

Effects of immunosuppression on alpha and beta cell renewal in transplanted mouse islets

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Abstract

Aims/hypothesis Immunosuppressive drugs used in human islet transplantation interfere with the balance between beta cell renewal and death, and thus may contribute to progressive graft dysfunction. We analysed the influence of immunosuppressants on the proliferation of transplanted alpha and beta cells after syngeneic islet transplantation in streptozotocin-induced diabetic mice.

Methods C57BL/6 diabetic mice were transplanted with syngeneic islets in the liver and simultaneously abdominally implanted with a mini-osmotic pump delivering BrdU alone or together with an immunosuppressant (tacrolimus,

sirolimus, everolimus or mycophenolate mofetil [MMF]). Glycaemic control was assessed for 4 weeks. The area and proliferation of transplanted alpha and beta cells were subsequently quantified.

Results After 4 weeks, glycaemia was significantly higher in treated mice than in controls. Insulinaemia was significantly lower in mice treated with everolimus, tacrolimus and sirolimus. MMF was the only immunosuppressant that did not significantly reduce beta cell area or proliferation, albeit its levels were in a lower range than those used in clinical settings.

Conclusions/interpretation After transplantation in diabetic mice, syngeneic beta cells have a strong capacity for self-renewal. In contrast to other immunosuppressants, MMF neither impaired beta cell proliferation nor adversely affected the fractional beta cell area. Although human beta cells are less prone to proliferate compared with rodent beta cells, the use of MMF may improve the long-term outcome of islet transplantation.

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Keywords Cell proliferation · Drug monitoring · Glucagon-secreting cells · Immunosuppression · Insulin-secreting cells · Islets transplantation · Mice

Abbreviations

IMPDH Inosine-5-monophosphate dehydrogenase
LSD Least significant difference
MMF Mycophenolate mofetil
mTOR Mammalian target of rapamycin
STZ Streptozotocin

Introduction

Human islet transplantation is an established treatment option for beta cell replacement in type 1 diabetic patients with poor

glycaemic control and frequent episodes of hypoglycaemia. Despite promising short-term results, a gradual impairment in glycaemic control is commonly observed in recipients of islet allografts [1]. This progressive graft dysfunction can result from several factors, including inflammation, allorejection and metabolic stress, as well as toxic and antiproliferative effects of the immunosuppressive therapy [2].

Data from several studies have suggested that the pancreatic beta cell mass changes dynamically according to metabolic demands. In addition, lineage-tracing experiments have shown that pre-existing beta cells are the major source of new beta cells in adult mice [3, 4]. Recent studies have suggested that the re-differentiation of alpha cells into beta cells is an additional mechanism for restoration of the beta cell mass [5, 6]. Common immunosuppressants used in human islet transplantation, such as sirolimus and tacrolimus, negatively affect beta cell replication [3, 7]. Consequently, long-term exposure to these drugs may hamper beta cell turnover and contribute to graft dysfunction in islet transplant recipients. Immunosuppressants that are compatible with beta cell regeneration may therefore prolong graft survival and function. In this study, we investigated the effects of several immunosuppressive drugs on alpha and beta cell proliferation in diabetic mice after syngeneic hepatic islet transplantation.

Methods

Animals Healthy 10- to 12-week-old C57BL/6 mice (Charles River, Bad Sulzfeld, Germany) were housed in cages at a constant temperature and with a standard 13 h light/11 h dark cycle. All mice were fed a 6% mouse chow diet (Altromin, Lage, Germany) and had free access to water. The Institutional Animal Care and Use Committee of the University of Dresden and the state government of Saxony approved all studies involving animal use.

Treatment groups Thirty-one C57BL/6 mice were assigned to five groups, each including five animals, except for the everolimus-treated group, which included 11 mice (see Results). A combination of BrdU (Sigma, St Louis, MO, USA) and an immunosuppressive drug was administered to the mice in each treatment group; the control mice received BrdU only. The concentrations of the immunosuppressants in the pumps were as follows: 1 mg (kg body weight)⁻¹ day⁻¹ everolimus (Sigma); 1 mg (kg body weight)⁻¹ day⁻¹ tacrolimus (Astellas Pharma, Tokyo, Japan); 20 mg (kg body weight)⁻¹ day⁻¹ mycophenolate mofetil (MMF; Roche Pharmaceuticals, Basel, Switzerland); 0.2 mg (kg body weight)⁻¹ day⁻¹ sirolimus (Sigma).

Induction of diabetes After overnight fasting, 180 mg/kg streptozotocin (STZ; Sigma) that had been freshly dissolved

in 100 µl citrate buffer was administered in a single i.p. injection.

Blood glucose and serum insulin Glycaemia was measured every third day between 09:00 and 11:00 hours in non-fasting conditions using a OneTouch Ultra glucometer (LifeScan, Milpitas, CA, USA). Insulinaemia was measured before surgery and at the end of weeks 2 and 4 post transplantation using an ultrasensitive mouse insulin ELISA kit (Crystal Chem, Downers Grove, IL, USA).

Drug monitoring Whole blood obtained from the tail vein was transferred onto filter paper (WS 903; Whatman International, Maidstone, UK) once per week after transplantation. The concentrations of tacrolimus, sirolimus and everolimus were determined in dried blood spots using HPLC-MS/MS (API 3000; AB SCIEX, Framingham, MA, USA) as previously described [8].

