T cell immunity to islets of Langerhans : relevance for immunotherapy and transplantation to cure type 1 diabetes

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CHAPTER 4B

Baseline factors associated with insulin independence and metabolic control in islet cell transplantation

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ABSTRACT

OBJECTIVE

To identify patient characteristics that are associated with less optimal posttransplant (PT) function and metabolic outcome after islet transplantation in type 1 diabetes.

RESEARCH DESIGN AND METHODS

We performed a retrospective analysis of base-line factors that may affect implant function, glycemic variability and gain of insulin-independence during the first 6 months after the initial islet transplantation. The patient cohort consisted of 30 non-uremic type 1 diabetic patients that received ATG induction and tacrolimus and mycophenolate mofetil maintenance immune suppression and 1-2 cultured β-cell grafts that were standardized in terms of purity and contained virtually no dead and acinar cells. Parameters that were found significantly associated in univariate analysis were included in a multivariate model.

RESULTS

Univariate analysis identified pre-ATG total lymphocyte count, T- and B-lymphocyte count and T-cell reactivity against islet autoantigens as factors that are negatively associated with gain of insulin-dependence, glycemic stability and β-cell graft function. Patients that did not become insulin-independent PT had significantly higher base-line total lymphocyte counts (p=0.007), T-lymphocyte counts (p=0.049), B-lymphocyte counts (p=0.023), and T-cell reactivity against islet autoantigens (p=0.046) than patients that became insulin-independent. Multivariate analysis maintained B-lymphocyte count and T-cel reactivity against islet autoantigens as independent factors associated with insulin independence (p=0.03; and p=0.06; respectively). During the first week PT, patients that did not become insulin-independent had significantly higher absolute B-lymphocyte counts (p=0.04) but not T-lymphocyte counts.

CONCLUSIONS

Higher baseline B- and T-lymphocyte counts and T-cell reactivity against islet autoantigens are associated with a less favourable short-term clinical outcome of islet cell transplantation. Prospective studies are under way that examine efficacy and safety of concomitant B-lymphocyte targeted therapy at initial implantation.

INTRODUCTION

Allogeneic islet cell tranplantation is since long considered a promising treatment for type 1 diabetic patients[1,2]. Insulin independence can be achieved during the first year posttransplantation in up to 80 percent of carefully selected patients in small, single center cohorts[3-7]. However, in multicenter trials[8,9] and studies including a larger number of patients[10] insulin independence is less frequently obtained. Several factors can account for this variability. Their identification is hindered by the difficulty in standardizing the protocols and by the small numbers of patients that have so far been included per protocol. Within these limitations, graft and recipient characteristics have been related with the outcome of clinical islet cell transplantation[10-13]. A minimal donor tissue mass was reported to induce insulin-independence but is in itself not sufficient[3,10,13]; administration of more potent immune suppressants can lower this treshold[14,15] which is lowest in autologous transplantation[16]. Using cultured β-cell preparations in an ATG-based protocol, we defined the minimal number of β-cells that reproducibly resulted in circulating signs of a surviving graft two months after transplantation[17]. In the latter study, achievement of insulin-independence also depended on the β-cell mass in the graft but appeared counteracted by the presence of an islet-specific autoreactivity as measured by lymphocyte stimulation tests against the islet autoantigens GAD and IA-2[18]. We have now analysed a cohort of thirty recipients for baseline characteristics that correlate with the clinical outcome of defined islet cell grafts under the same ATG-based protocol. Our findings extend the influence of the baseline immune status: in addition to the confirmed correlation between a less functional implant and an islet-specific autoreactivity, the present data show that this correlation also exists with higher T- and B-lymphocyte counts.

MATERIALS AND METHODS

GRAFT RECIPIENTS AND BASELINE CHARACTERISTICS

Between september 2000 and january 2006, 35 non-uremic type 1 diabetic patients received an islet β-cell transplant under ATG induction therapy and maintenance immune suppression with mycophenolate mofetil (MMF) and tacrolimus. They were all C-peptide negative, had large within-subject variation of fasted glycemia (CVfg) and one or more signs of diabetic lesions (hypoglycemic unawareness, microalbuminuria, retinopathy). The first twenty four patients had been included in a phase 1 dose finding study and the last eleven patients in a protocol that aims to assess influence of tapering of tacrolimus after month 12. Graft survival with this immune suppressive regimen was previously reported for the first 24 patients[17,18]. Informed consent had been obtained from all candidate recipients before they were listed as such by the Eurotransplant Foundation. Selection for transplantation occurred on basis of listing date, bloodgroup compatibility with the available graft and health status. At the time of transplantation, none presented

symptoms of acute infectious disease or inflammation. Analysis for CMV (PCR and serology) and Hepatitis A, B and C (serology) at baseline excluded active disease. Two patients tested positive for complement binding HLA antibodies pretransplantation, two patients discontinued immune suppression during the first 6 months and one patient died from a cerebral haemorrhage at 18 weeks PT. These five patients were excluded from the current analysis.

