CHAPTER 5

T cell alloreactivity in islet transplantation
Relevance of cytotoxic alloreactivity under different immunosuppressive regimens in clinical islet cell transplantation

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ABSTRACT

Islet or β-cell transplantation provides a promising cure for type 1 diabetes (T1D) patients, but insulin-independency decreases frequently over time. Immunosuppressive regimens are implemented attempting to cope with both auto- and alloimmunity after transplantation. We analyzed the influence of different immunotherapies on autoreactive and alloreactive T cell patterns and transplant outcome.

Patients receiving three different immunosuppressive regimens were analyzed. All patients received anti-thymocyte globulin induction therapy. 21 patients received tacrolimus-mycophenolate mofetil (MMF) maintenance immunosuppression, whereas the other patients received tacrolimus-sirolimus (TAC-SIR, n=5) or sirolimus only (SIR, n=5). Cellular autoreactivity and alloreactivity (CTL precursor frequency) were measured ex vivo. Clinical outcome in the first six months after transplantation was correlated with immunological parameters.

C-Peptide levels were significantly different between the three groups studied (p=0.01). We confirm that C-peptide production was negatively correlated with pre transplant cellular autoreactivity and low graft size (p=0.001, p=0.007 respectively). Combining all three therapies, cellular autoimmunity after transplantation was not associated with delayed insulin-independence or C-peptide production. In combined tacrolimus/sirolimus and sirolimus-treated patients, CTL alloreactivity was associated with less insulin independence and C-peptide production (p=0.03). The percentage of donors to whom high CTLp frequencies were measured was lower in insulin independent recipients (p=0.03).

In this cohort of islet cell graft recipients, clinical outcome in the first 6 months after transplantation correlates with the applied immunosuppressive regimen. An association exists between insulin-independence and lower incidence of CTL alloreactivity towards donor HLA. This observational study demonstrates the usefulness of monitoring T-cell reactivity against islet allografts to correlate immune function with graft survival.
INTRODUCTION

Pancreas or simultaneous pancreas-kidney transplantation are established and successful therapeutic options to cure type 1 diabetes patients with end-stage renal failure\(^1\,^2\). However, in patients not eligible for this major surgical procedure, transplantation of isolated \(\beta\)-cells from islets of Langerhans would be favored\(^3\). This method is associated with low morbidity while still restoring endogenous insulin production. Short-term results have been very promising as demonstrated in several studies, and with different isolation protocols and immunosuppressive regimens\(^4\,^8\). However, in a large number of patients the long-term outcome is disappointing, with lasting long-term insulin-independence achieved by less than 10 percent of recipients\(^9\) and occurrence of adverse events related to immunosuppression\(^10\).

Several factors related to donors, grafts, the transplantation procedure and the engraftment can be important for graft survival. The amount of \(\beta\)-cells injected was shown previously to correlate with C-peptide release 2 months after transplantation\(^8\). The successful Edmonton protocol applies daclizumab induction and sirolimus and tacrolimus maintenance immunosuppressive therapy. However, avoidance of tacrolimus may have some advantages, because of diabetogenic\(^11\) and nephrotoxic\(^12\) effects as well as its interference with tolerance induction\(^13\). The same holds true for sirolimus, that is associated with side effects such as mouth ulcers, acne and hypercholesterolemia as well as impaired engraftment and insulin resistance\(^9\,^10\,^14\,^16\). Induction with anti-thymocyte globulin (ATG) is as successful as daclizumab in simultaneous pancreas-kidney transplantation\(^17\) and is also reported to be effective in combination with monotherapy of sirolimus\(^18\). These arguments validate exploration and comparison of other immunosuppressive regimens in \(\beta\)-cell transplantation.

