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Rapamycin does not adversely affect intrahepatic islet engraftment in mice and improves early islet engraftment in humans

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Keywords: islet transplantation, engraftment, rapamycin, human, mouse

Objective: In this study we examined the effect of rapamycin (RAPA), a key component of the immunosuppressive regimen in clinical islet transplantation, on islet engraftment and function in vivo.

Methods and results: Diabetic C57BL/6 or BALB/C recipient mice were transplanted with 350 syngeneic islets through the portal vein (PV-Tx; C57BL/6 n = 60; BALB/C n = 22) and treated with once-daily oral RAPA (1 mg/kg) or vehicle. No differences in post-transplant blood glucose concentrations and glucose tolerance were observed between RAPA- and vehicle-treated mice. The impact of RAPA on human islet engraftment was assessed in 10 patients with type 1 diabetes treated with 0.1 mg/kg/day rapamycin before islet transplantation. Compared to non pre-treated islet transplant recipents (n = 12), RAPA pre-treated patients had increased blood RAPA concentrations ($p = 0.006$) and fasting C-peptide concentrations ($p = 0.005$) in the two weeks post-transplant. RAPA pre-treatment was associated with a reduction in chemokines CCL2 and CCL3 concentrations pre-transplant ($p < 0.01$), and a dampened chemokine response ($p = 0.005$) post-transplant. Concordantly, in vitro RAPA inhibited the secretion of CCL2 and CCL3 by monocytes.

Conclusion: Rapamycin does not adversely affect intrahepatic islet engraftment in the mouse, and potentially improves islet engraftment in humans by an anti-inflammatory mechanism.

Introduction

The Edmonton protocol was undoubtedly a major step forward in the history of islet transplantation.[1-3] Its immunosuppression regimen was largely based on the mTOR inhibitor rapamycin (RAPA), which remains the most frequently used immunosuppressive drug in clinical islet transplant protocols. As time reveals the somewhat disappointing long-term results of islet transplantation with this protocol,[2] a number of publications have appeared, addressing a possible deleterious role of rapamycin on islet or β-cell engraftment, function, survival and regeneration.[4-12]

During engraftment, due to its antiangiogenic activities,[13] RAPA could be detrimental for islet survival after transplantation. Islets are transplanted as single islets or islet clusters that are considered avascular after collagenase digestion and isolation. Although residual endothelial cells in isolated islets may contribute to islet revascularization,[14] adequate intraislet blood flow requires the formation of a functional microvascular network that links engrafted islets to the surrounding tissues.[15] Microvascular perfusion to newly transplanted islets does can take up to two weeks before a functional microvasculature in islet grafts is reestablished.[16-18] A delay in islet revascularization could potentially deprive islets of oxygen and nutrients, resulting in islet cell death. There is mounting evidence that impaired islet revascularization could be an independent factor that limits the success rate of islet transplantation.[17-19] Supporting the potential detrimental effect of RAPA on islet survival after transplantation, Zhang et al. reported that, in a syngeneic mouse model, RAPA adversely affected islet engraftment under the kidney capsule and compromised β-cell function, with reduced intragraft insulin content and decreased vascular density.[6] Cantaluppi et al. reported that RAPA inhibits the outgrowth of endothelial cells from human islet and the formation of capillary-like structures in vitro and in vivo after subcutaneous injection into the *SCID* mouse.[10] Finally, Ben et al. showed that RAPA affects the viability of islets by inducing islet cell apoptosis in vitro.[11]

While vascularization is likely to be important for long term islet function, other factors, such as inflammation at the time of transplantation, appear to affect early engraftment.[20-23] The predominant cell types infiltrating the islets after infusion are neutrophilic granulocytes and monocytes.[24] In this context, we reported that RAPA affects the survival of these cells and has important anti-inflammatory effects.[25] In vitro, RAPA induces monocyte death and in vivo significantly reduce peripheral monocytes, $CD14^+$ and $CD33^+$cells, and platelet counts. A role for RAPA on myeloid lineage survival was confirmed also at the bone marrow

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level where myeloid precursors such as $CD15^+$ and $CD15^+/CD16^+$ were selectively and significantly decreased during RAPA treatment.[25] Therefore, we postulated that RAPA could have a benefical effect on islet survival after transplantation.

The aim of our study was to evaluate the effect of RAPA in vivo on islet engraftment both in mouse and in man. In mice, we used syngeneic models of intraportal islet transplantation. In humans, we examined markers of β cell function and inflammation before and after transplantation in a unique group of pre-treated patients who were pretreated in monotherapy with RAPA before islet tranplantation. Our data indicate that RAPA does not adversely affect intrahepatic islet engraftment in mice and improves early islet engraftment in humans by dampening inflammatory profiles.

Results

RAPA did not significantly affect glucose tolerance in mice. To separate the effect of RAPA on islet engraftment from that on glucose metabolism,[26] we treated normal (n = 7) and diabetic (n = 23) *C57BL/6* mice with once daily oral administration of RAPA (1 mg/kg). Non-fasting glycemia profile was unaffected (**Fig. 1A**). No significant differences in weight, glycemia, glucose tolerance after IVGTT, HOMA-B and HOMA-IR were observed during and after RAPA treatment in normal mice (**Fig. 1B**). In diabetic mice a significant increase in fasting glycemia associated with a decrease of HOMA-B and glucose tolerance were observed during and after treatment with either RAPA or vehicle, suggesting that a worsening of β cell function is a consequence of the hyperglycaemic state itself rather than of RAPA treatment. Since after oral administration RAPA is absorbed via the portal vein reaching high portal concentrations, we also tested the effect of RAPA on the function of islets engrafted in the liver. Diabetic *C57BL/6* recipients were transplanted with syngeneic islets through the portal vein and, after three months, the transplanted mice that were normoglycaemic (non fasting glycemia 143±25 mg/dl, n = 7) were treated with oral RAPA (0.1 mg/kg day 0 to 10; 1.0 mg/kg day 11 to 20) (**Fig. 2**). Blood glucose profile, fasting glycemia and glucose tolerance were not significantly affected by RAPA treatment. Altogether, these data indicate that short to medium term treatment with RAPA does not affect glucose metabolism in mice.

