Consequences of Reducing Immunosuppressive Burden in Islet Transplantation
Chapter 5a

Immune Responses against Islet Allografts during Tapering of Immunosuppression - A Pilot Study in 5 Subjects


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ABSTRACT

Transplantation of isolated islet of Langerhans cells has great potential as a cure for type 1 diabetes but continuous immune suppressive therapy often causes considerable side effects. Tapering of immunosuppression in successfully transplanted patients would lower patients' health risk.

To identify immune biomarkers that may prove informative in monitoring tapering, we studied the effect of tapering on islet auto- and alloimmune reactivity in a pilot study in five transplant recipients in vitro. Cytokine responses to the graft were measured using Luminex technology. Avidity of alloreactive cytotoxic T Lymphocytes (CTL) was determined by CD8 blockade. The influence of immunosuppression was mimicked by in vitro replenishment of tacrolimus and MPA, the active metabolite of mycophenolate mofetil.

Tapering of tacrolimus was generally followed by decreased C-peptide production. T-cell autoreactivity increased in four out of five patients during tapering. Overall alloreactive CTL precursor frequencies did not change, but their avidity to donor mismatches increased significantly after tapering (p=0.035). In vitro addition of tacrolimus but not MPA strongly inhibited CTL alloreactivity during tapering and led to a significant shift to anti-inflammatory graft-specific cytokine production.

Tapering of immunosuppression is characterized by diverse immune profiles that appear to relate inversely to plasma C-peptide levels. Highly avid allospecific CTL that are known to associate with rejection increased during tapering, but could be countered by restoring immune suppression in vitro. Immune monitoring studies may help guiding tapering of immunosuppression after islet cell transplantation, even though we do not have formal prove yet that the observed changes reflect direct effects of immune suppression on immunity.

INTRODUCTION

Transplantation of islet cells isolated from a donor pancreas is a promising treatment for patients with type 1 diabetes with hypoglycaemic unawareness [229,256,257]. The proportion of patients becoming independent from exogenous insulin or preserving endogenous insulin production after transplantation has increased in recent years. The vast majority of transplant recipients show graft function and experience a significant decrease of serum glucose variation which ameliorates their quality of life [135,245]. Nevertheless, the need for continuous immunosuppressive therapy to prevent graft rejection and recurrence of autoimmunity imposes a heavy burden by impairing functionality of the immune system in general. This may lead to opportunistic infections, post-transplant lymphoproliferative disease and increased risk of malignancy [87,207,279]. Additionally, the functionality of regulatory T-cells promoting long-term graft acceptance may be impaired, while several drugs used in clinical practice affect islet engraftment, revascularization and beta-cell function and development [131,230,243,321]. Eventually these disadvantages may be more detrimental to the patient's condition and quality of life than regular intensive insulin treatment. In the European JDRF Center for Beta Cell Therapy in Diabetes, immunosuppressive medication is therefore routinely tapered off in recipients starting at least one year after transplantation.

Measures of auto- and alloreactivity may change upon tapering of immunosuppression, either by increased reactivity or ideally by the induction of operational tolerance. While demonstrated in kidney and liver transplantation [201,244], operational graft tolerance in the absence of immunosuppression has not yet been described in islet transplantation, where tolerance to both auto- and alloreactivity must be achieved. An additional challenge in islet transplantation is that no validated direct measure exists to monitor graft rejection, which is vital for controlled induction of tolerance and not to lose the graft [200]. We previously demonstrated that β-cell mass and autoimmune T-cell reactivity determine clinical outcome in patients transplanted under anti-thymocyte globulin (ATG) induction and tacrolimus/ mycophenolate mofetil (MMF) maintenance immunosuppression [124]. Alloreactive cytokine production in response to islet donor HLA was associated with outcome [127], while graft-specific cytotoxic T-cells (CTL) only proved an independent correlate of graft failure in immunosuppressive regimes containing sirolimus [234]. In case of kidney transplantation, the amount of CTL with high avidity for donor HLA (resistant to in vitro CD8 blockade) was able to discern permissible from non-permissible HLA mismatches and may prove more informative than the total amount of alloreactive CTL with regard to graft loss [233].