GRAFT CHARACTERISTICS AND TRANSPLANTATION PROCEDURE

Islet cells were isolated and cultured according to standardized protocols[17,19,20]. Preparations were analysed for their cellular composition and combined to grafts containing minimally 0.7 million β-cells per kg recipient bodyweight (BW). Grafts were infused into the portal vein using either a laparoscopic (n=16)[21] or subcutaneous transhepatic approach (n=14)[22]. Donor and graft characteristics are listed in Appendix 1.

IMMUNE THERAPY

Induction therapy consisted of rabbit ATG (Fresenius HemoCare, Redmond, WA) initiated 1-4 days before transplantation. A first dose of 9 mg/kg bodyweight was followed by 3 mg/kg for 6 days; no injection was given on days with a T-lymphocyte count under 50 cells/mm^3. Maintenance immunesuppression consisted of MMF (Cellcept, gift of Roche, Brussels, Belgium 2000 mg/d starting on the day of the first ATG injection) and Tacrolimus (Prograft, Fujisawa starting one day before the last ATG injection). Tacrolimus troughlevels were maintained between 8 and 10 ng/ml during the first 3 months after an islet cell transplantation and 6-8 ng/ml thereafter. This MMF-tacrolimus treatment was continued when a second islet cell transplant was given, without additional antibody course, but with one injection of 500 mg methylprednisolone 3 hours before transplantation.

ASSESSMENT OF BASELINE CHARACTERISTICS AND TRANSPLANTATION OUTCOME

Efficacy and safety criteria were examined weekly during the first 6 weeks PT, every two weeks between PT week 6 and 12, and monthly thereafter. The CVfg was assessed using home glucose monitoring. Plasma C-peptide (TRFIA, Perkin-Elmer, Turku, Finland) with corresponding glycemia, HbA_{1c} levels (HPLC; Pharmacia Biotech, Upsala, Sweden) and autoantibodies (islet cell antibody [ICA], insulinoma antigen 2 antibody [IA2A], glutamic acid decarboxylase antibody [GADA], insulin antibody [I(A)A]) were assayed in the central laboratory of the Belgian Diabetes Registry[23]. There was also a central measurement of lymphocyte subsets $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$, and NK cells ($CD3^-CD16^+CD56^+$) (EpicsXl flow cytometer, Beckman Coulter, Miami, FL). Total lymphocyte counts were determined by routine haematology in local centers. Baseline characteristics of graft recipients are shown in appendix 1. The majority of patients (n=24) were treated with a subcutaneous insulin pump for at least 2 months prior to transplantation. Insulin tapering was only considered in patients with plasma C-peptide values ≥ 1.0 ng/ml (at glycemia 120-220 mg/dl), CVfg < 25% and mean fasting glycemia <

125 mg/dl and was started after month 2 at a rate of minus 2IU every 3-5 days or faster when patients experienced hypoglycaemic episodes (< 70 mg/dl).

LYMPHOCYTE STIMULATION TEST TO DETERMINE BASELINE AUTOREACTIVITY AGAINST ISLET CELL ANTIGENS

Baseline T cell autoreactivity to islet cell antigens was assessed at the Leiden University Medical Center and data analysed blinded from clinical outcome. Blood was drawn before the first ATG administration, peripheral blood mononuclear cells (PBMCs) isolated and processed as described before[24]. Briefly, 150.000 fresh PBMCs were cultured in triplicate 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 presence of islet autoantigens IA-2 (10 µg/ml) or GAD65 (10 µg/ml), of IL-2 (35U/ml) or of medium alone. After 5 days, ^{3}H-thymidine (0.5 µCI per well) was added and its incorporation measured after 16 hours. Data were expressed as a stimulation index (SI) by comparison with the medium alone value. An SI ≥ 3 for any of the two antigens was considered as a sign of T cell autoreactivity against an islet cell antigen. In three patients cellular autoreactivitiy could not be assessed because autoantigens were not available for testing of baseline samples.

HISTOLOGY

On the occasion of a third islet infusion by laparoscopy 60 weeks after initial transplantation, a liverbiopsy was taken from a visible steatotic lesion on the liver surface. Immunohistochemistry was performed on semi-consecutive paraffin-embedded sections using insulin, CD3 and CD20 directed antibodies. Staining with monoclonal antibodies against CD3 (NeoMarkers, Freemont, Ca, USA) and CD20 (DAKO, Glostrup, Denmark) was performed with an automated immunostainer (Nexes, Ventana, Tuczon, AZ, USA). Tonsil was used as control tissue. For the detection of insulin, manual staining was performed using a polyclonal AB (gift of prof. Dr C. Van Schravendijk, Vrije Universiteit Brussel, Belgium, 1/10000). Normal pancreas was used as control. For epitope retrieval, the sections were heated to 98°C with citrate buffer, pH 6.0.