The immunosuppressive regimen used may also affect the success of \(\beta\)-cell replacement by its effect on T-cell mediated autoimmunity and allograft rejection. Therefore, analysis of auto- and alloreactivity before and after islet cell transplantation can contribute to identify possible markers for success. The importance of cellular islet-specific autoimmunity in tacrolimus-mycophenolate mofetil treated recipients was revealed previously\(^19\), and analysis of the alloreactive cytotoxic T response has proven useful in other cohorts involving both islet cell (islet alone and islet after kidney\(^20\,^21\)) and bone marrow transplantation\(^22\). Yet, alloreactive CTL responses towards donor human leucocyte antigen (HLA) antigens seemed non-informative in patients transplanted with islets under tacrolimus/mycophenolate mofetil immune suppression\(^19\). Since this might relate to the type of immunosuppression used, we performed a retrospective analysis of patients receiving standardized islet cell grafts and induction therapy (ATG) under three different maintenance immune suppressive protocols: tacrolimus-mycophenolate mofetil (TAC-MMF), sirolimus-tacrolimus (TAC-SIR), or sirolimus (SIR) monotherapy. TAC-MMF therapy has become standard practice in our centre in recent years, while TAC-SIR and SIR therapy were initiated to enable comparison with TAC-MMF in a homogeneous cohort in a single \(\beta\)-cell transplantation programme. Clinical outcome in the
different patient groups has been published previously\(^8,10\). The aim of the current study was to evaluate the effect of the different immunosuppressive regimens on clinical parameters as insulin independence and C-peptide release. Subsequently, the effect of the different immunosuppressive therapy was correlated with immunological data (auto- and alloreactivity).

**MATERIALS AND METHODS**

**PATIENT GROUPS**

Patients were recruited for islet cell transplantation after signing informed consent and met the following inclusion criteria: long-standing type 1 diabetes, between 18 and 65 years of age, plasma C-peptide <0.09 ng/ml, large variation in blood glucose levels (coefficient of variation of fasting glycemia (CVgl) ≥25%), Hba\(_1c\) concentration >7% and one or more chronic diabetes lesions. Exclusion criteria included body weight >90 kg, active smoking, pregnancy, disturbed liver function tests, history of hepatic disease, presence of HLA antibodies or negative Epstein-Barr virus (EBV) serostatus.

In this study 31 patients were analysed that received one (n=11) or two (n=20) islet cell grafts in the first 26 weeks after transplantation. Twenty-one patients analysed were transplanted under antithymocyte globulin (ATG) induction and tacrolimus and mycophenolate mofetil maintenance immunosuppression (TAC-MMF)\(^8,19\), five under ATG-tacrolimus-sirolimus (TAC-SIR) and five under ATG-sirolimus (SIR). All three groups have been described in detail before\(^8,10,19\). Given the number limitations inherent to β-cell transplantation programmes, group size for the TAC-SIR and SIR groups remained limited. For the TAC-MMF group, the cohort of 21 patients reported on earlier\(^8\) was included in the current study. Patients’ baseline characteristics were not different (Table 1). However, patients transplanted under TAC-MMF received a smaller total amount of β-cells per kg body weight (p=0.02), in accordance with the lower number of patients in this group receiving a second graft. The decision to inject a second islet cell graft in the TAC-MMF group was based on insufficient C-peptide levels.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>TAC-MMF (N=21)</th>
<th>TAC-SIR (N=5)</th>
<th>SIR (N=5)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>42 (37-49)</td>
<td>36 (35-40)</td>
<td>41 (33-47)</td>
<td>0.31</td>
</tr>
<tr>
<td>Gender (M-F)</td>
<td>13-8</td>
<td>4-1</td>
<td>4-1</td>
<td>0.60</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>69 (65-76)</td>
<td>75 (62-78)</td>
<td>78 (76-80)</td>
<td>0.17</td>
</tr>
<tr>
<td>Duration of disease (yr)</td>
<td>26 (19-33)</td>
<td>21 (20-29)</td>
<td>22 (6-23)</td>
<td>0.21</td>
</tr>
<tr>
<td>Age at onset (yr)</td>
<td>17 (12-24)</td>
<td>16 (10-19)</td>
<td>21 (18-25)</td>
<td>0.41</td>
</tr>
<tr>
<td>Hba(_1c) (%)</td>
<td>7.6 (6.9-8.1)</td>
<td>8.0 (7.9-8.5)</td>
<td>7.5 (7.0-7.9)</td>
<td>0.69</td>
</tr>
<tr>
<td>Insulin dose (IU/kg/d)</td>
<td>0.7 (0.5-0.9)</td>
<td>0.6 (0.5-0.6)</td>
<td>0.6 (0.5-0.7)</td>
<td>0.46</td>
</tr>
<tr>
<td>Total injected β-cells (10(^6)/kg BW)</td>
<td>3.8 (2.6-4.9)</td>
<td>7.0 (4.6-7.4)</td>
<td>5.4 (4.0-5.8)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Data present median and interquartile range. p-values are calculated by Kruskal-Wallis test.
T cell alloreactivity in islet transplantation