Islet transplantation Mouse islets were isolated from 8- to 14-week-old C57BL/6 littermates as previously described [9]. After overnight incubation, ~300 islets were manually handpicked and transferred into a 27-gauge butterfly needle attached to a 1 ml syringe. The recipient mice were anaesthetised by i.p. injection of 100 mg/kg ketamine (Pfizer International, New York, NY, USA) and 10 mg/kg xylazine (Sigma). After clamping the left portal vein, the islets were selectively transplanted into the right liver lobes by injection into the ileocecal vein. Osmotic mini-pumps (Alzet 1004; Palo Alto, CA, USA) were placed intraperitoneally to deliver 100 µg/µl BrdU in 100% DMSO (Merck, Darmstadt, Germany) at the rate of 0.11 µl/h for 28 days. At 30 days post surgery, the mice were killed by intracardial perfusion and their right liver lobes were harvested.

i.p. Glucose tolerance test Glucose tolerance was assessed at the end of weeks 2 and 4 post transplantation. Briefly, after overnight fasting, the mice received an i.p. glucose bolus (2 g/kg body weight), and glycaemia was monitored in the conscious mice at selected time points. Glucose disposal, expressed as the AUC, was used to calculate the change between the two tests (Δ AUC).

Immunohistochemistry and beta cell area measurement After intracardial perfusion with PBS and 4% paraformaldehyde (Merck), the right liver lobes were removed, fixed overnight and embedded in paraffin. Ten 5 µm thick paraffin sections separated ≥ 100 µm from each other were obtained from each specimen. After de-waxing and microwave antigen retrieval, the sections were first incubated in PBS with 16% goat serum and 0.1% saponin (PAA Laboratories, Pasching, Austria) and then incubated overnight with guinea pig anti-insulin (1:200; Abcam, Cambridge, MA, USA),

rabbit anti-glucagon (1:200; Millipore, Schwalbach, Germany) and mouse anti-BrdU (1:10; Roche Diagnostics, Mannheim, Germany) or rabbit anti-Ki67 (1:200; Abcam) antibodies followed by Alexa⁵⁶⁸-goat anti-guinea pig, Alexa⁴⁰⁵-goat anti-rabbit and Alexa⁴⁸⁸-goat anti-mouse or Alexa⁴⁸⁸-goat anti-rabbit (1:200; Invitrogen, Carlsbad, CA, USA) antibodies. The nuclei were counterstained using DRAQ5 or DAPI (1:1,000) (Enzo Life Sciences, Farmingdale, NY, USA). Images were acquired with a Zeiss LSM 510 confocal microscope (Carl Zeiss, Oberkochen, Germany). The numbers of BrdU⁺/Ki67⁺ beta cells among 1.2×10^3 beta cells/mouse were manually counted. The total numbers of alpha cells and BrdU⁺ alpha cells were recorded in parallel. Mistakes or counting of non-islet cells were ruled out by a predefined protocol: $\times 40$ magnification was used, and only nuclei with strong, complete BrdU⁺ staining and completely surrounded by an insulin- or glucagon-positive cytosol were counted. To measure the fractional alpha and beta cell area a total of 30 randomly selected insulin- and glucagon-positive areas per mouse were imaged at the final magnification of $\times 40$. Each area was quantified as a fraction of the total tissue area, corresponding to $255 \mu\text{m}^2/\text{image}$. For assessment of the mean beta cell size, we manually traced the contour of 100 randomly selected beta cells with clearly visible nuclei and cytoplasm in five islets/mouse. All morphometric analyses were performed using ImageJ software (NIH, Bethesda, MD, USA).

Statistics and graphics The values between the control and drug-treated mice at the same time point after islet transplantation were compared using the Mann–Whitney *U* test, and the values within the same group at different time points were compared using the Wilcoxon signed-rank test. Because of multiple testing, the significance level (α) was adjusted according to the Holm–Bonferroni method. For the statistical tests without multiple comparisons, the significance level was $p < 0.05$. One-way ANOVA was calculated on datasets of BrdU and Ki67 labelling of beta cells. Post hoc analysis was performed using Fisher's least significant difference test (LSD). The correlations between pairs of variables were calculated using Spearman's rank correlation test. The results are presented as mean \pm SD unless otherwise stated. The histograms were prepared using Microsoft Excel.

Results

Mouse population and drug monitoring The STZ-treated diabetic mice in which approximately 300 syngeneic islets were transplanted in the liver continuously received one of several immunosuppressants together with BrdU via i.p. osmotic mini-pumps for 2 or 4 weeks, and the mice were subsequently killed (Fig. 1a). Doses of immunosuppressive drugs were chosen based on the established serum concentration ranges