STATISTICS

All values are expressed as median and interquartile range (IQR) unless indicated otherwise. Baseline and postransplant characteristics were related with status of insulin-independence at month 6 as well as β-cell graft function and glycemic variability during the first 6 months. To assess differences between subgroups we used non-parametric Mann-Whitney U test for continuous data and Fisher exact test for categorical data. Correlations between baseline characteristics and CVfg or mean C-peptide during the first 6 months after transplantation were assessed by calculating Pearson's correlation coefficient.

To determine independent predictor ability of the variables we used forward stepwise binary logistic regression analysis for insulin independence and a stepwise linear regression

model for both CVfg and mean C-peptide. The analysis was performed on twenty seven subjects. Three subjects could not be included because of missing data. In our multivariate model we included parameters with p value <0.05 in univariate analysis.

All analysis were performed using SPPS (version 16.0), graphics were computed by GraphPad Prism (version 4.0). All reported P values are two sided and p<0.05 was considered significant.

RESULTS

METABOLIC OUTCOME OF ISLET CELL TRANSPLANTATION

All thirty recipients became C-peptide positive after transplantation, but one patient returned to C-peptide negativity before PT month 6. At PT month 6, fifteen patients were insulin independent while the other fifteen were on low-dose insulin therapy (Table 1). Both groups had similar HbA_{1c} concentrations and fasting mean glucose levels levels (Table 1). However, insulin-independent recipients had significantly higher basal C-peptide levels and exhibited a lower variability of fasting glycemia (Table 1).

TABLE 1 Metabolic outcome 6 months after islet cell transplantation

	At posttransplant month 6		
	Ins. independent	**Ins. dependent**	**p***
C-peptide positive (n)	15/15	14/15	
C-peptide (ng/ml)	2.3 (1.9-3.0)	1.0 (0.4-1.2)	<0.001
Fasting glycemia			
Mean (mg/dl)	127 (115-135)	132 (127-153)	0.12
CVfg (%)	9 (8-10)	19 (15-39)	<0.001
HbA_{1c} (%)	6.1 (5.8-6.4)	6.1 (5.7-6.7)	0.66
Insulin dose (IU/kg/d)	0	0.27 (0.20-0.46)	<0.001
Percentage of baseline insulin dose (%	0	43 (34-69)	<0.001

Data represent median (interquartile range); Fasting glycemia was measured at home and CVfg was calculated during the preceding month. *Statistical analysis was done with Mann-Whitney U test

COMPARISON OF INSULIN-INDEPENDENT AND INSULIN-TREATED RECIPIENTS FOR THEIR BASELINE GRAFT AND RECIPIENT CHARACTERISTICS

No differences between both patient groups were noticed in terms of graft characteristics (Table 2). Respectively 87 and 80 percent of subjects had received at least 2 million β-cells per kg BW in the first graft in the insulin-independent and insulin-dependent group.

Baseline recipient characteristics such as age, gender, body weight-BMI, duration of disease, autoantibody-positivity, metabolic control and insulin dose were also similar (Table 2). Patients that did not become insulin-independent after islet cell transplantation tended to have higher HbA_{1c} concentrations at base-line (p=0.054).

TABLE 2 Baseline graft and recipient characteristics according to insulin need at 6 months posttransplantation

	At posttransplant month 6		
	Ins. independent N=15	Ins. dependent N=15	p*
Grafts			
β-cell number (million/kg)			
First graft	3.0 (2.2-4.2)	2.5 (2.0-2.9)	0.16
Total	4.5 (3.5-5.7)	3.7 (2.6-4.7)	0.11
Cellular composition			
β-cells	32 (21-38)	30 (20-37)	0.87
α-cells	9 (4-11)	7 (5-10)	0.34
Nongranulated cells	46 (41-63)	45 (34-62)	0.28
Acinar cells	1 (1-4) 1 (1	1 (1-5)	0.80
Dead cells	8 (6-11)	10 (7-12)	0.31
Culture time (days)	6 (4-9)	6 (4-10)	0.66
Recipient			
Age (years)	45 (41-49)	40 (32-52)	0.14
Gender (M/F)	10/5	7/8	0.46
Body weight (kg)	68 (64-74)	68 (62-78)	0.72
BMI (kg/m^2)	23 (21-26)	25 (22-26)	0.55
Duration of disease (years)	27 (21-33)	24 (17-32)	0.30
HbA_{1c} (%)	7.0 (6.5-7.9)	7.8 (7.4-8.1)	0.05
Insulin dose (IU/kg/d)	0.52 (0.39-0.74)	0.67 (0.58-0.85)	0.13
Fasting glycemia			
Mean (mg/dl)	146 (129-174)	166 (123-199)	0.65
CoV (%)	43 (38-46)	46 (35-50)	0.42
Immune status before ATG			
Total lymphocyte count	1579 (1380-1885)	2065 (1869-3005)	0.007
$CD3^+$ count (cells/mm^3)	1142 (916-1342)	1419 (1160-1867)	0.049
$CD19^+$ count (cells/mm^3)	247 (141-271)	318 (227-514)	0.023†
Leucocyte count	5300 (4400-6000)	6400 (5700-8100)	0.040
T-cell reactivity against IA2 and/ or GAD	6/13	12/14	0.046†
Positivity for ICA/GAD/IA2-A	4/8/6	2/5/7	0.65/0.46/1.0
Positivity for ≥2 autoAb	5	3	0.68