and/or variation of fasting glycemia (CVgl>25%) after the first engraftment 8. Patients in the TAC-SIR and SIR group always received a second transplant regardless of C-peptide levels or CV. We also compared those patients in the TAC-MMF group receiving two transplants (n=10) with the TAC-SIR and SIR groups (all receiving two transplants).

PREPARATION OF ISLET CELL GRAFTS

Pancreases from brain-dead heart-beating donors were procured by hospitals affiliated with the Eurotransplant Foundation (Leiden, The Netherlands) according to local medical, legal, and ethical guidelines for organ donation. Islet cell-enriched fractions were cultured as described previously by using serum-free Ham’s F10 medium/0.5% human albumin/135 mg/dl glucose/2 mM glutamine (50 μl of tissue in 45 ml of medium suspended in a T175 Starsted culture flask with a vented cap). After 2–20 days (median 6 days; IQR 3–11 days) the preparations were analyzed for their β-cell number and purity5,8,10. Data were used to select preparations that, after combination, would constitute a graft with 0.5–5 × 10^6 β-cells per kilogram of recipient body weight (BW) suspended in 40–85 ml of Ham’s F10 medium with 0.5% human albumin. The final cellular composition of each β-cell graft was determined on samples that were taken just before implantation5,8,10. For each preparation, whether taken at the start of culture or during culture, or from the final graft, triplicate samples for DNA assay were taken, each being assayed in duplicate; when calculated for 30 consecutive grafts, the CV among these aliquots was 9% (5–14%), and that among duplicate samples was <5%. The total number of cells in a fraction was calculated by dividing its DNA content (in picograms) by 6.5 pg per cell, the average cellular DNA content measured in sorted single human adult β-cells and duct cells. The number of β-cells was then determined on the basis of the percentage of insulin-positive cells counted in duplicate samples of this fraction. The number of donors per graft was four (median; IQR of three to five). Compared with freshly isolated islet fractions4, these preparations exhibit a higher percent β-cells and contain virtually no acinar cells. Standardized grafts were injected into the portal vein of the recipient, as described previously5,8,23,24.

IMMUNOSUPPRESSION AND CLINICAL FOLLOW-UP

ATG induction therapy (ATG, Fresenius, Fresenius Hemocare, WA, USA) was administered to all patients and started with a single infusion of 9 mg/kg and subsequently with 3 mg/kg for 6 days or until T-lymphocyte count was under 50/mm^3. Tacrolimus maintenance immunosuppression (Prograf, Astellas Pharma Benelux) was dosed according to trough level: 8-10 ng/ml in months 0-3 post transplantation, 6-8 ng/ml thereafter. Sirolimus (Rapamune, Wyeth Pharmaceuticals) was administered orally at 0.2 mg/kg/day as a loading dose, 0.1 mg/kg/day thereafter, to achieve through levels of 10-15 ng/ml. Standard Mycophenolate mofetil (MMF, Roche) dosage was 2000 mg/day. Three hours before an islet cell graft implant, one dose of 500 mg methylprednisolone was given intravenously.
Graft recipients were regularly followed up regarding plasma C-peptide levels (at glycemia 120-200 mg/dl) as well as percentage HbA1c. The C-peptide level over 26 weeks was calculated by the area under the curve (AUC) of available plasma C-peptide values. Insulin dose was adjusted to avoid symptomatic hypoglycaemia, maintain blood glucose levels between 70 and 180 mg/dl and HbA1c levels below 7.0%.