The aim of the current pilot study was to identify immune parameters correlating with islet graft function in islet transplantation during tapering of immune suppressive therapy that may guide tapering in the future. A pilot cohort of five patients transplanted under ATG - tacrolimus/ MMF immunosuppression in whom immunosuppression was tapered differentially was selected. The patterns of auto- and alloreactive parameters during tapering were evaluated blinded from clinical outcome and beta-cell function and subsequently related to clinical and metabolic follow-up.
Chapter 5A

MATERIALS AND METHODS

PATIENTS AND CLINICAL FOLLOW-UP

Five patients were studied in whom tacrolimus was tapered off >52 weeks after transplantation and graft function was monitored according to the approved protocol [124]. All patients received islet cell grafts characterized for cellular composition as described before [135] and were transplanted after ATG induction and under tacrolimus/ MMF maintenance immunosuppressive therapy. The patients have all been part of the cohort previously studied for immune factors influencing graft survival in the first year after transplantation [124]. ATG (Fresenius, Fresenius Hemocare, WA, USA) was given as a single infusion of 9 mg/kg and subsequently at 3 mg/kg for 6 days except when T-lymphocyte count was under 50/mm³. Tacrolimus (Prograf, Fujisawa/Pharma Logistics) was dosed according to trough level: 8-10 ng/ml in the first three months post transplantation, 6-8 ng/ml thereafter. MMF (Roche) was initially dosed at 2000 mg/day and had already been decreased at the end of the first year in some patients (clinical and immunological patient characteristics in week 0-52 after transplantation are shown in Table 5.1). Patients continued to be followed up regularly regarding plasma C-peptide level (at glycaemia between 6.7-16.6 mmol/l) and %HbA1c. If two consecutive HbA1c measurements exceeded 7.0%, insulin treatment was reintroduced.

FOLLOW-UP OF CELLULAR AUTOACTIVITY

Cellular immune responses were determined blinded from clinical results. Cellular autoreactivity was determined at regular intervals during tapering, as described before [242]. Briefly, 150,000 fresh PBMC well were cultured in 96-well round-bottom plates in Iscove’s Modified Dulbecco’s Medium with 2 mmol/l glutamine (Gibco, Paisley, Scotland) and 10% pooled human serum in the presence of antigen, IL-2 (35 U/ml) or medium alone in triplicates. After 5 days [H-thymidine (0.5µCi per well) was added and [H]-thymidine incorporation was measured after 16 hours on a beta-plate counter. Antigens analyzed included IA-2 (10µg/ml), GAD65 (10µg/ml), insulin (25µg/ml) and tetanus toxoid (‘third party’ antigen, 1.5 LF/ml). Results were interpreted as stimulation index (SI) compared to medium value.

All samples were tested for islet cell autoantibodies (ICA), autoantibodies against insulin (IAA), IA-2 protein (IA-2A) and glutamate decarboxylase (GADA), as described before [65]. Briefly, ICA were determined by indirect immunofluorescence and end-point titers expressed as Juvenile Diabetes Foundation (JDF) units. IAA, IA-2A and GADA were determined by liquid phase radiobinding assays, and expressed as percent tracer bound. Cutoff value determination amounted to ≥12 JDF units for ICA, ≥0.6% tracer binding for IAA, ≥0.44% for IA-2A and ≥2.6% for GADA.

FOLLOW-UP OF ALLOACTIVITY

The CTLp assay to determine CD8+ T-cell mediated cytotoxic alloreactivity was described before [34]. For this study the assay was performed on cryopreserved PBMC drawn from patient before, during and after tapering. Briefly, PBMC were cultured in a limiting dilution assay (40,000 to 625 cells/well, 24 wells per concentration) with