Data represent median (interquartile range); *Statistical analysis was done with Mann-Whitney U test for continues variables, Fisher exact test for dichotomous variables.†These variables were confirmed as independently associated with insulin independence by multivariate analysis (Binary Logistic Regression).

On the other hand, a significant difference was noted in the baseline immune state as expressed by the absolute number of lymphocytes, $CD3^+$ and $CD19^+$ cells, with higher initial counts in the recipient group that would not become insulin-independent after transplantation (Table 2). Of the four patients with a baseline lymphocyte count above 3000 cell/mm^3 (range 3005-3455), none became insulin-independent. More specifically, the higher number of T-lymphocytes in insulin-requiring patients was related to a higher number of $CD8^+$ lymphocytes (547/mm^3, IQR 462-657 versus 393/mm^3 in insulin-independent patients,

IQR 305-503; p=0.021) but not of $CD4^+$ T-lymphocytes (850/mm^3, IQR 742-1134 and 716/mm^3, IQR 570-936 resp.; p=0.093). There was no difference in NK cells counts (184/mm^3, IQR 147-302 vs 174/mm^3, IQR 144-271 resp.; p=0.72).

Both groups also differed in their baseline T-cell autoreactivity. This in vitro test could be performed in 27 of the 30 subjects listed in Table 2. In the group that not became insulin-independent, 12 out of 14 patients scored positive for IA2 and/or GAD65 whereas this was only the case in 6 out of 13 patients who would become insulin-independent (p=0.046; Table 2). On the other hand, no difference was detected in baseline autoantibody status or the presence of multiple autoantibodies prior to transplantation (Table 2). Of the 19 patients positive for IA2 and/or GAD65 antibodies at baseline, 10 became insulin-independent whereas this was the case for 5 out of 10 patients that were negative for these antibodies (p=1.0 by Fisher exact test). Similar results were obtained for ICA-positivity (data not shown).

UNIVARIATE AND MULTIVARIATE ANALYSIS OF ASSOCIATIONS BETWEEN BASELINE RECIPIENT AND GRAFT CHARACTERISTICS AND CLINICAL OUTCOME PARAMETERS

The observed correlations between baseline recipient and graft characteristics and the achievement of an insulin-independent state at PT month 6 were examined by multivariate analysis (Table 3). Baseline B-lymphocyte count and T-cell autoreactivity were found to be independently correlated with the ability to achieve insulin independence (OR 0.989; 95% CI 0.979-0.999 and OR 0.101; 95% CI 0.009-1.067 resp.). Of the nine patients without T cell

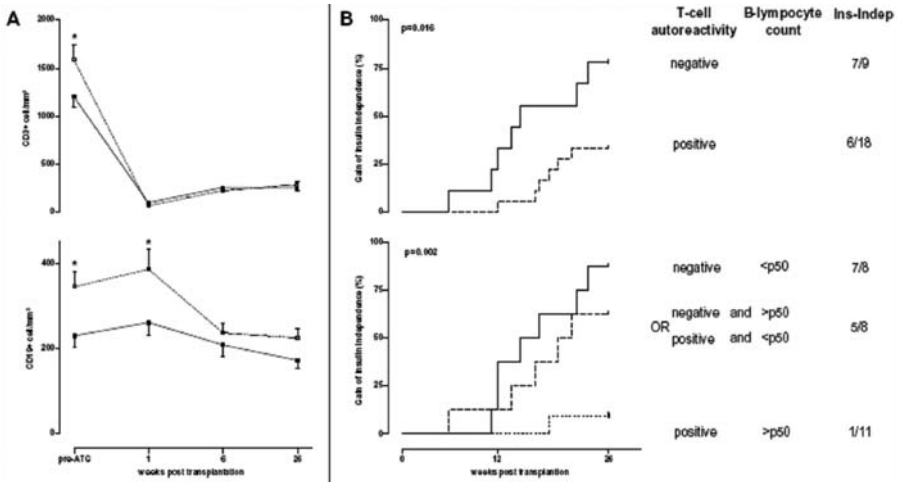

FIGURE 1 T- and B-lymphocyte count at baseline and during 6 months after transplantation (panel A) in insulin requiring (dotted line) and insulin independent (solid line) islet graft recipients. Data represent mean and SEM, * p <0.05. Panel B: Gain of insulin independence according to baseline in vitro T-cell reactivity against islet autoantigens. The lower panel shows gain of insulin independence in three different groups according to both T-cell autoreactivity and B-lymphocyte count.