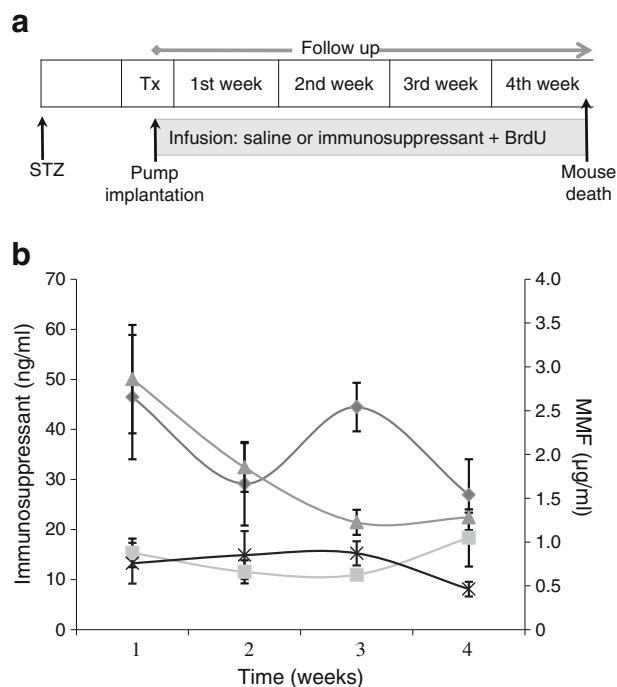


Fig. 1 (a) Experimental timeline. (b) Average serum concentration of immunosuppressants (mean \pm SD). Tx, treatment. Symbols: diamonds, everolimus; squares, tacrolimus; crosses, sirolimus; triangles, MMF

used in humans undergoing islet or pancreas transplantation (Table 1). Because everolimus has greater plasma protein binding and a shorter half-life in mice than in humans, we aimed for higher serum levels in this group. Moreover, it has been shown that a larger dosage of everolimus than sirolimus is required to maintain the same blood concentrations in pancreatic islet-transplanted patients [10]. Weekly monitoring indicated that the serum concentrations of MMF and sirolimus were within the therapeutic levels, while the concentrations of everolimus and tacrolimus were above the clinical concentration ranges (Fig. 1b and Table 1). Notably, six of the 31 islet-transplanted mice, all of which were treated with everolimus, were excluded from any further analysis. Two of these mice had very high everolimus levels (1,340 ng/ml and 1,630 ng/ml), conceivably because of a malfunction of the pump, and died 7 and 9 days, respectively, after transplantation. A third everolimus-treated mouse was excluded because the drug concentration was 159 ng/ml. In the remaining three mice, the everolimus levels were closer to the accepted therapeutic range (10–15 ng/ml), but the mouse blood glucose levels remained >20 mmol/l during the first week post transplantation, indicating a failure of the islet engraftment. In all other mice, the blood glucose levels dropped from >20 mmol/l (mean 26.9 ± 4.4 mmol/l) after STZ treatment to <15.3 mmol/l (mean 9.01 ± 3.4 mmol/l) within 1 week after the islet transplantation (Fig. 2a).

Immunosuppressants interfere with glycaemic control after transplantation The average body weight of the transplanted

Table 1 Metabolic profiling and morphometric islet analysis of control and drug-treated mice

Variable	Controls (n=5)	Everolimus (n=11)	Tacrolimus (n=5)	MMF (n=5)	Sirolimus (n=5)
Drug concentration (ng/ml) ^a		35.3±9.19	14.06±2.48	1.89±0.62 ^b	12.89±6.12
Body weight (g)					
Week 2	21.17±4.22	18.81±2.65	25.87±1.97	25.2±1.97	19.13±2.62
Week 4	22.13±4.6	20.13±2.08	26.8±1.59	25.07±1.09	20.56±2.43
Blood glucose (mmol/l)					
Week 2	8.04±1.22	10.54±4.13	9.19±0.81	10.89±2.08**	9.49±1.76
Week 4	6.41±0.83 ^{††}	10.70±4.77**	8.63±1.45**	9.78±1.11**	8.51±2.22**
Serum insulin (pmol/l)					
Week 2	87.3±19.1	57.6±31.5	61.5±28.9	66.3±38.2	68.5±36.9
Week 4	124.5±34.1 [†]	68.3±33.1*	57.8±32.4**	72.0±42.8	72.7±16.9*
Glucose tolerance (ΔAUC)	7.12±2.21	1.08±1.02**	3.03±2.71*	2.22±2.11*	0.76±1.9**
BrdU ⁺ beta cells (%)	41.39±5.41	22.15±8.02**	31.6±5.65	43.79±10.57	21.33±11.5*
Ki67 ⁺ beta cells (%)	0.98±0.21	0.43±0.27**	0.52±0.14**	0.73±0.22	0.38±0.23**
Beta cell area (%)	5.60±0.55	3.22±1.13*	5.38±1.21	5.74±0.84	3.26±0.64**
Beta cell size (μm ²)	106.45±6.52	100.35±10.8	99.08±3.96	109.64±9.04	104.34±1.31
BrdU ⁺ alpha cells (%)	3.58±1.51	1.51±0.75	2.78±1.79	2.66±1.26	1.27±0.43
Alpha cell area (%)	0.84±0.34	0.41±0.23	0.62±0.28	0.69±0.28	0.51±0.12

^a Clinical concentration ranges: everolimus 10–15 ng/ml; tacrolimus 8–12 ng/ml; MMF 1–3.5 μg/ml; sirolimus 10–15 ng/ml

