cells were undetectable prior to 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 allo-reactive cells can be to solid organ transplantation (36;37). We cannot conclude on any order of reactivity between islet autoantigens, since 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, 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 in recent-onset diabetic subjects, an inverse pattern compared to 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 newly diagnosed patients. We have no clear explanation for this trend, other then patients being slightly younger than control subjects. Yet, this finding suggests that the increases of antigen-specific CD8 T-cells in T1D are not a general phenomenon reflecting hyper-immune reactivity per se, but seem more specific for islet autoreactive T-cells. Even though the gate settings will be largely similar between individuals and between 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 LSRII. Importantly, the minor differences in background staining did not distinguish patients from healthy subjects. We recommend having longitudinal series of blood samples from a given subject be analyzed on the same day to minimize inter-assay 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 distinguishing background from positive staining of low avidity TCR. It will be interesting to employ our new technology to assess functional and phenotypic differences of islet autoreactive T-cells between T1D patients and other subjects (siblings, healthy unrelated subjects, patients with other diseases). Preliminary results suggest that CD8 T cells recognizing the PPI15-24 epitope in an islet-cell transplanted patient are largely of a memory phenotype (supplementary Figure 2). In conclusion, our novel, highly sensitive, detection system allows for direct assessment of circulating auto-reactive CD8 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 to 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 employment 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 employed Qdot-MHC multimer technique allows a highly sensitive, combinatorial assessment of multiple islet epitope specific CD8 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

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REFERENCES

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### Table I. Combinations of Q-dot labeled HLA-A2 multimers

<table>
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<tr>
<th>Origin</th>
<th>Position/protein</th>
<th>Sequence</th>
<th>Signal</th>
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<tr>
<td>CMV</td>
<td>pp65</td>
<td>NLVPMVATV</td>
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### Table II. Selectivity of the tested epitopes

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<th>Epitope</th>
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<th>Recent onset T1D patients (n=20)</th>
<th>Sensitivity</th>
<th>Specificity</th>
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<td>IGRP 265-273</td>
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Table III. Clinical outcome. 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 received graft. In the resulting prediction of clinical outcome, also the presence of CD8 T-cells against novel HLA-A3 and HLA-B7-restricted epitopes were considered. Clinical outcome is defined as insulin independence or remaining insulin requiring, the coefficient of variation in fasting glycemia in the first 6 months (CoV), and time to reach insulin independence.

<table>
<thead>
<tr>
<th>Pat #</th>
<th>CD8 T-cell reactivity against islet epitopes</th>
<th>Prediction</th>
<th>Clinical outcome&lt;sup&gt;6&lt;/sup&gt;</th>
<th>Pat #</th>
<th>CD8 T-cell reactivity against islet epitopes</th>
<th>Prediction</th>
<th>Clinical outcome&lt;sup&gt;6&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>CD8 Diab-Q-kit</td>
<td>CD4 Proliferation</td>
<td>Insulin needs</td>
<td>CoV (%)</td>
<td>time to ins. indep.</td>
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<tr>
<td></td>
<td>0 w</td>
<td>6 w</td>
<td>26 w</td>
<td>52 w</td>
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<tr>
<td>Bet # 1</td>
<td>Tx1 A2 A3 B7</td>
<td>Tx2 A2 A3</td>
<td>Ins Req</td>
<td>-</td>
<td>Ins Req</td>
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<tr>
<td>HLA-A2</td>
<td>ppIAPP</td>
<td>PPI</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>HLA-A3</td>
<td>PPI&lt;sub&gt;76-84&lt;/sub&gt;</td>
<td>PPI&lt;sub&gt;76-84&lt;/sub&gt;</td>
<td>PPI&lt;sub&gt;76-84&lt;/sub&gt;</td>
<td>PPI&lt;sub&gt;79-88&lt;/sub&gt;</td>
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<tr>
<td>HLA-B7</td>
<td>PPI&lt;sub&gt;79-88&lt;/sub&gt;</td>
<td>PPI&lt;sub&gt;79-88&lt;/sub&gt;</td>
<td>PPI&lt;sub&gt;4-13&lt;/sub&gt;</td>
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<td>Bet # 2</td>
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<td>Ins Req</td>
<td>Ins Req</td>
<td>Ins Req</td>
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<td>GAD65</td>
<td>PPI</td>
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<tr>
<td>HLA-B7</td>
<td>PPI&lt;sub&gt;4-13&lt;/sub&gt;</td>
<td>PPI&lt;sub&gt;4-13&lt;/sub&gt;</td>
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<td>Bet # 3</td>
<td>Tx1 A2</td>
<td>Tx2 A2</td>
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<td>PPI</td>
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<td>Tx2 A2</td>
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<td>-</td>
<td>Ins Indep</td>
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<td>Bet # 5</td>
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<td>Tx2 A2</td>
<td>-</td>
<td>Ins Req</td>
<td>Ins Indep</td>
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<td>15.0 weeks</td>
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<td>PPI</td>
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<tr>
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<tr>
<td>HLA-B7</td>
<td>PPI&lt;sub&gt;4-13&lt;/sub&gt;</td>
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<td>Bet # 6</td>
<td>Tx1 A2</td>
<td>Tx2 A2</td>
<td>Ins Req</td>
<td>Ins Req</td>
<td>Ins Req</td>
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<td>B10-18</td>
<td>PPI</td>
<td>PPI</td>
<td>IA-2</td>
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<td>HLA-B7</td>
<td>GAD65</td>
<td>ppIAPP</td>
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<td>Bet # 7†</td>
<td>Tx1 A2</td>
<td>Tx2 A2</td>
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<td>-</td>
<td>Ins Indep</td>
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<td>B10-18</td>
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<td>† This patient CMV-converted upon transplantation.</td>
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<td>* All experiments were performed blinded from clinical outcome.</td>
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Figure Legends.