TABLE 3 Univariate and multivariate analysis of recipient characteristics associated with clinical outcome

	Insulin independence		CVfg 0-6months		Mean C-peptide 0-6months	
	Univariate analysis*	Multivariate analysis†	Univariate analysis‡	Multivariate analysis§	Univariate analysis‡	Multivariate analysis§
	p	p	p	p	p	p
Age (years)	0.14		0.02	0.06	0.17	
Gender (M/F)	0.46		0.44		0.48	
Body weight (kg)	0.72		0.91		0.95	
BMI (kg/m^2)	0.55		0.86		0.83	
Duration of disease (years)	0.30		0.55		0.41	
HbA1c (%)	0.05		0.71		0.48	
Insulin dose (IU/kg/d)	0.13		0.02	0.05	0.04	0.16
Fasting glycemia						
mean (mg/dl)	0.65		0.70		0.11	
CVfg (%)	0.42		0.78		0.94	
Immune status before ATG						
Total lymphocyte count	0.007	0.21	0.001	0.001 (β 0.542)	0.006	0.001 (β -0.433)
CD3$^+$ count	0.05	0.32	0.06	0.08	0.06	0.08
CD19$^+$ count	0.02	0.03 (OR 0.989)	0.08		0.13	
Leucocyte count	0.04	0.14	0.007	0.36	0.04	0.35
T-cell reactivity against IA2 and/ or GAD	0.05	0.06 (OR 0.101)	0.01	0.009 (ß 0.408)	0.001	<0.001 (ß -0.658)
Presence of autoantibodies pre-Tx (yes/no)	0.70		0.86		0.88	
Immune status PT day 1-7						
Median CD3$^+$ count	0.38		0.82		0.76	
Median CD19$^+$ count	0.04	0.54	0.02	0.81	0.03	0.83
Median CD4/CD8 ratio	0.59		0.29		0.39	

*Mann-Whitney U test for continuous variables, Fisher exact test for categorical data; †Independent predictor ability of the variables studied by forward stepwise binairy logistic regression analysis; ‡Pearson's correlation for continuous variables and Mann-Whitney U test for categorical varaibles; §Independent predictor ability of the variables studied by stepwise linear regression analysis, inclusion criteria P<0.05.

autoreactivity at start, seven became insulin-independent whereas this was only the case for six out of the 18 patients that tested positively (Figure 1B). When these patients were further stratified according to their baseline B lymphocyte count, ie under or above the 50th percentile (259 B cells/mm^3), insulin-independence was seen in seven out of eight patients without baseline T cell autoreactivity and a B lymphocyte count <p50 while only one of the 11 patients with T cell autoreactiviy and a B lymphocyte count >p50 became insulin-independent (p=0.001 by Fisher exact test).

In univariate analysis, similar correlations were found with other clinical outcome parameters, such as the average coefficient of variation of prebreakfast glycemia (CVfg) and the mean plasma C-peptide levels during the first 6 months (Table 3). After multivariate analysis, base-

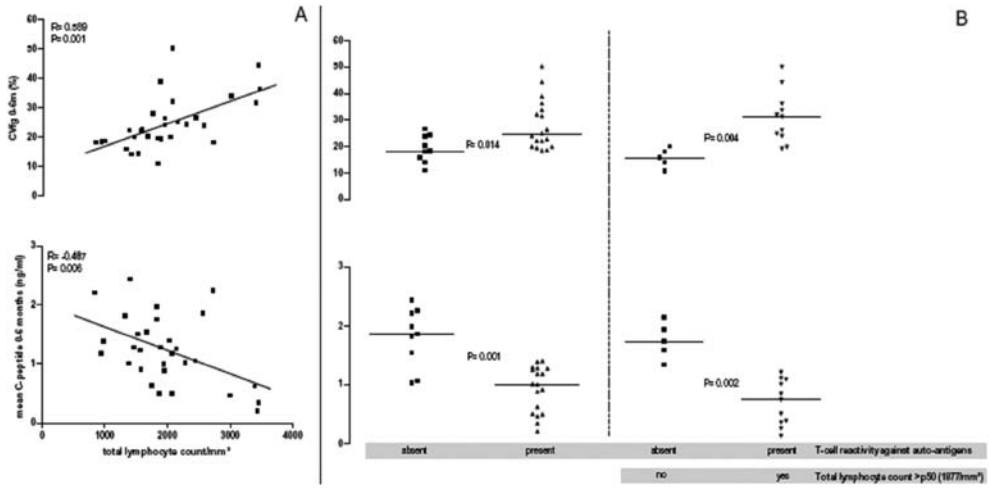

FIGURE 2 The effect of baseline total lymphocyte count (panel A), in vitro T-cell reactivity against islet autoantigens or both (panel B) on glycemic stability and C-peptide release.