^b μg/ml

* $p < 0.05$; ** $p < 0.01$, Holm-adjusted between treated and control animals

[†] $p < 0.05$; ^{††} $p < 0.01$, between week 2 and week 4 post transplantation

mice did not differ significantly between the control and drug-treated mice (Table 1). Throughout the follow-up period, glycaemia was significantly improved in the control animals ($p = 0.006$; Fig. 2a). At week 2 of follow-up, the average blood glucose levels of the mice receiving MMF were significantly higher compared with the control animals ($p = 0.001$). However, at week 4, all drug-treated mice displayed significantly higher blood glucose levels (Fig. 2a and Table 1). As expected, in all groups, the serum insulin concentrations increased after transplantation, although they did not reach pre-STZ treatment levels (Fig. 2b). Insulinaemia was further increased between weeks 2 and 4 post transplantation in the control mice (87.3 vs 124.5 pmol/l; $p = 0.046$), suggesting the

recovery of beta cell function (Table 1). Although the insulin levels of treated animals did not differ significantly from those of untreated controls at week 2 post transplantation, at week 4 they were significantly lower in the mice treated with everolimus ($p = 0.015$), tacrolimus ($p = 0.009$) or sirolimus ($p = 0.015$). Glucose tolerance was assessed using i.p. glucose tolerance tests at the end of weeks 2 and 4 post transplantation. We subsequently calculated the ΔAUC within each group. The ΔAUC was significantly reduced in all drug-treated mice compared with the control mice (Fig. 2c).

Transplanted beta and alpha cells replicate The proliferative capacities of the transplanted beta and alpha cells that

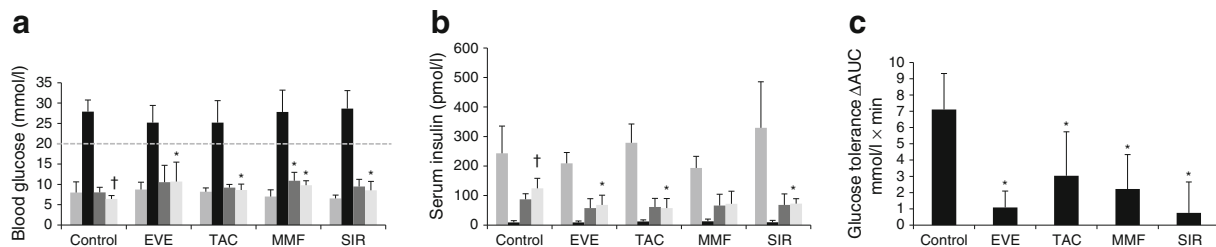


Fig. 2 Average blood glucose levels (a) and serum insulin concentrations (b) at the indicated time points. The grey horizontal line indicates the threshold value of 20 mmol/l glucose; the mice above this threshold were regarded as diabetic. (c) Glucose tolerance at weeks 2 and 4 post transplantation represented by ΔAUC. †Indicates a significant

difference ($p < 0.05$) between week 2 and week 4 post transplantation; *indicates a significant difference ($p < 0.05$, Holm-adjusted) between the treated and control animals. EVE, everolimus; SIR, sirolimus; TAC, tacrolimus. Mid-grey bars, pre-STZ; black bars, STZ; dark grey bars, week 2; light grey bars, week 4

were not subjected to immunosuppressive therapy were examined after 2 and 4 weeks post transplantation (Fig. 3). After 2 weeks, $14.8\pm 3.9\%$ of beta cells were BrdU⁺ compared with $41.4\pm 5.4\%$ after 4 weeks ($p=0.014$) (Fig. 4a, b). In the case of the alpha cells, the increase in cumulative BrdU⁺ labelling was not significant, changing from $2.65\pm 0.19\%$ after 2 weeks to $3.6\pm 1.5\%$ after 4 weeks ($p=0.18$).

Not all immunosuppressants inhibit beta cell proliferation In the mice treated with MMF for 4 weeks, the BrdU⁺ beta and alpha cells accounted for $43.8\pm 10.6\%$ and $2.7\pm 1.3\%$ of all beta and alpha cells, respectively, which was comparable to those of the control mice ($p=0.69$ and $p=0.42$, respectively) (Fig. 4c, d). In the tacrolimus-treated mice, 31.6 ± 5.0 of beta cells ($p=0.032$, Holm-adjusted significance level $\alpha=0.025$) and $2.8\pm 1.8\%$ of alpha cells ($p=0.42$) were BrdU⁺. However, this result was not significantly different from that of untreated controls. The number of BrdU⁺ beta cells was significantly reduced in the mice treated with the mammalian target of rapamycin (mTOR) inhibitors everolimus ($22.1\pm 8.0\%$; $p=0.008$, Holm-adjusted significance level