Figure 1. Flow cytometric analysis of epitope specific CD8+ T-cells using the combinatorial Qdot-approach. **A: Gating strategy:** viable CD8+ 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), CD8-APC positive but dump-channel-FITC (CD4 + CD14 + CD16 + CD20 + CD40) negative cells were gated, of which the 7-AAD positive cells were gated out. **B: Quantum dot staining:** within the viable CD8+ single T-cells, the cells recognizing the epitopes in the viral mix (Qdot 585+655) and insulin B10-18 (Qdot605+655) are shown as a typical example for a healthy control, a recent onset diabetes patient and a pre-transplantation islet cell recipients.

Figure 2. Frequencies of epitope specific CD8+ T-cells in recent onset diabetes patients, their siblings and healthy controls. The frequencies of CD8+ T-cells recognizing the epitopes HLA-A22115-2124, the viral mix, insulinB10-18, PPI15-24, GAD65114-123, IA-2797-805, IGRP265-273 and ppIAPP5-13 in HLA-A2 as determined by flow cytometry are depicted. First, the frequency detected in recent onset diabetes 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 frequency detected in all recent onset patients (RO, n=20) and controls (Con, n=15) were compared (right panels). Statistical analysis was performed using the unpaired t-test with Welch correction (for HLA-A2 peptide and PPI15-24) or the Mann-Whitney test (all other epitopes).

Figure 3. Frequencies of epitope specific CD8+ T-cells in islet cell recipients over time. The frequencies of CD8+ T-cells recognizing the epitopes HLA-A22115-2124, the viral mix, insulinB10-18, PPI15-24, GAD65114-123, IA-2797-805, IGRP265-273 and ppIAPP5-13 in HLA-A2 as determined by flow cytometry are depicted. The frequency of CD8+ T-cells was measured at four different time-points: prior to transplantation, 6 weeks thereafter (reconstitution of the T-cell compartment after ATG-induction treatment), 26 weeks and 52 weeks after transplantation. Changes in epitope-reactivity of islet cell transplant recipients over time were tested using the Friedman-test followed by the Dunn’s multiple comparisons test with *p < 0.05 values considered statistically significant. Data points of recent onset diabetes patients are depicted to allow easy comparison of ‘recent onset-reactivity’ and ‘islet cell transplant-reactivity’.

Figure 4. Cumulative frequencies of epitope specific CD8+ T-cells in islet cell recipients at different time-points. The cumulative frequencies of CD8+ T-cells recognizing the epitopes HLA-A22115-2124, the viral mix, insulinB10-18, PPI15-24, GAD65114-123, IA-2797-805, IGRP265-273 and ppIAPP5-13 in HLA-A2 as determined by flow cytometry, are depicted for each islet cell recipient individually. Please note the different axis for patient #6 and patient #7.
Detection of circulating autoreactive T-cells

Figure 1.

A

B

Healthy

Recent onset T1D

Islet recipient

Viral Mix

Ins_{B10.18}
Detection of circulating autoreactive T-cells

Figure 2.

- HLA-A2_{2115-2124}
- Viral Mix
- InsB_{10-18}
- PPI_{15-24}
- IA-2_{797-805}
- GAD65_{114-123}
- IGRP_{265-273}
- ppIAPP_{5-13}
Detection of circulating autoreactive T-cells

Figure 3

- HLA-A2\textsubscript{2115-2124} 0.29
- Virus mix 0.02
- Ins\textsubscript{B10-18} 0.75
- PPI\textsubscript{15-24} 0.06
- IA-2\textsubscript{797-805} 0.11
- GAD65\textsubscript{114-123} 0.09
- IGRP\textsubscript{265-273} 0.04
- ppIAPP\textsubscript{5-13} 0.07
Figure 4.