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<th>Table 5.1. Patient characteristics. Tx: transplantation.</th>
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<td>Median Tacrolimus level (ng/ml) 0-12 months</td>
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<td>Median MMF dosage (mg/day) 0-12 months</td>
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<td>Pre-transplant cellular autoimmunity</td>
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<td>Average plasma C-peptide (mmol/l) 0-52 weeks</td>
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three to four different irradiated stimulator PBMC expressing HLA-class I antigens also expressed on injected beta-cell grafts (50,000 cells/well). Cells were cultured for 7 days at 37°C in 96-well round-bottom plates in RPMI 1640 medium with 3 mmol/l L-glutamine, 20 U/ml IL-2 and 10% pooled human serum. All culture plates were split in two at day 7 and supplemented with either culture medium (for total CTLp frequency) or culture medium + anti-CD8 (3.0µg/ml final concentration) for 1 hour (for high avidity CTLp frequency). Next, Europium-labelled graft HLA-specific target cells (5,000 cells/well) were added for 4 hours. Wells were scored positive if the Europium release through target cell lysis exceeded spontaneous release +3 SD. Quantification of CTLp frequencies was performed by computer software developed by Strijbosch et al. [274].

For MLC experiments determining graft-specific CD4+ T-cell mediated proliferation the same stimulator and responder cells were used as for CTLp assays. One-way MLC were set up in triplicates in 96-well V-bottom plates (Costar, MA, USA) in 150µl RPMI with 2 mmol/l L-glutamine (Gibco, Paisley, Scotland) and 10% pooled human serum. Responder cells (40,000) were incubated with 50,000 irradiated (3000 rad) stimulator cells per well at 37°C/5%CO₂. After 5 days, [H]-thymidine (1.0µCi per well)
was added and $^3$H-thymidine incorporation was measured on a betaplate counter after 16 hours. Proliferation in response to phytohaemagglutinin was used as positive control. Results were interpreted as stimulation index (SI) compared to background value (responder only + stimulator only). The fraction of recipient-donor mismatches that was tested is shown per patient in Table 5.1.

To test the influence of immunosuppression on in vitro alloreactivity, mycophenolic acid (MPA, the active metabolite of MMF) and/or tacrolimus was added in CTLp and MLC assays at 250 ng/ml and 0.3 ng/ml, respectively. These concentrations were determined on basis of earlier experiments [236] as well as titration experiments using mismatched healthy panel donor PBMC as stimulator and responders. Such titrations were essential, since MPA levels used in vitro and found in human serum reported in the literature differ greatly [103,172,177]. In each subsequent experiment, a healthy donor control sample was included to validate the inhibitory capacity.

Screening for the presence of HLA-class I and class II-specific antibodies was performed on all available samples by ELISA (LAT class I&II, One Lambda, CA). When positive, the specificity of HLA-class I antibodies was determined by complement-dependent cytotoxicity (CDC) assay against a selected panel of 52 HLA typed donors.

**ANALYSIS OF CYTOKINE PRODUCTION IN MLC**

Production of different cytokines was measured with Luminex technology using a human Th1/Th2 Bio-plex cytokine kit (BioRad, Veenendaal, the Netherlands), including IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-13, GM-CSF, IFNγ and TNFα, according to the manufacturer’s protocol.

**STATISTICAL ANALYSIS**

Friedman test was used to analyze paired observations of multiple groups with correction for multiple comparisons by Dunn’s post-hoc test. For normally distributed data (proven by D’Agostino & Pearson omnibus normality test), repeated measures ANOVA with Bonferroni’s test for multiple comparisons was used. Statistical analyses were performed using GraphPad Prism version 5.01. p<0.05 was considered significant.

**Figure 5.1. Overview of clinical and immunological parameters of five islet cell transplant recipients during tapering of immunosuppression.** Shown are immunosuppression levels: tacrolimus trough level (green dots), MMF dosage (blue dotted line); Plasma C-peptide levels (black squares) and period of insulin use (gray bar); Cellular autoimmunity: stimulation index of autoreactive proliferative response to IA-2 (green squares) and GAD (blue open circles); Total cellular alloreactivity: CTLp frequency in response to 3-4 different graft specific stimulator cells; Highly avid cellular alloreactivity: CTLp frequency after addition of anti-CD8; Antibodies: serum levels of allo-antibodies (black squares) and auto-antibodies ICA (red inverse triangles), IAA (gray diamonds), GADA (blue circles), IAZA (green triangles).
RESULTS

GRAFT FUNCTION DURING TAPERING

Three out of five patients studied started tapering of tacrolimus at 50 weeks after transplantation while insulin independent and with stable graft function (Figure 5.1). One patient required exogenous insulin but had stable graft function (C-peptide >0.17 nmol/l) and started after 70 weeks. The fifth patient (MR) started tapering 125 weeks after transplantation, with only minimal detectable C-peptide levels and on complete insulin replacement therapy.