line positivity for T cell autoreactivity and total lymphocyte counts correlated positively with the values of CVfg and negatively with the mean C-peptide levels (Fig 2A). Recipients with baseline T-cell reactivity had significantly higher CVfg (24%; IQR 20-35) and lower C-peptide (1.0 ng/ml; IQR 0.5-1.3) than those without (18%; IQR 15-24; p=0.014 and 1.9 ng/ml; IQR 1.3-2.2; p=0.001 resp.) (Fig 2B). These differences became more pronounced when a further stratification was made according to presence or absence of a baseline total lymphocyte count above p50. Thus, a negative test for T cell autoreactivity and a lymphocyte count <p50 was associated with a lower CVfg and a higher C-peptide, whereas a positive test and a count >p50 appeared to predispose for a high CVfg and a low C-peptide (Figure 2A and 2B). The number of β-cells transplanted per kilogram bodyweight in the first graft had a tendency to correlate with CVfg in univariate analysis (R= -0.34; p=0.07) but less with C-peptide levels during the first 6 months PT (R=0.27; p=0.15). This could not be retained when it was added to the multivariate model (p=0.56 and p=0.42 resp.). The total number of β-cells transplanted or the number of donors used did not affect CVfg nor C-peptide levels.

COMPARISON OF INSULIN-INDEPENDENT AND INSULIN-TREATED RECIPIENTS IN TERMS OF POSTTRANSPLANTATION LYMPHOCYTE COUNTS

One week after start of the immune therapy, the number of $CD3^+$ cells had markedly dropped in both groups, reaching similar low values, that were maintained till PT month 6 (Figure 1A). On the other hand, baseline $CD19^+$ counts did not decrease during the first week, thus remaining higher in the patient group that did not become insulin-independent (Figure 1A); at later time points, $CD19^+$ counts had decreased to similar levels in both groups. Total ATG dose in insulin independent patients was similar to insulin-requiring patients (21.9 mg/kg

BW IQR 20.0-24.7 and 23.6 IQR 22.0-26.2 resp. p=0.11). Mean tacrolimus troughlevels (9.2 ng/ml IQR 8.6-9.5 and 9.2 IQR 7.4-9.7) and MMF dose (2000 mg/d IQR 1500-2000 and 2000 IQR 1500-2000) during the first 6 months PT were also comparable between both groups (p=0.66 and p=0.45 resp.). Similar results were obtained in relation to CVfg and mean C-peptide levels during the first 6 months (data not shown).

HISTOLOGICAL EVIDENCE FOR THE PRESENCE OF B-LYMPHOCYTES IN ISLET CELL GRAFTS (FIGURE 3)

Insulin staining on the liver biopsy identified one islet in a portal tract. Inflammatory cells were located around the islet and consisted predominantly of $CD20^+$ lymphocytes. $CD3^+$ lymphocytes are also present but far less abundantly. A few $CD3^+$ cells were present along blood vessels, within the islet itself, but the $CD20^+$ lymfocytes remained localized in the peri-islet region in a cluster-like structure. This patient had received two islet infusions with a total of 2.4 million β-cells per kg BW and did not achieve insulin independence. Mean C-peptide levels during the first 6 months were 0.62 ng/ml and CVfg was 32%. Autoreactivity against IA2, high lymphocyte counts (3393/mm^3) of which 536/mm^3 B-lymphocytes were measured before transplantation. The mean B-lymphocyte count until the time of biopsy was 421/mm^3.