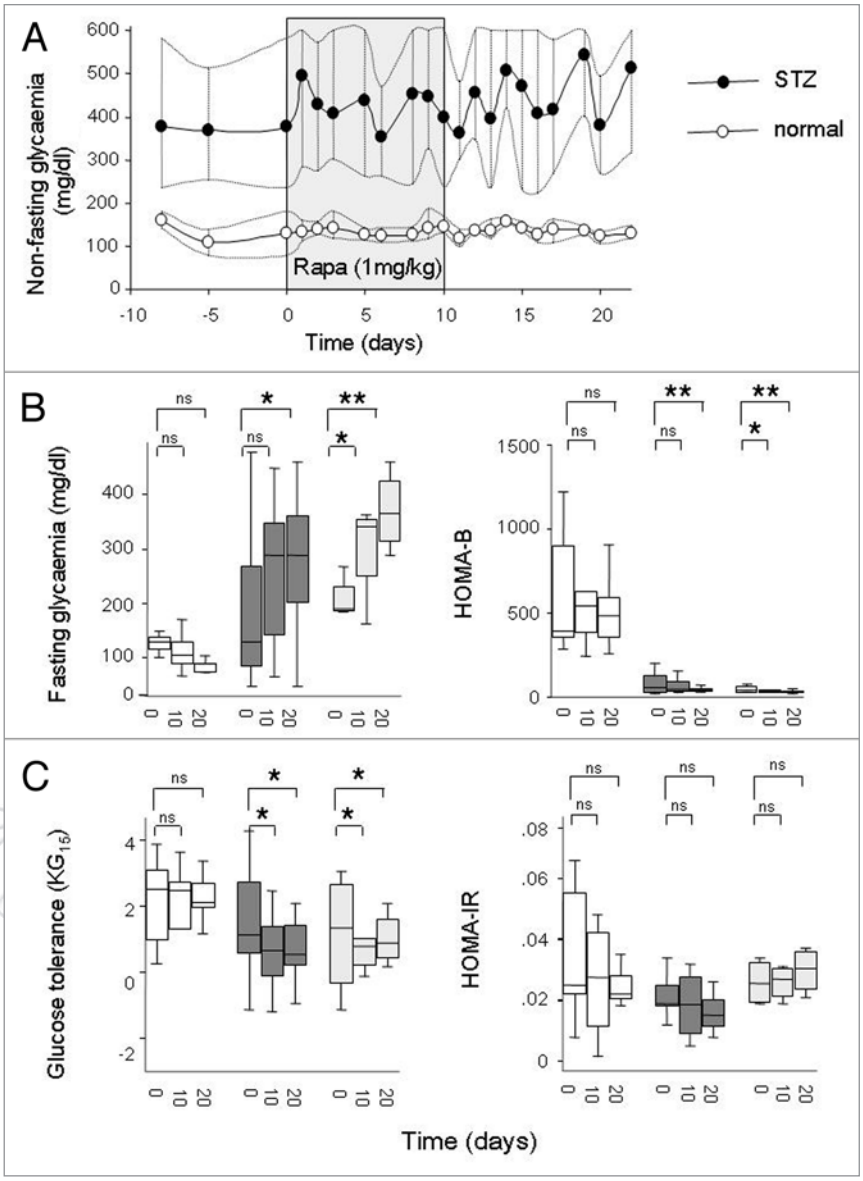

Figure 1. Effect of RAPA treatment on islet function in vivo. (A) blood glucose profile of normal (n = 7) or STZ-induced diabetic (n=23) *C57BL/6* mice before, during and after RAPA treatment (1 mg/kg). Data are expressed as mean and range. (B) and (C) fasting glycemia, glucose tolerance after IVGTT (KG_{15}), β-cell function (HOMA-B) and insulin resistance (HOMA-IR) in normal (white boxplots; n = 7) and STZ-diabetic (dark gray boxplots; n = 23) *C57BL/6* mice before (day 0) during (day +10) and after (day +20) 1 mg/kg RAPA treatment. A group of STZ-diabetic *C57BL/6* mice was treated with vehicle, and are shown as control (light-gray boxplots; n = 7). Statistical analysis was performed by ANOVA with the Dunnett Test. NS=not statistically significant; *p<0.05; **p<0.01.

RAPA did not impair islet engraftment after portal vein injection. To specifically study RAPA effect on islet engraftment, diabetic *C57BL/6* or *BALB/C* recipients were transplanted with 350 syngeneic islets through the portal vein (PV-Tx; *C57BL/6* n = 60; *BALB/C* n=22) and treated with RAPA (1.0 mg/kg) for 14 days (**Fig. 3**). RAPA treatment did not significantly affect the function of islets transplanted in the liver through portal vein injection. In both *C57BL/6* mice and in *BALB/C* mice, RAPA treatment had no effect on post transplant blood glucose profile or glucose tolerance post-transplant. Moreover, this was observed in conditions where blood glucose concentrations were reduced, but still elevated, conditions similar to those of islet transplantation in man.