Tacrolimus trough levels were decreased by at least 67% in all patients. MMF was decreased partially in two patients (VMW, BM) and stopped in MR, which was after the last immunologically analyzed time point. All patients with remaining graft function displayed a gradual decrease in plasma C-peptide levels and had to resume insulin injection during the tapering period (week 91, 106 and 79 for VMW, SV and VF respectively). This pattern largely followed the decrease in tacrolimus trough levels (Figure 5.1). Three patients had preserved beta-cell function more than two years after start of tapering.

IMMUNE RESPONSES DURING TAPERING

Immunological follow-up during tapering showed differential patterns between patients (Figure 5.1). Four patients showed profound cellular autoimmune reactivity to GAD and/or IA-2, even though these changes did not accurately mirror changes in immune suppressive therapy. In the fifth patient (having least residual beta-cell function at the start of tapering of immune suppression) no increases in autoimmune responses were observed. No significant changes in in vitro immune reactivity were observed in response to whole insulin and tetanus toxoid (not shown). One patient (VF) showed a sudden increase in islet and GAD autoantibodies halfway throughout the tapering period, approximately half a year after peaking cellular autoimmune responses.

Overall, alloreactive CTLp frequencies increased in two patients (VF and BM) during tapering to at least one donor specific stimulator and decreased in two other patients (VMW and SV) to donor specific stimulators with an initially high response. In patient MR CTLp frequencies peaked to one stimulator, but declined thereafter. Highly avid (CD8 co-stimulation independent) CTLp frequencies roughly followed total CTLp frequencies. No alloantibodies were observed throughout the observed period.

In individual patients, changes in graft function followed changes in immune reactivity. In patient VMW decrease in C-peptide was accompanied by cellular autoimmune responses to IA-2 and later also GAD. High alloreactive CTLp frequencies preceded reduction in graft function. In patient SV high CTLp (including highly avid CTLp) frequencies preceded the fall in C-peptide production. Patient VF first had high cellular autoimmune to IA-2 and alloreactive total CTLp frequencies, followed by increased highly avid CTLp frequencies and drop in C-peptide production by the graft. Thereafter a sudden rise in GAD antibodies occurred. Patient BM lost graft function after high cellular autoreactivity to IA-2 and with increasing alloreactive total and highly avid CTLp frequencies. Temporarily detectable levels of C-peptide (>0.03 nmol/l) in MR coincided with peaking cellular autoreactivity to both IA-2 and GAD as well as alloreactive total and highly avid CTLp frequencies.

All CTLp assays were performed in parallel with addition of anti-CD8 to determine the frequency of high-avidity CTL, as these T-cells display the strongest association with allograft rejection in different transplantation settings. When analyzing all stimulator-responder combinations with complete data (n=19) before, during and after tapering, no significant changes in total or highly avid CTLp frequency were observed (Figure 5.2A and B). However, the percentage inhibition by anti-CD8 significantly decreased during tapering (p=0.0013, Figure 5.2C), indicating a relatively higher proportion of highly avid alloreactive CTL. MLC proliferation and graft-specific cytokine production did not change significantly (not shown).

IN VITRO EFFECT OF IMMUNOSUPPRESSION ON ALLOREACTIVITY

To test a possible relationship between changing alloreactivity and reduced immunosuppression, tacrolimus and/or MPA were added in pharmacological concentrations in vitro. These experiments were performed on stimulator-responder combinations that previously displayed high levels and clear changes in CTLp frequency over time.