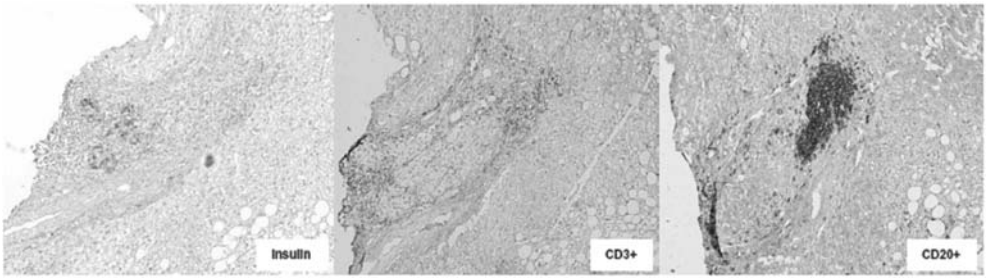

FIGURE 3 Consecutive sections of an islet implant in the liver of a recipient unable to stop insulin injections. The infiltration is dominated by $CD20^+$ B-lymphocytes. The patient presented with cellular autoreactivity against IA2, high total (3393/mm^3) and B-lymphocyte counts (536/mm^3) before transplantation. Biopsy was taken 60 weeks after the first transplantation. Mean B-lymphocyte counts until the time of biopsy were 421/mm^3. At the time of biopsy tacrolimus troughlevel was 6.8 ng/ml and MMF dose was 2000 mg/d.

DISCUSSION

The present study aimed to identify baseline factors that can affect clinical outcome after islet cell transplantation. We were able to show that differences in baseline blood composition and immune state affect the ability to achieve insulin independence as well as glucose control and C-peptide release in the first 6 months after transplantation under ATG, tacrolimus and MMF immunosuppression. Our findings may help select for patients with optimal

clinical outcome and provide possible new targets for future immunosppressive regimens to improve clinical outcome in a subgroup of patients with less favorable pretransplant conditions.

We observed significantly higher numbers of B-lymphocytes at baseline and in the peritransplant period in patients that were unable to stop insulin injections which was also reflected in worse glucose control and lower C-peptide release in the first 6 months after transplantation. This observation raises questions about the role of B-lymphocytes in islet cell transplantation which might involve different mechanisms including autoimmunity, alloimunity or both. First, participation of B-lymphocytes in the pathogenesis of type 1 diabetes has since long been described in animal studies[24-28]. However, their role in human disease is not yet fully understood. The production of islet-specific autoantibodies has been thouroughly investigated and presents an important marker to identify first degree relatives of type 1 diabetics at increased risk for developing disease[29-31]. Yet, there is no evidence that these antibodies are pathogenic, while type 1 diabetes has been described in a patient with severe hereditary B-cell deficiency[32]. In islet cell transplantation, autoantibodies can identify recurrence of autoimmunity and in some cases be correlated to clinical outcome[8,33-36] but this is not a consistent finding[18,20]. In a recent study, autoantibody status before the last islet cell injection was correlated with the attainment of insulin independence under the Edmonton protocol[8], something we did not observe in our study. Importantly, we did not find any correlation between the number of B-lymphocytes and autoantibody prevelance or titers. Second, B-lymphocytes are able to impair graft function through production of donor-specific alloantibodies in solid organ transplantation[37]. The development of these alloantibodies after islet transplantation has been described[38,39] but their role in islet graft dysfunction remains unclear. Third, B-lymphocytic infiltrates have been described in renal recipients with allograft rejection without evidence of humoral rejection[40,41]. The mechanism by which B-lymphocytes may impair graft function is still unknown but it has been suggested that besides antibody production, the role of B-lymphocytes in both auto- and alloimmunity could be related to their capacity for antigen presentation and interaction with T-cells[42-45]. This may be particularly important in the setting of islet transplantation where, in contrast with solid organ transplantation, the tissue is directly injected into the bloodstream. Since about sixty percent of the transplanted tissue may be lost during the first 2-3 weeks after implantation[46], circulating lymphocytes are exposed to a large pool of degraded auto- and alloantigens. Although ATG may suppress T-cell function in this phase, B-lymphocytes may retain there pathogenic role and initiate auto- and/or allo immune responses ultimately leading to acute or chronic graft destruction.

To our knowledge, we are the first to describe histological evidence of a B-lymphocyte dominated infiltrate after islet cell transplantation. Though the infiltration is located in the periph-

ery without direct evidence of β-cell destruction it does demonstrate that B-lymphocytes are involved in the immuneresponse following allogeneic islet cell tansplantation. If these cells are involved in auto- or alloimmune responses remains an important question. In this context it is important to note that thourough histological studies investigating insulitis in recent onset type 1 diabetic patients have revealed infiltrates dominated by T- rather than B-lymphocytes. On the other hand, infiltrating B-lymphocytes have been described in renal allografts and related to corticoid resistent allograft rejection[40]. Interestingly, this patient presented high B-lymphocyte counts before and after transplantation. If this is correlated to the infiltration we observed is unclear in the current study.