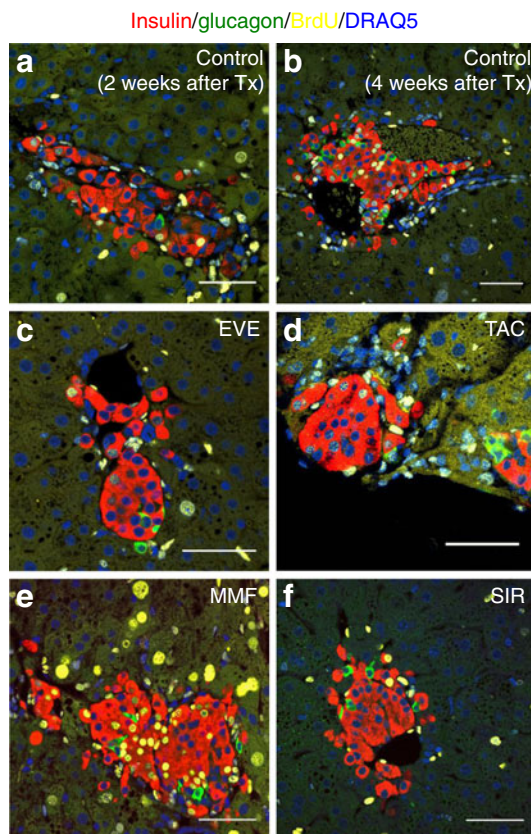


Fig. 3 Representative confocal images of liver-transplanted islets in control mice (a) 2 and (b) 4 weeks post transplantation, and in mice treated with the indicated drugs (c–f) 4 weeks post transplantation. BrdU labelling appears in yellow, insulin in red, glucagon in green and DRAQ5 (nuclei) in blue. Scale bars, 50 μm . EVE, everolimus; SIR, sirolimus; TAC, tacrolimus; Tx, transplantation

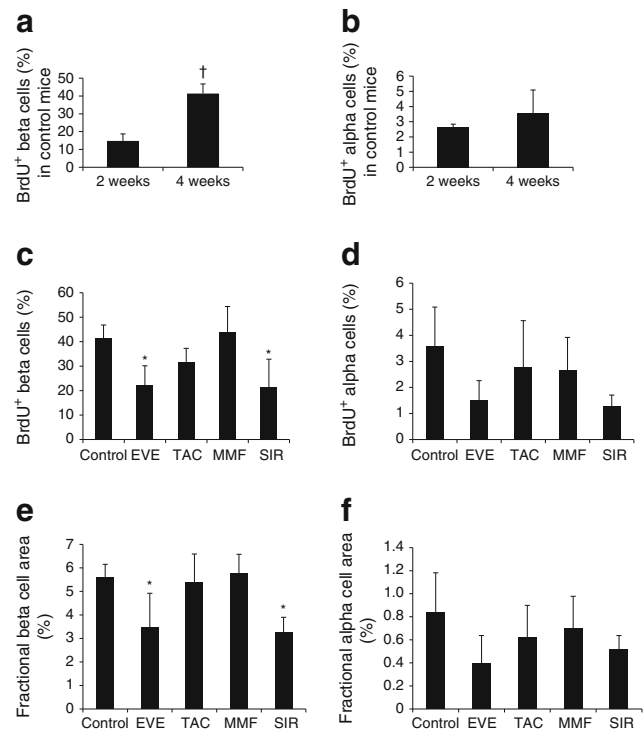


Fig. 4 Percentage of BrdU⁺ (a) beta and (b) alpha cells in the control mice 2 and 4 weeks post transplantation. †Indicates a significant difference ($p<0.05$) between week 2 and week 4 post transplantation. Percentage of BrdU⁺ (c) beta and (d) alpha cells 4 weeks post transplantation and treatment with immunosuppressants. Fractional area of (e) beta and (f) alpha cells. *Indicates a significant difference ($p<0.05$, Holm-adjusted) between the treated and control animals. EVE, everolimus; SIR, sirolimus; TAC, tacrolimus

$\alpha=0.0125$) and sirolimus ($21.3\pm 11.5\%$; $p=0.016$, Holm-adjusted significance level $\alpha=0.0167$), while the fraction of BrdU⁺ alpha cells did not differ significantly (everolimus $1.5\pm 0.75\%$, $p=0.056$; sirolimus $1.3\pm 0.4\%$, $p=0.032$, Holm-adjusted significance level $\alpha=0.0125$). One-way ANOVA was calculated on BrdU labelling of beta cells. This analysis was significant: $F(4/20)=6.6$, $p=0.001$. Fisher's LSD test of BrdU labelling found that MMF was the only drug that was comparable relative to control but differed significantly from each of the other immunosuppressants (Table 2).

To verify differences found by BrdU accumulation, we determined the frequency of proliferation by immunohistochemical detection of Ki67. Consistent with the BrdU labelling, the number of Ki67⁺ beta cells in the mice treated with MMF and the untreated controls did not differ significantly (MMF $0.73\pm 0.22\%$, controls 0.98 ± 0.21 ; $p=0.286$) (Fig. 5). Likewise, the number of Ki67⁺ beta cells was significantly reduced in the mice treated with the mTOR inhibitors everolimus ($0.43\pm 0.27\%$; $p=0.009$, Holm-adjusted significance level $\alpha=0.025$) and sirolimus (0.38 ± 0.23 ; $p=0.008$, Holm-adjusted significance level $\alpha=0.0125$). However, in tacrolimus-treated mice $0.52\pm 0.14\%$ of beta cells were Ki67⁺, which was significantly different from the untreated

Table 2 Results of ANOVA with post hoc test for multiple comparisons (Fisher's LSD test) of BrdU and Ki67 labelling