Tacrolimus monotherapy and tacrolimus plus MPA were able to reduce alloreactive donor HLA-class II-restricted mixed lymphocyte reaction (MLC), CTLp and highly avid CTLp frequencies in the selected patient versus donor specific graft combinations (Figure 5.3). MPA alone seems less able to do so. Tacrolimus effectively reduced T-cell responses in vitro to pre-tapering levels.

Tacrolimus or tacrolimus and MPA combined, but not MPA alone, effectively decreased IL-2, IFNγ and IL-13, but increased IL-10 and TNFα production in response to alloantigens of the islet donors throughout tapering in all five recipients.
**Consequences of Reducing Immunosuppressive Burden in Islet Transplantation**

Chapter 5A

Figure 5.3. Effect of addition of tacrolimus and/or mycophenolic acid on CD4+ T-cell proliferation (A), CD8+ CTLs (B) and highly avid CTLs (C) in high responsive patient and graft-specific stimulator combinations. Data represent stimulator-responder combinations of five patients before, during (median if multiple values) and after tapering of immunosuppression (mean+SEM) without addition of immunosuppression (○), with tacrolimus (■), with mycophenolic acid (▲) or tacrolimus and mycophenolic acid (▼). *p<0.05, **p<0.01, ***p<0.001 by Friedman test. In all significant tests with p<0.05, the difference between no immunosuppression and tacrolimus/mycophenolic acid remains significant after Dunn’s correction for multiple comparison.

(5A)

**DISCUSSION**

In this pilot study we comprehensively investigated auto- and alloimmune responses in the cellular and humoral immune compartment during tapering of immunosuppression in five islet transplanted patients. The patients received similar islet grafts under the same immunosuppressive protocol, but they were heterogeneous with respect to timing and indication for tapering. We aimed to identify immune correlates that in the future may be validated as biomarkers to guide tapering of immunosuppression. Different patterns of immune reactivity were observed that in each case could explain changes in graft function. In vitro experiments indicate that tacrolimus is a potent inhibitor of alloreactivity, which may partly function by shifting immune reactivity to more anti-inflammatory responses, and tapering does not impair its suppressive capacity.

A large variety of factors may influence long-term islet graft survival. These factors can be both auto- and alloimmune mediated as well as beta-cell related (e.g. to graft composition, insulin-producing capacity and graft size) [5,136,245,273]. Furthermore, immunosuppression itself can be unfavorable for islet cell engraftment [321] and function [131,230]. However, the trend of tacrolimus trough levels changing with plasma C-peptide levels that was observed in the current study is intriguing and warrants substantiation, as it suggests a role for tacrolimus to protect beta-cell function.

Different patterns of alloreactivity developed during tapering that followed C-peptide levels and tacrolimus trough levels in most patients. CTLp frequencies seem to rise in case of minimal residual graft function. Decreases in CTLp frequencies with

Figure 5.4. Graft-specific production of cytokines after addition of tacrolimus and/or mycophenolic acid. Data represent IL-2, IFNγ, IL-13, IL-10 and TNFα production by PBMC of five patients in high responsive patient and graft-specific stimulator combinations before, during (median if multiple values) and after tapering of immunosuppression (mean+SEM) without addition of immunosuppression (○), with tacrolimus (■), with mycophenolic acid (▲) or tacrolimus and mycophenolic acid (▼). *p<0.05, **p<0.01, ***p<0.001 by Friedman test. For all comparisons significant differences remain after Dunn’s correction for multiple comparison: for IL-10, pre and post tapering; no immunosuppression - tacrolimus and mycophenolic acid - tacrolimus; during tapering: mycophenolic acid - tacrolimus/mycophenolic acid; for IL2, pre tapering: no immunosuppression - tacrolimus - tacrolimus; during tapering: no immunosuppression - tacrolimus and mycophenolic acid - tacrolimus; post tapering: mycophenolic acid - tacrolimus/mycophenolic acid; for TNFα, during tapering: no immunosuppression - tacrolimus.