LYMPHOCYTE STIMULATION TEST TO DETERMINE CELLULAR AUTOREACTIVITY
All cellular reactivity tests were performed blinded from clinical results. Blood was drawn from patients before transplantation and on regular intervals post transplantation (once every two to six weeks). Peripheral blood mononuclear cells (PBMCs) were isolated and processed as described before. Briefly, 150,000 fresh PBMCs/well were cultured in 96 well round-bottomed plates in Iscove’s Modified Dulbecco’s Medium (IMDM) with 2 mMol/l glutamine (Gibco, Paisley, Scotland) and 10% pooled human serum in the presence of antigen, IL-2 (35U/ml) or medium alone in triplicates. Antigens analyzed included IA-2 (10 μg/ml), GAD65 (10 μg/ml), insulin (25 μg/ml) and tetanus toxoid (‘third party’ antigen, 1,5LF/ml). Results were interpreted as stimulation index (SI) compared to medium value, where SI<3 was considered negative and SI≥3 positive. After transplantation, positivity in the case of incidental SIs between 3 and 5 was defined based on the pattern and frequency of autoreactivity over time, blinded from clinical outcome.

CYTOTOXIC T LYMPHOCYTE PRECURSOR (CTLP) ASSAY TO DETERMINE THE NUMBER OF ALLOREACTIVE T CELLS
The CTLp assay has been described in detail previously. Briefly, cryopreserved PBMCs from recipients from before and different time points after transplantation were cultured in a limiting dilution assay (40,000 to 625 cells/well, 24 wells per concentration) with different irradiated stimulator PBMCs expressing HLA class I antigens that are also expressed on the injected islet cell grafts (50,000 cells/well, 3 to 8 different stimulators depending on the number of donors and mismatches). Cells were cultured for 7 days at 37°C in 96-well round-bottomed plates in RPMI 1640 medium with 3 mM L-glutamine, 20 U/ml IL-2 and 10% pooled human serum. Next, Europium-labelled graft HLA-specific target cells (5,000 cells/well, 4 to 8 different targets) were added to the stimulator/responder combinations for 4 hours. Wells were scored positive if the Europium release through target cell lysis exceeded spontaneous release +3 standard deviations (SD). Quantification of CTLp frequencies was performed by computer software developed by Strijbosch et al. Cytotoxic alloreactivity in the first 26 weeks after transplantation was analyzed blinded from clinical outcome and classified initially as either low or increased, based on the CTLp frequencies against the different mismatch combinations and their evolution over time.
STATISTICS
Analysis of dichotomous data was performed by two-tailed Fischer’s exact test and χ² test. Quantitative differences between groups were analyzed by Mann-Whitney U test as well as Kruskal Wallis analysis. Correlations between quantitative variables were calculated by Spearman’s rank correlation test. Analyses were performed using GraphPad Prism (version 4.0) and SPSS (version 14.0) software. p<0.05 was considered significant.

RESULTS

CLINICAL OUTCOME
Factors possibly influencing transplant outcome were analysed with respect to two clinically relevant parameters: independence from exogenous insulin and C-peptide production over 26 weeks. C-peptide levels were significantly different between the three groups studied (Figure 1, p=0.01). Furthermore, a significant difference in achievement of insulin independence was observed between the groups (p=0.04; Table 2).

INFLUENCE OF β-CELL MASS, CELLULAR AUTO- AND ALLOREACTIVITY
In the total patient group the known predictive factors, pre-transplant cellular autoreactivity and injected β-cell mass, were significantly associated with clinical outcome of islet cell transplantation (Table 2). Post transplant cellular islet autoreactivity against GAD and/or...
**TABLE 2** Influence of immune parameters on outcome in 31 islet transplant recipients.