Dependent variable	Group (I)	Group (J)	Mean difference (I–J)	Significance
Ki67 labelling of beta cells	Controls	Everolimus	0.55	0.001*
		Tacrolimus	0.46	0.004*
		MMF	0.257	0.096
		Sirolimus	0.606	0.000*
	Everolimus	Controls	-0.556	0.001*
		Tacrolimus	-0.096	0.497
		MMF	-0.299	0.056
		Sirolimus	0.05	0.722
	Tacrolimus	Controls	-0.46	0.004*
		Everolimus	0.096	0.497
		MMF	-0.203	0.183
		Sirolimus	0.146	0.305
	MMF	Controls	-0.257	0.096
		Everolimus	0.299	0.056
		Tacrolimus	0.203	0.183
		Sirolimus	0.349	0.028*
	Sirolimus	Controls	-0.606	0.000*
		Everolimus	-0.05	0.722
		Tacrolimus	-0.146	0.305
		MMF	-0.349	0.028*
BrdU labelling of beta cells	Controls	Everolimus	18.12	0.004*
		Tacrolimus	8.66	0.139
		MMF	-3.52	0.538
		Sirolimus	18.94	0.003*
	Everolimus	Controls	-18.12	0.004*
		Tacrolimus	-9.46	0.108
		MMF	-21.64	0.001*
		Sirolimus	0.82	0.885
	Tacrolimus	Controls	8.66	0.139
		Everolimus	9.46	0.108
		MMF	12.18	0.042*
		Sirolimus	10.28	0.082
	MMF	Controls	3.52	0.538
		Everolimus	21.64	0.001*
		Tacrolimus	12.18	0.042*
		Sirolimus	22.46	0.001*
	Sirolimus	Controls	-18.94	0.003*
		Everolimus	-0.82	0.885
		Tacrolimus	-10.28	0.082
		MMF	-22.46	0.001*

*Indicates a significant difference between groups (I) and (J)

controls ($p=0.008$, Holm-adjusted significance level $\alpha=0.0167$). One-way ANOVA of Ki67 labelling of beta cells was significant: $F(4/19)=6.4$, $p=0.002$. Fisher's LSD test of Ki67 labelling found that MMF was the only drug that was

comparable to control mice. However, MMF significantly differed only from sirolimus (Table 2).

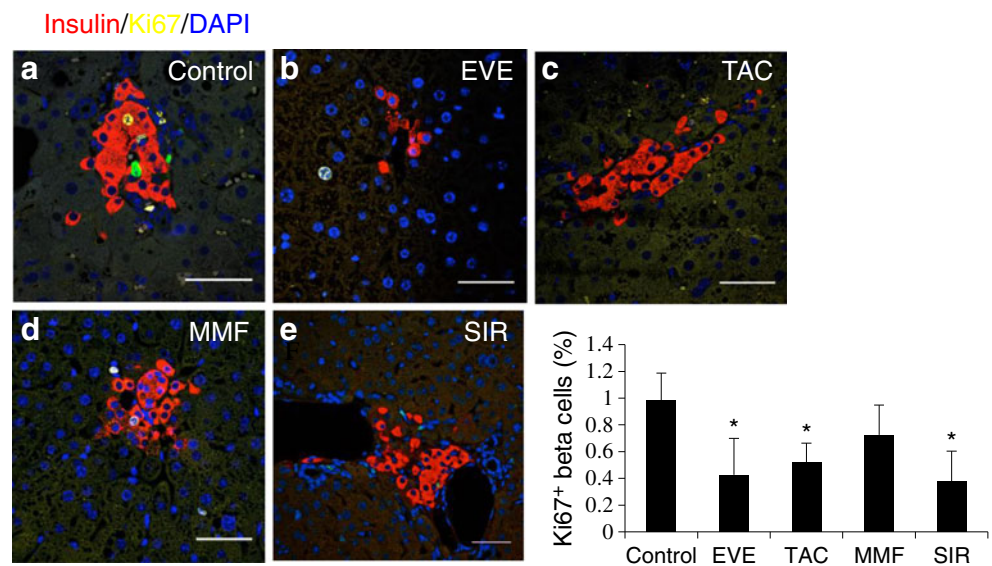
Fractional beta and alpha cell area and beta cell size The cumulative beta cell fraction of the liver areas and the cumulative proliferation rates of beta cells were positively correlated ($R=0.72$; $p=0.01$), indicating a direct influence of beta cell proliferation on the beta cell mass of the islet graft (Fig. 6). There was no significant correlation between the fractional area and BrdU accumulation of alpha cells ($R=0.33$; $p>0.11$). The fractional beta and alpha cell areas of MMF-treated animals were comparable to those of the control mice ($p=0.84$ and $p=0.69$) (Fig. 4e, f). Likewise, the fractional beta and alpha cell areas of the tacrolimus-treated animals did not differ significantly from those of the control mice ($p=0.84$ and $p=0.31$, Holm-adjusted significance level $\alpha=0.0167$). In the everolimus- and sirolimus-treated mice, the fractional beta cell areas decreased significantly ($p=0.016$ and $p=0.008$, respectively, Holm-adjusted significance level $\alpha=0.0167$), while the fractional alpha cell areas were comparable to those of the control mice ($p=0.056$ and $p=0.151$, respectively). Four weeks after transplantation, the beta cells in all groups exhibited comparable sizes (Table 1), which is consistent with previous findings [11].