Table 1. Recipient, donor and islet characteristics in RP and ED groups

		RP group (n = 10)		ED group (n = 12)	
Recipient:					
Age (yr)		36.5 (32-39)		37 (29-41.5)	
Sex (M/F)		6/4		6/6	
Diabetes duration (yr)		24.5 (19-37)		20.5 (14.5-26.5)	
Equivalent islet/kg at first infusion		5694 (4528-6251)		5043 (3909-5828)	
	Time 0	**Pre RAPA**	**Pre Tx**	**Time 0**	**Pre Tx**
Hba1c (%)	8.9 (7.9-9.7)	8.9 (8-9.3)	8.7 (8-9.3)	8.1 (7.4-8.8)	8 (7.8-9)
C-pep (ng/ml)	0.02 (0.02-0.19)	0.02 (0.02-0.3)	0.09 (0.02-0.3)	0.03 (0.01-0.05)	0.06 (0.02-0.16)
EIR (U/kg/die)	0.64 (0.58-0.91)	0.70 (0.62-0.91)	0.58 (0.51-0.77) *	0.64 (0.56-0.82)	0.63 (0.57-0.69)
Weight (kg)	64 (55-70)	64 (55-70)	66 (56-70)	63.5 (59-68.5)	63 (59-68.5)
Crea (ng/ml)	0.79 (0.63-0.95)	0.80 (0.69-0.96)	0.83 (0.72-0.91)	0.81 (0.59-0.95)	0.72 (0.62-082)
Glomerular filtration rate (ml/min)	108 (91-127)	98 (89-115)	102 (94-107)	106 (94-126)	124 (103-134)

Data are expressed as median with 25th and 75th percentiles in parenthesis. *p < 0.05 vs pre RAPA

Effect of RAPA on islet engraftment in vivo in human. RAPA pre-treatment did not affect HbA1c, fasting C-peptide and kidney function during the pre-transplant period (**Table 1**). A small (19%) but significant (p = 0.03) decrease in exogenous insulin requirement (EIR) was observed. RAPA concentrations were higher in the RP group than in the ED group at the time of islet infusion and throughout the two weeks post-transplant (mean RAPA concentration at day 7: 13.8±5 vs 7.8±4 ng/ml, RP and ED group, respectively; p = 0.006; **Fig. 4B**). Target blood RAPA concentrations were not reached in the first week after islet transplantation in the ED group. No difference in blood FK506 concentrations was observed between the two groups. With respect to islet function, fasting C-peptide concentrations were significantly higher during the first two weeks after transplantation in the RP group than in the ED group (p = 0.005). C-peptide concentrations continued to rise in ED group and reached those in the RP group by two weeks post-transplant.

RAPA pre-treatment is associated with a down regulation of the inflammatory markers CCL2/MCP-1 and CCL3/MIP-1α. We monitored the concentrations of CCL2/MCP-1 and CCL3/MIP-1α, two inducible inflammatory chemokines reported to be associated with islet survival after transplantation. Treatment with RAPA prior to transplantation was associated with a decrease in the circulating concentrations of both chemokines after 21 days (CCL2: 62.3±14 pg/ml vs 101±14 pg/ml P=0.03; CCL3: 223±67 pg/ml vs 383±55 pg/ml, p = 0.01; **Fig. 5A**). Post-transplant, there was a further reduction of CCL2 and CCL3 in the RP group, whereas in the ED group there was a transient rise in CCL2 and little or no change in CCL3 concentrations (CCL2 dAUC first week post-transplant: 406±403 vs –856±570 pg/week/ml, for the ED and RP group, respectively; p = 0.05; CCL3 dAUC first week post-transplant: -546±1443 vs –9747±3602 pg/week/ml, for the ED and RP group, respectively; p = 0.05; **Fig. 5B**).

CCL2 and CCL3 are predominantly released by monocytes. To test the hypothesis that RAPA could directly modulate the release of these two chemokines, human monocytes from healthy donors were cultured in the presence of RAPA (0.1–5 ng/mL) after LPS-mediated TLR4 activation. A significant (p < 0.01, n = 6) dose-dependent decrease of CCL2 and CCL3 production was detected starting from drug concentrations of 1 ng/mL (**Fig 6**). Other inflammatory cytokines induced by LPS stimulation, such as IL-1β and IL-6, were unaffected by RAPA treatment, suggesting a selective rather than a general downregulation of inflammatory mediators.

Discussion

Successful islet transplantation depends on the infusion of sufficiently large quantities of islets, but only a fraction of transplanted islets can survive and become engrafted, and yet the underlying mechanism remains partially unclear. In this study, we examined the effect of RAPA, a key component of the immunosuppressive regimen in clinical islet transplantation, on islet engraftment and function in vivo. We demonstrated that RAPA administration to normal, diabetic and euglycaemic islet transplanted mice does not modify glycaemic control, indicating at most a marginal impact of