Though taken together our data suggests a possible role of B-lymphocytes in islet allograft dysfunction, it does however not provide sufficient prove for a causal connection. Only intervention studies including B-cell depleting strategies will be able to address this question. One posibility might be the use of rituximab, which selectively targets $CD20^+$ B-lymphocytes. A study in non-human primates by Liu et al. demonstrated better long-term survival of islet allografts after adding rituximab to an induction with rabbit ATG followed by rapamycin maintenance immunotherapy[47]. Furthermore, it has been suggested that B-lymphocyte mediated immune regulation by IL-10 secretion may play an important role in controling autoimmune pathology. This regulation may be lost in autoimmune diseases but can possbily be restored by B-lymphocyte depleting immunotherapy[27,48]. Thus, targeting B-lymphocytes definitely needs further investigation in clinical islet transplantation. It is however important to note that about fifty percent of our patients did achieve insulin independence with the currently used, well tolerated, immunosuppressive regimen[17]. Thus, possibly, prospective studies targeting B-lymphocytes should be carried out in carefully selected patients. Our data may help identify these patients.

In the present study, we confirm our previous findings that the presence of baseline T-cell autoreactivity hinders the attenuance of insulin independence[18]. Our current study extends this finding in a larger cohort. Baseline T-cell autoreactivity also correlates with worse glucose control and low C-peptide release in the first 6 months after transplantation providing additional evidence for its clinical relevance at baseline in islet transplantation under our immunosuppressive protocol. In a recent publication Monti et al demonstrated that homeostatic proliferation of T-lymphocytes in response to lymphopenia after daclizumab or ATG can induce *in vivo* proliferation of autoreactive memory T-cell in islet graft recipients[49]. After pancreas transplantation, memory $CD4^+$ T-cells specific for GAD65 can be detected in patients with recurrent hyperglycemia[50]. These observations are in line with our findings and provides additional insight why persisting T-cell autoreactivity before transplantation affects clinical outcome.

Only one recipient showed complete graft loss in our study. It is interesting to note that this subject presented with baseline T-cell reactivity against both GAD and IA2, a combination that was previously shown to be associated with the least favourable clinical outcome[18]. Of the seven patients with both GAD and IA2 in the current analysis only one subject achieved insulin independence but had to resume insulin treatment within 25 weeks after withdrawl.

Whatever the actual mechanism, our findings identified markers of a more active immune system in patients that do not achieve insulin independence. This is further illustrated by the fact that within eleven individuals with both the presence of baseline cellular autoreactivity and high B-lymphocyte counts only one achieved insulin independence (9%) whereas among those with low lymphocytes without autoreactivity independence was reached in seven out of eight (88%, p=0.001). Both B- and T-lymphocytes are higher in insulin requiring subjects with a predominance of $CD8^+$ T-cells and the same is true for leucocyte counts but not for NK cells.

In conclusion, our data are the first to show that differences in immune state in type 1 diabetic patients, as supported by the presence of higher numbers of circulating lymphocytes and cellular reactivity against autoantigens affect clinical outcome. A higher number of B-lymphocytes in the peri-transplant period in patients unable to stop insulin injections and their presence in a biopsy of an islet graft recipient raises interesting questions about the role of these lymphocytes in islet cell transplantation which will need further exploration.

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Appendix 1. Recipient and graft characteristics. Data represent median (interquartile range) for 30 patients.

Recipients	
n	30
Age (years)	44 (38-50)
Gender (M/F)	17/13
Body weight (kg)	68 (64-76)
BMI (kg/m^2)	24 (22-26)

Diabetes	
Duration of disease (years)	26 (20-33)
Age at onset (years)	16 (11-23)
Positivity for ICA/GADA/IA2-A	6/13/13
$HbA1_c$ (%)	7.6 (7.0-8.1)
Insulin dose (IU/kg/d)	0.6 (0.5-0.8)
Fasting glycemia	
Mean (mg/dl)	151 (129-182)
CoV (%)	44 (38-47)
Retinopathy	22
Microalbuminuria	13

Donor tissue (per graft)	
Donor pancreata (n)	4 (3-5)
Donor age (years)	49 (44-53)
Pancreas cold ischemia time (h)	9 (8-10)
Pancreas weight (g)	91 (78-100)

Grafts	
Culture time (days)	6 (4-10)
Cellular composition	
β-cells	30 (21-37)
α-cells	8 (5-11)
Nongranulated cells	45 (37-62)
Acinar cells	1 (1-4)
Dead cells	9 (6-11)
β-cell number (million/kg)	
First graft	2.6 (2.1-3.3)
Total	4.2 (3.2-5.1)

Immune suppression	
Total ATG dose (mg/kg BW)	23 (21-25)
Tacrolimus troughlevels (ng/ml) 0-6 months	9.2 (8.3-9.6)
MMF dose (mg/day) 0-6 months	2000 (1500-2000)

All recipient data were measured before transplantation, except glycemia, which represents measurements at home during the preceding year. 13 patients received one graft and 17 patients received a second graft 12 (9-14) weeks after the initial transplantation.