Discussion

Our data indicate that transplanted mouse pancreatic beta cells that are not exposed to a toxic insult or to auto- or alloreactivity have a strong capacity for replication. We found 14.8% BrdU⁺ beta cells in the control group 2 weeks after transplantation, which was less than Nir et al [3] found 2 weeks after beta cell ablation (36% of beta cells were BrdU⁺). Zahr et al [7] reported that in pregnant mice receiving BrdU via drinking water for 1 week 22% of the beta cells were BrdU⁺ at gestational day 14. Previously, we found that continuous BrdU administration to mice for 1 week after 70–80% pancreatectomy led to the labelling of 18% of the beta cells [12]. The lower percentage of BrdU⁺ beta cells reported in the current study may reflect the reduced ability of the beta cells within transplanted islets to replicate compared with beta cells in the native pancreas.

In human islet transplantation the number of donor islets is usually insufficient to restore the beta cell mass in recipients. In our study, we decided to transplant an almost marginal islet mass in order to mimic the clinical setting of islet transplantation as closely as possible. The use of a larger islet mass would reduce the stimulus for proliferation because of a smaller metabolic demand per islet. Consequently, differences in proliferation rates would be harder to detect and interference with beta cell function would

Fig. 5 Representative confocal images of liver-transplanted islets in (a) control mice and in mice treated with the indicated drugs (b–e) 4 weeks post transplantation. Ki67 labelling appears in yellow, insulin in red and DAPI (nuclei) in blue. The bar chart shows the percentage of Ki67⁺ beta cells 4 weeks post transplantation. *Indicates a significant difference ($p < 0.05$, Holm-adjusted) between the treated and control animals. Scale bar, 50 μm . EVE, everolimus; SIR, sirolimus; TAC, tacrolimus



probably be attenuated. Therefore, in the control mice treated with STZ and transplanted with islets, the non-fasting glucose levels were slightly elevated compared with the untreated control mice; their average insulinaemia was significantly reduced and their glucose tolerance was impaired. Conceivably, the reduction in the endogenous beta cell mass stimulated the replication of the transplanted beta cells.

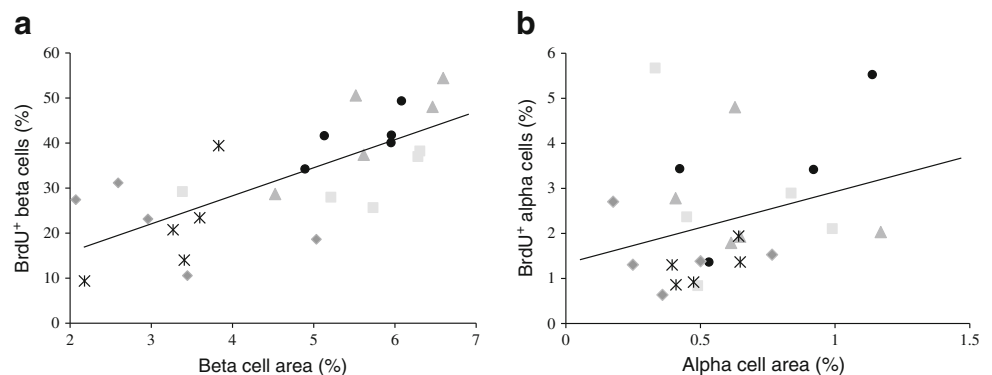
Notably, the BrdU labelling of the beta cells positively correlated with the fractional beta cell area. In our model, however, we cannot exclude that other pancreatic (endocrine, acinar or ductal) or progenitor cells contributed to beta cell regeneration and the restoration of glycaemic control [13].

The relative importance of beta cell generation vs cell death is difficult to discern using histological analysis [13]. No reliable procedures for the cumulative *in vivo* measurement of apoptosis or necrosis exist. Morphological methods, such as TUNEL or the detection of highly condensed and/or fragmented nuclei, can be applied to determine the frequency of apoptosis at a given time point. However, these methods are not suitable for measuring the cumulative rate of apoptosis, especially under conditions such as islet transplantation, in which the frequency of apoptosis is likely to

vary greatly over time. Interestingly, some data imply that spontaneous beta cell regeneration results from accelerated formation rather than the reduced apoptosis of beta cells [3, 11]. Moreover, the frequency of beta cell apoptosis does not increase following the combined administration of sirolimus and tacrolimus *in vivo* [3, 7]. In our study, we consistently found no increase in beta cell or hepatocyte apoptosis in random samples of liver sections of mice treated with immunosuppressants and untreated controls 4 weeks post transplantation using tetramethylrhodamine (TMR) red labelling (data not shown).

The mTOR inhibitors sirolimus and everolimus have a negative effect on beta cell replication and glucose homeostasis [3, 14, 15]. In this study, their detrimental effect on beta cell replication can be explained by considering that the inhibition of mTOR decreases the activity of the translational regulators 4EBP1 and S6K1, which has a negative effect on the expression of cyclins and associated factors, thereby inhibiting the progression of the cell cycle from the G1 to the S phase [16, 17]. The impairment of glucose homeostasis may be secondary to the reduction of the beta cell mass [18] and the inhibition of mTOR downstream of insulin signalling [19].