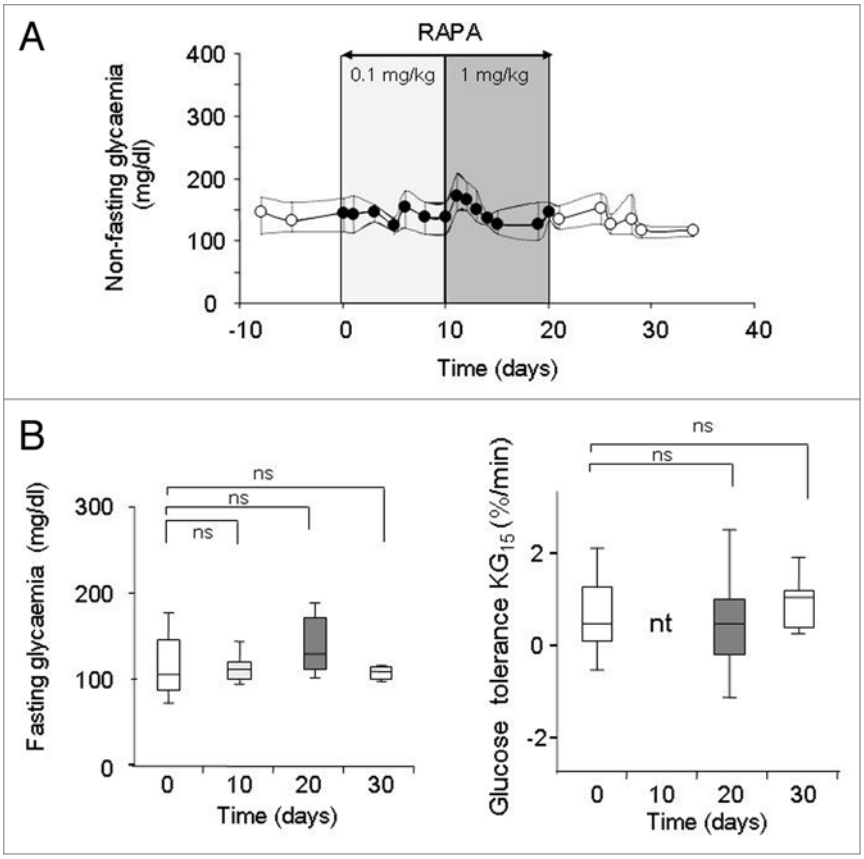

Figure 2. Effect of RAPA treatment on already engrafted islet in vivo. (A) Blood glucose profile of engrafted islet during RAPA treatment. Diabetic *C57BL/6* recipients were transplanted with 350 syngeneic islets through the portal vein. Three months post-transplantation, normoglycaemic transplanted mice (non-fasting glycemia 143±25 mg/dl, n = 7) were treated with once daily oral administration of RAPA at 0.1 mg/kg for 10 days and then 1.0 mg/kg for additional 10 days. Data are expressed as mean and range. (B) Fasting glycemia and glucose tolerance after IVGTT (KG15) in *C57BL/6* mice with engrafted islet before (day 0) during (day +10 0.1mg/kg; day +20 1mg/kg) and after (day +30) RAPA treatment. Blood glucose data are expressed as mean ±SE; glucose tolerance data are expressed as KG15 box plot. NT = not tested. Statistical analysis was performed by ANOVA with the Dunnett Test. NS = not significant. *$p < 0.05$; **$p < 0.01$.

the drug on glucose metabolism in this model. This first observation allow us studying the effect of RAPA on islet engraftment. By using a syngeneic islet transplantation model, we demonstrated that RAPA does not affect islet engraftment after portal vein injection in mice. Finally, in a unique group of patients with type 1 who were treated with RAPA as monotherapy before islet transplantation, we confirmed that RAPA does not impair islet engraftment and was associated with increased C-peptide concentrations in the first two weeks after islet infusion. These findings, together with a blunted inflammatory profile, suggest that pre-treatment with RAPA could improve islet engraftment.

The immunosuppressive mechanism of RAPA is based on the selective blockade of the mammalian target of RAPA activation (mTOR), a molecule known to play a pivotal role in cell cycle progression from late G_1 into S phase in response to T-cell growth factor stimulation.[27,28]

Considering the ubiquitous expression of mTOR in different cell types, the effects of RAPA are not restricted to the immune system, but affect different physiopathological processes involved in tissue repair and neovasculogenesis. Recent reports suggest that RAPA may not be optimal for the survival and revascularization of newly transplanted islets. In contrast to our findings, Zhang et al. reported that RAPA adversely affects islet engraftment in mouse and compromises β-cell function, with reduced intragraft insulin content and decreased vascular density.[6] Similarly Cantaluppi et al. reported the anti-angiogenic activity of RAPA as particularly detrimental in the early engraftment phase of islet in mouse.[10] Of note is that the site of islet implantation in the study of Zhang was the kidney capsule and in the study of Cantaluppi was the subcutaneous tissue. In most rodent models, islets are transplanted under the kidney capsule, whereas islet transplantation in man is performed by percutaneous intrahepatic infusion through the portal vein.[29] Intrahepatic islet infusion is associated with an immediate blood-mediated inflammatory reaction, thrombosis and hepatic tissue ischemia.[20,30-41] Kidney subcapsular transplantation or subcutaneous implantation are not intravascular and are, therefore, devoided of these complications. Thus, the mechanism of engraftment and islet survival are different and, although transplantation under the kidney capsule may be a useful model to study mechanisms of immune tolerance or neovasculogenesis, unlikely provides information relevant to islet engraftment in man.

As we recently reported RAPA plays a role in the physiology and survival of monocyte/myeloid lineage cells.[25] This could have a benefical effect on islet engraftment, since neutrophilic granulocytes and monocytes are the predominant cell types infiltrating the islets after transplantation,[24] exerting direct and indirect cytotoxic effects.[20,21] Furthermore, our study suggests a new mechanism by which RAPA can improve islet survival after transplantation, i.e., the inhibition of CCL2/MCP-1 and CCL3/MIP-1α secretion. CCL2/MCP-1 is a prototypic inflammatory chemokine, which targets monocytes, T lymphocytes and other cells expressing the C-C chemokine receptor (CCR2).[42] Remarkably, CCL2/MCP-1 not only provides chemotactic cues for the recruitment of monocytes from the bloodstream to the inflamed tissue, but it is also responsible for monocyte activation and induction of the respiratory burst.[42] CCL2/MCP-1 has been implicated in the development of acute or chronic inflammation in different scenarios, and our group has previously described a detrimental effect on human islet survival.[23] An involvement of CCL3/MIP-1α in islet survival after transplantation was also previously suggested.[43,44] CCL3/MIP-1α is crucial for T-cell chemotaxis from the circulation to inflamed tissue and also plays an important role in the regulation of