Tapering have previously been reported for tapering of MMF in kidney transplantation and might be explained by specific T-cell apoptosis, regulation or migration of cells to the tissue [293]. This last mechanism could cause destruction of the graft. Conversely, the rise in CTLp frequencies in minimal residual graft function might be because of increased recognition of beta-cells by upregulated HLA in beta-cell stress [261]. The different patterns observed between patients may explain why the frequency of CTL did not change significantly when patients were analyzed collectively. Yet, their increasing avidity implies that the quality of the reaction may be more important than the actual frequencies of alloreactive CTL confirming earlier data in heart and corneal transplantation [202,235] and should be taken into account when further investigating immunity for guidance of tapering. Tapering of immunosuppression did not lead to sensitization with alloantibodies, in contrast to what has been reported in other studies and could have detrimental consequences for later kidney transplantation [45,227]. While our screening strategy for alloantibodies may have missed low level donor-specific alloantibodies, the vast majority of clinically relevant alloantibodies will be detected.

Autoimmune T-cell reactivity may be insufficiently affected by immunosuppressive strategies currently employed in clinical islet transplantation [124]. In this study, autoreactivity before tapering did not invariably lead to increasing autoreactivity thereafter. However, clear increases in autoreactivity were observed in most patients and may have contributed to loss of graft function during tapering. These findings are in line with concerns
that current immunosuppressive protocols might insufficiently affect established autoimmunity in pre-immunized individuals, such as those suffering autoimmune conditions [118,124,305]. Increase in autoantibodies was seen in one patient only after the patient had lost most graft function and therefore seems to fall short as candidate biomarker.

The relationship between high or increasing alloreactive T-cell responses against islet donors with decreasing immunosuppression was assessed by in vitro addition of immunosuppressive agents.

In most patients tacrolimus was tapered primarily. The tacrolimus concentration elected in vitro (0.3 ng/ml) resembles 6 to 15 ng/ml trough levels in vivo, since 95 to 98% of tacrolimus is bound to erythrocytes in the circulation [187,236]. This concentration of tacrolimus could inhibit most of the alloreactivity at any stage of tapering regardless of addition of MPA, the active metabolite of MMF. The MPA concentration in vitro (250 ng/ml) mimicked the in vivo effective concentration (1 g MMF twice a day giving trough levels of 2.5-4.5µg/ml and 97% albumin bound) [170], but by itself did not inhibit alloreactive T-cell responses as potently as tacrolimus. This may be partly explained by the minimal tapering of MMF in vivo, retaining its effect in PBMC when tested in vitro. We speculate that alloreactivity would have remained at baseline levels in those patients if tacrolimus had not been tapered. The stable inhibitory capacity throughout tapering further suggests that any increasing alloimmune responses may be countered by returning to full immunosuppression, in spite of the increased avidity of donor-specific CTL supports. Functionally, tacrolimus reduced IL-2 production by lymphocytes responding to islet donor alloantigen, as expected [97,104]. Interestingly, tacrolimus reduced other pro-inflammatory cytokines (IFNγ, IL-13), but increased anti-inflammatory IL-10, as well as TNFα that has been reported to contribute to tissue specific immune regulation, pointing to a pro regulatory T-cell effect endorsed by tacrolimus [122,144,253].

Tapering of immunosuppression in transplantation is presently guided mainly by clinical markers of sustained graft function since immune markers for long-term clinical graft acceptance are not sufficiently reliable [246]. Tapering management could be improved by earlier detection of processes leading to deteriorating graft function. In islet cell transplantation, both auto- and alloreactivity need to be inhibited and should therefore be monitored. With the limited and varied population, no clear predictors of loss of C-peptide could be identified so far. Knowledge on long-term graft acceptance after tapering may increase by assessment in larger patient cohorts and analysis of other immune response markers such as autoreactive cytotoxic T-lymphocytes [302] and different types of regulatory T-cells [154,288,309]. The current data suggest that tapering of tacrolimus leads to differential changes in immune reactivity with an increasing role for high avidity alloreactive CTL, any of which may cause loss of graft function. More extensive research is needed to verify which (immune) parameters will be useful to guide tapering of immune suppression in order to achieve a stable state where both loss of graft function due to immunological causes and the burden of immunosuppressive medication are minimal.