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Variable (n)</th>
<th>N (%)</th>
<th>p*</th>
<th>Median (range)</th>
<th>p**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin Independence</td>
<td>TAC/MMF (21)</td>
<td>13 (62%)</td>
<td>0.04</td>
<td>40.43 [3.56 – 68.63]</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>TAC/SIR (5)</td>
<td>3 (60%)</td>
<td>0.02</td>
<td>33.46 [5.12 – 51.97]</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>SIR (5)</td>
<td>0 (0%)</td>
<td>0.004</td>
<td>8.77 [2.99 – 20.91]</td>
<td>0.01</td>
</tr>
<tr>
<td>C-peptide production (AUC) over 26 weeks (wks*ng/ml)</td>
<td>All injections ≥ 2.0x10^6 β-cells/kg</td>
<td>No (10)</td>
<td>2 (20%)</td>
<td>15.22 [2.99 – 40.78]</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Yes (21)</td>
<td>14 (67%)</td>
<td>0.004</td>
<td>51.97 [2.99 – 68.63]</td>
<td>0.01</td>
</tr>
<tr>
<td>Pre transplant cellular autoactivity</td>
<td>no reactivity (11)</td>
<td>9 (82%)</td>
<td>0.02</td>
<td>31.18 [14.59-40.78]</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>IA2 or GAD (6)</td>
<td>4 (67%)</td>
<td>0.004</td>
<td>12.39 [3.56 – 42.79]</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>IA2 and GAD (6)</td>
<td>0 (0%)</td>
<td>0.004</td>
<td>12.39 [3.56 – 42.79]</td>
<td>0.01</td>
</tr>
<tr>
<td>Post transplant cellular autoactivity</td>
<td>no reactivity (9)</td>
<td>5 (56%)</td>
<td>0.02</td>
<td>35.45 [22.81 – 65.85]</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>IA2 or GAD (12)</td>
<td>5 (42%)</td>
<td>0.15</td>
<td>33.83 [3.30 – 68.63]</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>IA2 and GAD (6)</td>
<td>2 (33%)</td>
<td>0.15</td>
<td>26.33 [2.99 – 51.97]</td>
<td>0.74</td>
</tr>
<tr>
<td>Overall post transplant cellular alloreactivity (CTLp)</td>
<td>Low (17)</td>
<td>10 (65%)</td>
<td>0.46</td>
<td>31.75 [3.56 – 64.19]</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>High (13)</td>
<td>5 (38%)</td>
<td>0.46</td>
<td>33.83 [2.99 – 68.63]</td>
<td>0.74</td>
</tr>
<tr>
<td>% donors with high CTLp frequency</td>
<td>(30)</td>
<td>15 (50%)</td>
<td>0.03**</td>
<td>33.46 [2.99 – 68.63]</td>
<td>0.53***</td>
</tr>
</tbody>
</table>

Autoreactivity data was unavailable for eight patients, alloreactivity data for one patient. * p-values calculated by χ² test or Fischer’s exact test, ** calculated by Mann-Whitney U or Kruskal Wallis test, *** p-value calculated by Spearman’s correlation, r=-0.12. AUC: area under the curve; CTLp: cytotoxic T lymphocyte precursor assay; GAD: glutamic acid decarboxylase; IA-2: protein–tyrosine–phosphatase; MMF: mycophenolate mofetil; SIR: sirolimus; TAC: tacrolimus.

IA-2, assessed blinded from clinical outcome, did not correlate with insulin independence or C-peptide production (p=0.15 and 0.62, respectively).

Alloreactivity was analyzed by determination of graft HLA-specific CTLp frequencies as well as the percentage of islet donors inducing alloreactivity. The total number of islet donors per patient ranged from 2 to 10 (mean 6) representing 9-29 (mean 18) HLA class I mismatches.

![Coverage of islet transplant HLA class I mismatches by Cytotoxic T Lymphocyte precursor assay (CTLp).](image)

**FIGURE 2** Coverage of islet transplant HLA class I mismatches by Cytotoxic T Lymphocyte precursor assay (CTLp).
per patient (mismatches expressed on more donors were counted separately). In our CTLp analysis we were able to evaluate 79% (429 out of 542) of the HLA mismatches using extensive mismatch combinations of a large panel of HLA typed blood donors. Consequently, we covered alloreactivity to 96% of the grafts. With respect to 59% of the donors, coverage of all HLA mismatches was reached (Figure 2).