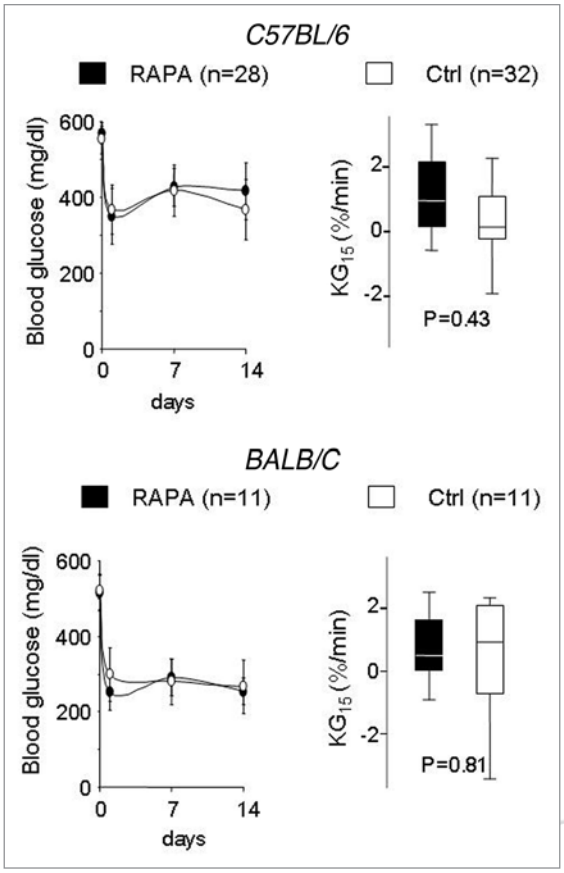

Figure 3 Effect or RAPA on islet engraftment in mice. Diabetic *C57BL/6* or *BALB/C* recipients were transplanted with 350 syngeneic islets through the portal vein (PV-Tx; *C57BL/6* n = 60; *BALB/C* n = 22) After islet transplantation, recipient mice were treated with once-daily oral RAPA at 1.0 mg/kg or vehicle (Ctrl) for 14 days. Blood glucose profile (mean±SE) and two weeks glucose tolerance after IVGTT (KG15) are reported. Comparisons between groups were performed by Mann Whitney test.

transendothelial migration of monocytes and dendritic cells and other cells expressing the C-C chemokine receptors CCR1 and CCR5.[45]

A limitation of our study is the short term follow up data in clinical islet transplant. In fact, the association we report in the present work is between RAPA pre-treatment and increased C-peptide concentrations in the first two weeks after islet infusion. Even if we have evidence that the RP group has maintained a better performance over time (data not shown), some confounding factors do not allow to definitively conclude for a positive role of the RAPA pre-treatment on long-term islet function. In fact RAPA blood level starting from two weeks after the first islet infusion did not differ between RP and ED groups and since many patients in both groups were subsequently reinfused in the presence of RAPA, the direct contribution of first islet infusion on overall function can not be dissected.

In summary, this study indicates that RAPA does not adversely affect intrahepatic islet engraftment in mouse. The lack of detrimental effect of RAPA in animal models permitted us to plan a clinical study with RAPA pre-treatment in human islet recipients showing that RAPA improves islet engraftment in man and is associated with a blunted inflammatory reaction. Thus RAPA treatment appears to be not solely beneficial in controlling lymphocyte responses, but also as an anti-inflammatory therapy at the time of early islet engraftment.

Material and Methods

Islet function in normal and diabetic mice. To address the effect of RAPA on islet function in vivo we treated normal (n = 7) or diabetic (STZ induced, n = 23) *C57BL/6* mice with once daily oral administration of RAPA at the dose of 1.0 mg/kg for 10 days. As control, one group of diabetic *C57BL/6* mice (n = 7) was treated with vehicle. Non-fasting glycemia, fasting glycemia, glucose tolerance after IVGTT (KG_{15}), serum insulin levels, insulin resistance and β cell function (calculated by HOMA) and weight were evaluated at day 0 (before RAPA treatment), at day 10 (last day of RAPA treatment) and at day 20 (ten days after withdrawing RAPA treatment). IVGTT was performed after a 16-hour fast; mice in each experimental group were given glucose (0.5 g/kg) by tail vein bolus injection. Blood samples were obtained at 0, 1, 5, 10, 15, 20, 30 and 60 minutes after glucose injection and were used to measure glucose concentrations. Glucose tolerance was quantified from the glucose elimination constant (KG; expressed as percent elimination of glucose per minute) as the reduction in circulating glucose between 1 and 15 min (KG_{15}) after logarithmic transformation of plasma glucose values.[46] Serum insulin was determined by radiobinding assay (INSIK-5 RIA Kit, Linco Research, St. Charles, MO). The minimal modeling of insulin and glucose data from non-fasting blood sample was used to assess the β-cell function. HOMA provided equations for estimating β-cell function and insulin resistance.[47]

Islet transplantation in mice. Islet isolation and culture. Pancreatic islets were isolated from *C57BL/6* or *BALB/c* mice (nine weeks old, 20–22 g; Charles River, Calco, Italy) by a collagenase digestion method. Briefly, 2 ml of cold Hank's buffer/collagenase type V solution (1 mg/ml; Sigma, St. Louis, MO) was infused into the pancreatic duct in situ, and the removed pancreas was digested at 37°C for 15 min. Islets were purified on a discontinuous Ficoll gradient (Sigma). The islets (250 islet/ml) were cultured free-floating (37 °C, 5% CO_2) in medium RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with L-glutamine (Sigma), penicillin-streptomycin (1000 U/ml-10 mg/ml; Sigma) and 10% (vol/vol) fetal calf serum (HyClone, Celbio, Logan, UT) for 20–24 h before the transplant. Islet purity was >90%.

Recipient mice. Male *C57BL/6* or male *BALB/c* mice (9-week-old, 20–22 g; both from Charles River) were used as recipients. Mice were made diabetic (non-fasting blood glucose levels between 400 and 600 mg/dl) with intravenous STZ injection (175 to 200 mg/kg; Sigma) one to two weeks prior to transplantation. Blood glucose measurements were performed using a Glucometer Elite (Bayer Canada, Toronto, Ontario, Canada). The animals had free access to tap water and pelleted food throughout the course of the study. The local animal ethics committee approved all experiments.