Fig. 6 Scatter blots representing the relationship between the fractional areas and cumulative percentages of BrdU⁺ (a) beta and (b) alpha cells. Circles, control; diamonds, everolimus; squares, tacrolimus; triangles, MMF; crosses, sirolimus



The negative impact of tacrolimus on beta cell proliferation and insulin secretion on the inhibition of the calcineurin/cytoplasmic nuclear factor of activated T cells complex has also been well described *in vitro* [20]. In our study, tacrolimus interfered with insulin secretion. Surprisingly, the cumulative BrdU labelling and fractional area of beta cells of the tacrolimus-treated mice did not significantly differ from those of the control mice, although, the Ki67⁺ beta cells were significantly reduced 4 weeks after transplantation. These data imply only faint antiproliferative effects of tacrolimus that did not lead to a significant reduction of the cumulative BrdU labelling and fractional area of beta cells. The lack of indirect drug actions that interfere with islet engraftment such as the inhibition of islet angiogenesis may also be an explanation.

MMF strongly inhibits inosine-5-monophosphate dehydrogenase (IMPDH), which converts IMP into xanthosine monophosphate, and thus the *de novo* synthesis of guanine nucleotides. Guanine nucleotides are required for the biosynthesis of GTP and GDP, whose ratio regulates both beta cell growth and insulin secretion [21, 22]. In rodent islets, however, the major supply of guanine nucleotides is an IMPDH-independent salvage pathway rather than the *de novo* synthesis pathway [23]. This may explain why MMF did not significantly affect the proliferation of transplanted beta cells. However, it is unclear why MMF-treated mice also presented with impaired glycaemic control. Blood glucose levels not only reflect beta cell function but also may depend on the responsiveness of other cell types relevant for glucose homeostasis. The design of the present study cannot address why MMF-treated mice were as hyperglycaemic as mice treated with immunosuppressants that inhibited beta cell proliferation. The present study, however, suggests that hyperglycaemia in MMF-treated mice was not the result of impaired beta cell proliferation.

In recent clinical trials, the tacrolimus–MMF protocol was shown to improve islet graft survival when compared with treatments using tacrolimus–sirolimus or sirolimus alone [1, 24, 25]. The authors suggested that alloreactivity was better controlled using tacrolimus–MMF. However, they could not exclude the possibility that impaired beta cell replication, poor islet engraftment or the induction of insulin resistance affected their outcomes. In our study, beta cell replication was reduced by sirolimus but not significantly affected by MMF, which may partly explain the superiority of the tacrolimus–MMF protocol.

In our opinion, it is of particular importance to monitor serum drug concentrations in order to draw conclusions on the possible effects of a drug. In principle, we would have preferred to use concentrations that are proven to be equally effective and well tolerated in mice as in humans, as pharmacokinetics and pharmacodynamics differ among species. In particular, it is known that binding of everolimus to

plasma proteins is greater in mice (99.9%) than in humans (70%) [26, 27]. Furthermore, the plasma half-life of everolimus is ~4–8 h in mice compared with ~30 h in humans [26, 28]. By contrast, the binding of sirolimus, tacrolimus and MMF to erythrocytes and plasma proteins is comparable in mice and in humans [29, 30]. Due to the paucity of this information, however, we aimed at serum concentrations of immunosuppressants within the therapeutic ranges for humans, since these are well known and established [24, 25, 28, 31]. To our knowledge, this is the first study in rodent islet transplantation in which the levels of immunosuppressants have been measured.

Our data indicate that at defined serum concentrations everolimus, sirolimus and tacrolimus reduce beta cell replication *in vivo*. The average level of tacrolimus was above the clinical concentration range, which could account for the observed inhibition of insulin secretion and beta cell proliferation. Similar considerations apply to everolimus, the levels of which were also above its clinical concentration range. Notably, however, we found that its derivative sirolimus, whose levels were within the therapeutic range, comparably inhibited beta cell proliferation. Hence, the detrimental impact of everolimus on beta cell replication may not only refer to supratherapeutic levels. Finally, the levels of MMF were at the lower end of its clinical therapeutic range, which may have attenuated antiproliferative effects on beta cells in these mice. Since MMF is commonly used at the upper therapeutic range, the results of our study cannot be applied to the clinical setting. However, we can conclude that MMF at a mean serum concentration of $1.89 \pm 0.62 \mu\text{g/ml}$ does not affect proliferation.

In summary, our results demonstrate that the beta cells of transplanted islets have a strong capacity for self-renewal when they are not affected by immune or toxic insults. The increased proliferation and concurrent improvement in glycaemic control after transplantation support the proposition that proliferation is critical for the preservation of the transplanted beta cell mass. This result emphasises the importance of protecting islet grafts from factors that compromise cell proliferation and viability as early as possible.

In addition, our data indicate that MMF is the only drug among those we tested that did not significantly reduce BrdU and Ki67 labelling of beta cells and total fractional beta cell area compared with control mice. Multiple comparisons of BrdU labelling also point to MMF as the only drug that was comparable relative to control but differed significantly from each of the other immunosuppressants. These data suggest that among the tested drugs MMF is less detrimental for beta cell proliferation. This conclusion, however, should be tempered in view of the fact that the concentrations of MMF, unlike those of the other tested compounds, were at the lower end of its clinical therapeutic range. Hence, we cannot exclude the possibility that in the

clinical setting MMF may also affect beta cell proliferation. Additional studies with inter-drug comparisons and longer follow-up periods should be conducted to verify these results.

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