The donor HLA-specific cytotoxic T cell precursor frequency was analyzed blinded over time in the first 26 weeks after transplantation. The general pattern of overall CTLp frequencies after transplantation proved not to indicate clinical outcome in the total patient group (Table 2), nor in the TAC-MMF treated patients (Figure 3A and 19). However, in patients receiving sirolimus (TAC-SIR/SIR, n=10), a high donor alloantigen specific CTLp frequency was associated with significantly lower C-peptide production compared to patients with a low CTLp frequency (p=0.03; analysed in TAC-SIR/SIR combined).

ALLOREACTIVITY AFTER SECOND TRANSPLANT

In patients receiving a second transplant, exposure to new β-cell antigens and foreign HLA could lead to changes in auto- and alloimmune reactivity. All patients treated with sirolimus as immunosuppression (SIR/TAC-SIR) and 10 of the 20 patients in the TAC/MMF group received a second islet infusion. In patients with an increased CTLp frequency after the second transplant under TAC-SIR or SIR immunosuppression (examples in Figure 4), a significantly lower C-peptide production was observed (p=0.02, data not shown). Additionally, only one of seven patients with an increased CTLpf became insulin independent versus eight out of 13 of the patients with stable CTLpf (p=0.07 by Fischer exact test).

Not every stimulator-target combination induced CTL alloreactivity in every patient (Figure 4; 2 out of 5 in panel A, vs. all in panel B). For this reason selective donor-specific correlations between CTL alloreactivity and clinical outcome were further assessed by calculating the
fraction of donors against which alloreactive CTLs were induced (‘targeted donors’), ranging from 0% (increased CTLp frequency against none of the donors tested) to 100% (increased

![Graph A](image1.png)

**FIGURE 4** Different patterns of CTLp frequency development after first and second islet implantation. The single lines in each graph represent different HLA mismatch-specific stimulator-responder combinations. Straight lines depict combinations specific for the first transplant only, striped lines for the second transplant only and dotted lines for both. Interpretation of cellular alloreactive pattern per patient was performed based on all mismatch combinations and blinded from clinical outcome. Shown are representative examples of patients with low CTLp before and high after second transplant (A) or with high CTLp frequency both before and after (B).

![Graph B](image2.png)

**FIGURE 5** Percentage of islet donors causing high CTLp reactivity in the first 26 weeks after islet transplantation, stratified for patients who were (n=15) or were not (n=15) insulin independent after 26 weeks. Black rounds depict patients transplanted under TAC-MMF immunosuppression, open rounds patients transplanted under TAC-SIR and grey rounds patients under SIR only. P-value TAC-MMF: p=0.35, non-TAC-MMF: p=0.03.
CTLp frequencies against all donors tested). In the total patient group, the proportion of targeted donors differed significantly between insulin-dependent and insulin-independent recipients (p=0.03, Figure 5).

**DISCUSSION**

The TAC-MMF protocol has been shown previously to result in considerable islet graft survival in our clinical trial. We recently reported a pilot study evaluating SIR alone or TAC-SIR, which resulted in poor islet graft survival compared to TAC-MMF therapy. In the current study we analyzed pre- and post-transplant immune reactivity in these three groups with different immune suppression and investigated their correlation with insulin independence and C-peptide production.

Analysis of three different immunosuppressive protocols led to a number of potentially valuable observations. First, the applied immunosuppressive regimen was associated significantly with outcome of islet cell transplantation. Furthermore, we confirmed that next to β-cell mass, pre-transplant cellular autoreactivity correlated with worse transplant outcome in the total cohort of 31 patients. Thirdly, the assessment of the alloreactive CTL response against donor HLA antigens is not an indicator of clinical outcome in the patients receiving TAC-MMF, but alloreactive CTLs may mark poor clinical outcome in patients receiving sirolimus regardless of combination with tacrolimus. However, as this association was significant only when the TAC-SIR and SIR patient populations were combined, definitive conclusions regarding this matter are precluded. An increased CTL alloreactivity after second transplantation correlated with clinical outcome under TAC-SIR or SIR as well.