Islet transplantation via the portal vein route.[48] The recipient mice were anesthetized with isoflurane (Forane 1–2%; Abbott

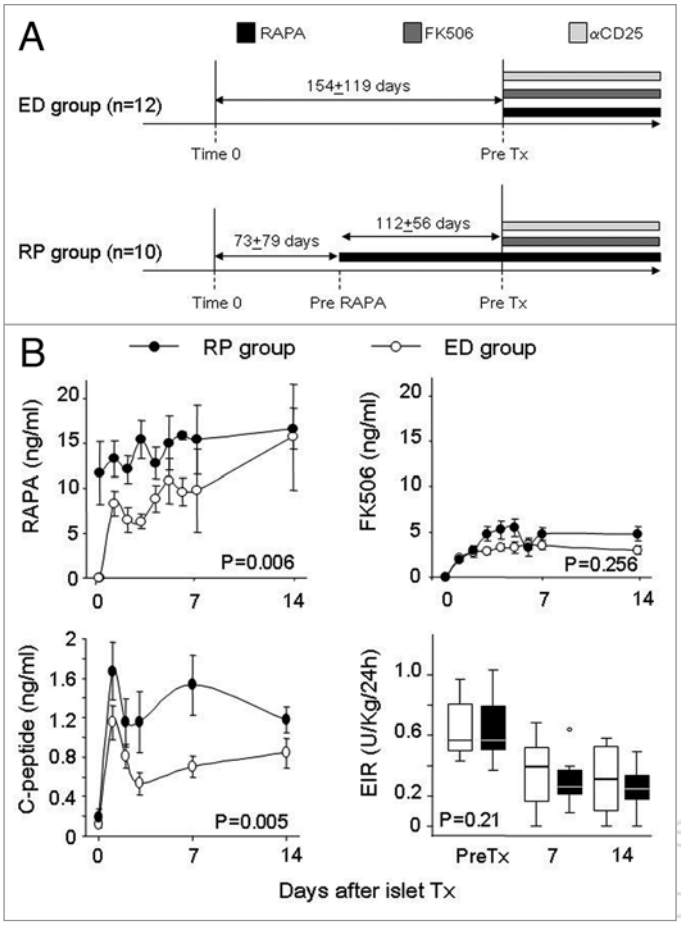

Figure 4. Effect of RAPA treatment on islet engraftment in human. (A) Schematic representation of clinical islet transplantation protocols. Patients received immunosuppression according to the Edmonton protocol (ED group, n = 12) or a preconditioning therapy with rapamycin pre-transplant followed by immunosuppression according to the Edmonton protocol (RP group, n = 10). In the RP cohort, RAPA was given at a dose of 0.1 mg/kg/day for a mean period of 112±56 days. At the time of transplant, both RP and ED groups received: αCD25 as induction therapy (1 mg/kg intravenously, and then every 2 weeks for a total of five doses); FK506 (given orally at an initial dose of 1 mg/Kg, then to maintain serum levels of 3–6 ng/mL); RAPA (given orally at a loading dose of 0.2 mg/kg, followed by a dose of 0.1 mg/kg to achieve a range level of 12–15 ng/mL for the first 90 days, and 7–10 ng/mL thereafter). (B) RAPA blood concentration, FK506 blood concentration, fasting C peptide and EIR in RP and ED groups during 14 days post-islet Tx. Data are expressed as mean±SE and boxplots. Statistical analysis was performed by using mixed-effect models.

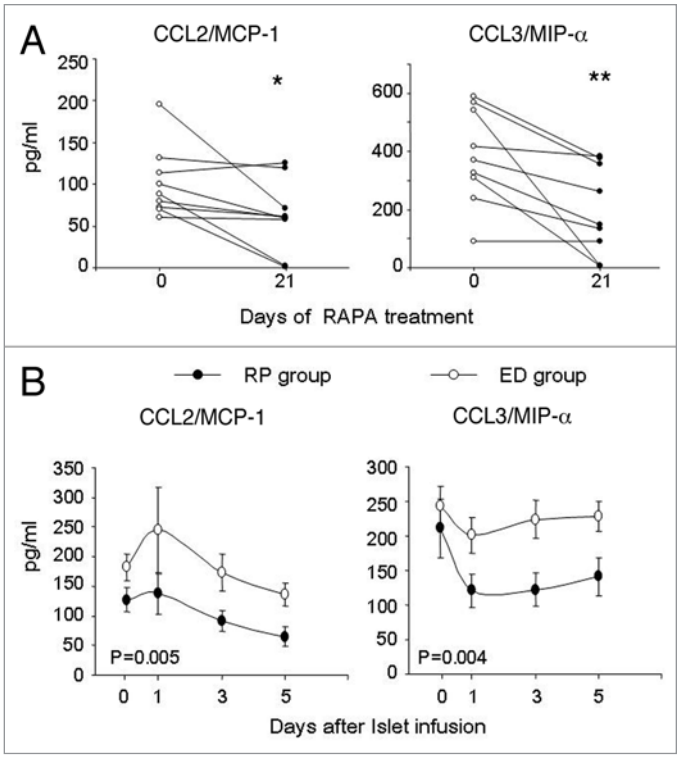

Figure 5 RAPA modulated CCL2/MCP-1 and CCL3/MIP-1α secretion in vivo (A) Blood CCL2/MCP-1 and CCL3/MIP-1α concentrations before and 21 days after RAPA (0.1 mg/kg/day) monotherapy treament in nine patients with type 1 diabetes. The value for each patient are shown and the mean before and after RAPA treatment were compared using the Mann-Whitney test. * p<0.05. ** p<0.01 (B) CCL2/MCP-1 and CCL3/MIP-1α blood concentration after first islet infusion. Data are expressed as mean+SE. Statistical analysis was performed by using mixed-effect models.

Laboratories, Toronto, Canada) and then prepared for abdominal surgery. A 2 cm midline incision was made slightly below the xyphoid. The intestines were gently reflected leftward from the peritoneal cavity onto dampened gauze and the portal vein was exposed. The islets were suspended in 0.3–0.5 ml RPMI media aspirated from a 1.5 ml plastic tube into a heparinized plastic 1ml syringe with a 29-gauge 12.5 mm needle. The needle was then inserted into the exposed portal vein and the plunger was gently advanced to expel the islets into the portal vein. A small piece of gelform was placed directly over the hole as the needle was being removed from the portal vein and then pressure was applied until all bleeding stopped. The peritoneal cavity was closed. Three-hundred-fifty islets were injected.

Immunosuppression. Recipient mice were treated as previously described.[49] The treatment of the transplanted mice began the day before transplant. Rapamycin (Rapamune; Wyeth-Ayerst, Pearl River, NY) was diluted in peanut oil (Sigma) and administered once daily for 14 consecutive days at a dose of 1 mg/kg by gavage.

Evaluation of graft function. Blood sugar levels were measured 15, 30 and 60 minutes after the end of surgical procedure, daily for the first week and then every second day post-transplantation. Surgical death was defined as death within the first seven days after transplantation. In syngeneic models, recipient mice were classified as having full function if non-fasting blood glucose concentrations were less than 50% of pre-transplant concentration for at least one week. An intravenous glucose tolerance test (IVGTT) was performed 2 weeks after transplantation to demonstrate the function of the grafted islets.

Islet transplants in patients with type 1 diabetes. From January 2001 to March 2005, 22 patients with type 1 diabetes received islet transplantation and were immunosuppressed by either the Edmonton protocol (ED, n = 12) or a protocol that included RAPA administration before the first islet infusion (RP;

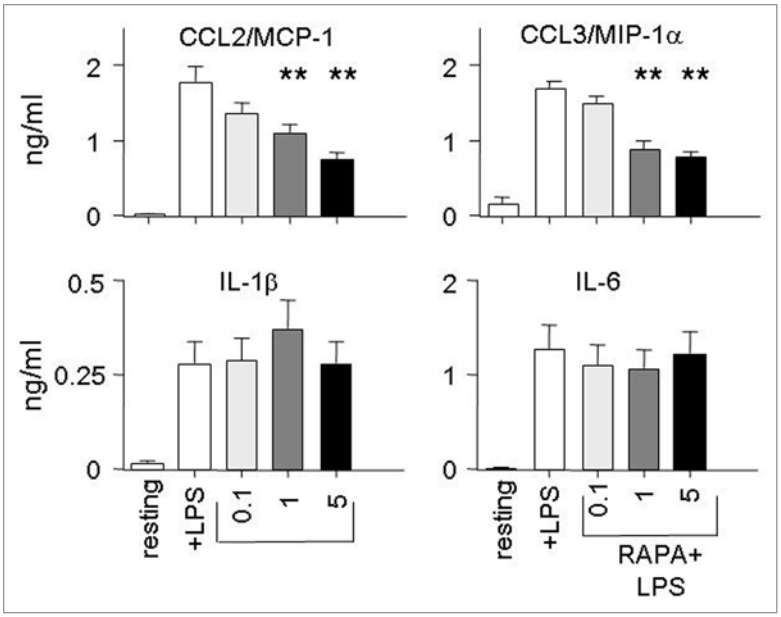

Figure 6 RAPA modulated CCL2/MCP-1 and CCL3/MIP-1α secretion in vitro CCL2/MCP-1, CCL3/MIP-1α, IL-1β and IL-6 secretion by freshly isolated monocytes after TLR4 stimulation. Human monocyte cells were isolated by Ficoll/Percoll gradients and adherence. Freshly isolated monocytes (1x10^6/ml CD14$^+$/CD68) were cultured with RAPA (0–5 ng/ml) in the presence of LPS for 24 h. CCL2/MCP-1, CCL3/MIP-1α, IL-1β and IL-6 concentration were analyzed in the supernatants. Results are expressed as mean ± SD (n = 6). Statistical analysis was performed by ANOVA with the Dunnett Test. *p < 0.05; **p < 0.01

n = 10). All patients had reduced awareness of hypoglycemia, brittle diabetes or progressive complications, despite optimization of insulin therapy. Patients in the RP group received RAPA (Rapamune, Wyeth-Ayerst) orally at least one month prior to the transplant, at a dose of 0.1 mg/kg/day. At the time of transplant, both RP and ED groups received Daclizumab (Zenapax, Roche; 1 mg/kg intravenously) as induction therapy, which was repeated every 2 weeks for a total of 5 doses; FK506 (Prograf, Fujisawa, Japan) was given orally at an initial dose of 1 mg/kg, then to maintain serum levels of 3–6 ng/ml; RAPA was given orally at a loading dose of 0.2 mg/kg, followed by a dose of 0.1 mg/kg to achieve levels within the range of 12–15 ng/ml for the first 90 days, and 7–10 ng/ml thereafter. Patients in the ED and RP groups were similar with respect to age, gender, weight, duration of diabetes, number of islet infusions received, and number of islet equivalents per kg of body weight received (**Table 1**). The ethical committee of the Scientific Institute San Raffaele approved the protocol, and all patients signed an informed consent before entering the study.

Monocyte culture. Highly enriched monocytes (>95% CD14$^+$) were obtained from buffy coats of blood donors (through the courtesy of Centro Trasfusionale, Ospedale San Raffaele, Milan, Italy) using Ficoll and Percoll gradients and purified by adherence. Monocytes were cultured at 10^6/mL in six-well multiwell tissue culture plates (Falcon, Becton Dickinson, Somerville, NJ, USA) in RPMI (Biochrom, Berlin, Germany) 10% FCS (Hyclone, Logan, UT) with or without RAPA. For Toll-Like Receptor 4 (TLR4) activation, LPS 10 ng/mL was added for 24 hr of culture. All cultures were tested for the presence of endotoxin (<0.03 U/mL; Lymulus Test).