SIR monotherapy in particular led to lower C-peptide levels that correlated inversely with cytotoxic alloreactivity. Therefore the differences between the cohorts may result from the fact that monotherapy with SIR is insufficient to suppress immune reactivity after islet transplantation. However, we cannot exclude that worse islet engraftment and induction of insulin resistance, that were recently shown to be associated with SIR therapy, also affected clinical and immunological outcome. SIR monotherapy following ATG has, none the less, proven to be successful in kidney transplantation. The inadequacy of immunosuppression in the SIR only group is supported by the significantly higher numbers of CD4+ cells in this group compared to the TAC-SIR group, which confirms previous claims that CD4+ counts are reduced by calcineurin inhibitors but not by SIR. Calcineurin inhibitors have been reported to be more potent inhibitors of memory effector T cells that survive depletion regimens and therefore useful against acute rejection. Furthermore, it is known that different immunosuppressive therapies can have differential effects on antigen presentation. For instance, SIR does not inhibit major histocompatibility complex (MHC)-restricted antigen presentation whereas TAC can. Although T cells are the main targets of the calcineurin inhibitors, antigen
presentation is also affected. Production of tumour necrosis factor (TNF)-α by plasmacytoid dendritic cells (PDCs), a type of antigen-presenting cells, is inhibited by TAC which leads to an impaired T cell response.

There are various examples of the value of CTLp measurement in clinical transplantation in the islet cell transplantation setting (both islet alone and islet after kidney) as well as in bone marrow transplantation. The presence of CTL against donor HLA class I appears relevant and informative on graft function and survival in the combined SIR protocols, but not in the TAC-MMF group. Although in the separate groups the relationship between CTL measurement and clinical outcome was not significant, this lack of association seems to be mainly attributable to small group size. An alloreactive response after the second transplant correlated with a significantly lower rate of insulin independence and lower C-peptide levels. This effect again was mainly attributable to the SIR and TAC-SIR groups. Patients treated with TAC and MMF may harbour alloreactive CTLs, but the applied immunosuppression apparently appears capable of suppressing these sufficiently in vivo. This would also explain why two patients with increased CTLp frequencies to all donors still managed to achieve insulin independence. None the less, highly avid CTL may prove particularly important, as has been shown in pre-transplant and post-transplant renal and cardiac transplantation settings.

The combined usage of TAC and MMF as maintenance therapy appears the best option to cope with alloreactivity, as it leads to good clinical outcome regardless of the presence of alloreactive CTLs and often without a need of a second islet transplant. Both TAC-MMF and TAC-SIR contain a calcineurin inhibitor reported to be diabetogenic. Both drugs also contain a cell cycle inhibitor (inhibiting mTOR and mycophenolate, respectively). However, in contrast to MMF, SIR has potential side effects that may limit its feasibility in islet cell transplantation, as it was shown to impair islet engraftment. Furthermore, sirolimus-based therapies are associated with considerable side effects that largely disappear after conversion to TAC-MMF. Yet, even though SIR is not well tolerated by the patients, it may aid the generation of regulatory T cells.

In conclusion, ATG induction and TAC-MMF maintenance therapy seem effective to counter alloreactivity in islet cell transplantation, apart from their limited effect on pre-existent autoimmunity. Donor-specific CTL alloreactivity may be related to islet graft function and clinical outcome, especially in patients receiving SIR or TAC-SIR. The determination of alloreactivity may be useful to predict or guide safe tapering of immunosuppression, but future studies are required to define its feasibility.
REFERENCES


33. Roelen D, Datema G, van Bree S, et al. Evidence that antibody formation against a certain HLA alloantigen is associated not with a quantitative but with a qualitative change in the cytotoxic T cells recognizing the same antigen. *Transplantation* 1992;53:899-903