Chemokine and cytokine assays. Chemokines and cytokines were measured by SearchLight Human Proteoma Array (Pierce Endogen, Woburn, MA) according to the manufacturer's instructions.

Statistical analysis. General characteristics of study participants were described using summary statistics. Comparison between two groups was conducted using paired or unpaired Student t-test, Mann-Whitney test, as appropriate. Comparisons between more than two groups was conducted using analysis of variance or Kruskall-Wallis test, as appropriate.

Longitudinal data such as rapamycin, FK506, fasting C-peptide and cytokines levels overtime were analyzed using mixed-effect models. Treatment group and time were included in the models and the interaction between treatment and time also was assessed. For all analyses, a two-tailed P value of 0.05 was considered significant. Statistical analyses were performed using Stata, version 10 (Stata Corporation, College Station, TX).

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References

1. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 2000; 343:230-8.
2. Ryan EA, Paty BW, Senior PA, Bigam D, Alfadhli E, Kneteman NM, et al. Five-year follow-up after clinical islet transplantation. Diabetes 2005; 54:2060-9.
3. Shapiro AM, Ricordi C, Hering BJ, Auchincloss H, Lindblad R, Robertson RP, et al. International trial of the edmonton protocol for islet transplantation. N Engl J Med 2006; 355:1318-30.
4. Laugharne M, Cross S, Richards S, Dawson C, Ilchyshyn L, Saleem M, et al. Sirolimus toxicity and vascular endothelial growth factor release from islet and renal cell lines. Transplantation 2007; 83:1635-8.
5. Bussiere CT, Lakey JR, Shapiro AM, Korbutt GS. The impact of the mTOR inhibitor sirolimus on the proliferation and function of pancreatic islets and ductal cells. Diabetologia 2006; 49:2341-9.
6. Zhang N, Su D, Qu S, Tse T, Bottino R, Balamurugan AN, et al. Sirolimus is associated with reduced islet engraftment and impaired beta-cell function. Diabetes 2006; 55:2429-36.
7. McDaniel ML, Marshall CA, Pappan KL, Kwon G. Metabolic and autocrine regulation of the mammalian target of rapamycin by pancreatic beta-cells. Diabetes 2002; 51:2877-85.
8. Cross SE, Richards SK, Clark A, Benest AV, Bates DO, Mathieson PW, et al. Vascular endothelial growth factor as a survival factor for human islets: effect of immunosuppressive drugs. Diabetologia 2007; 50:1423-32.
9. Kneteman NM, Lakey JR, Wagner T, Finegood D. The metabolic impact of rapamycin (sirolimus) in chronic canine islet graft recipients. Transplantation 1996; 61:1206-10.
10. Cantaluppi V, Biancone L, Romanazzi GM, Figliolini F, Beltramo S, Ninniri MS, et al. Antiangiogenic and immunomodulatory effects of rapamycin on islet endothelium: Relevance for islet transplantation. Am J Transplant 2006; 6:2601-11.
11. Bell E, Cao X, Moibi JA, Greene SR, Young R, Trucco M, et al. Rapamycin has a deleterious effect on MIN-6 cells and rat and human islets. Diabetes 2003; 52:2731-9.
12. Zahr E, Molano RD, Pileggi A, Ichii H, Jose SS, Bocca N, et al. Rapamycin impairs in vivo proliferation of islet beta-cells. Transplantation 2007; 84:1576-83.

13. Guba M, von Breitenbuch P, Steinbauer M, Koehl G, Flegel S, Hornung M, Bruns CJ, et al. Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: Involvement of vascular endothelial growth factor. Nat Med 2002; 8:128-35.
14. Linn T, Schneider K, Hammes HP, Preissner KT, Brandhorst H, Morgenstern E, et al. Angiogenic capacity of endothelial cells in islets of langerhans. FASEB J 2003; 17:881-3.
15. Lai Y, Schneider D, Kidszun A, Hauck-Schmalenberger I, Breier G, Brandhorst D, et al. Vascular endothelial growth factor increases functional beta-cell mass by improvement of angiogenesis of isolated human and murine pancreatic islets. Transplantation 2005; 79:1530-6.
16. Menger MD, Yamauchi J, Vollmar B. Revascularization and microcirculation of freely grafted islets of langerhans. World J Surg 2001; 25:509-15.
17. Mattsson G, Jansson L, Nordin A, Carlsson PO. Impaired revascularization of transplanted mouse pancreatic islets is chronic and glucose-independent. Transplantation 2003; 75:736-9.
18. Jansson L, Carlsson PO. Graft vascular function after transplantation of pancreatic islets. Diabetologia 2002; 45:749-63.
19. Zhang N, Richter A, Suriawinata J, Harbaran S, Altomonte J, Cong L, et al. Elevated vascular endothelial growth factor production in islets improves islet graft vascularization. Diabetes 2004; 53:963-70.
20. Barshes NR, Wyllie S, Goss JA. Inflammation-mediated dysfunction and apoptosis in pancreatic islet transplantation: Implications for intrahepatic grafts. J Leukoc Biol 2005; 77:587-97.
21. Montolio M, Biarnes M, Tellez N, Escoriza J, Soler J, Montanya E. Interleukin-1beta and inducible form of nitric oxide synthase expression in early syngeneic islet transplantation. J Endocrinol 2007; 192:169-77.
22. Bottino R, Fernandez LA, Ricordi C, Lehmann R, Tsan MF, Oliver R, et al. Transplantation of allogeneic islets of langerhans in the rat liver: Effects of macrophage depletion on graft survival and microenvironment activation. Diabetes 1998; 47:316-23.
23. Piemonti L, Leone BE, Nano R, Saccani A, Monti P, Maffi P, et al. Human pancreatic islets produce and secrete MCP-1/CCL2: Relevance in human islet transplantation. Diabetes 2002; 51:55-65.
24. Moberg L, Korsgren O, Nilsson B. Neutrophilic granulocytes are the predominant cell type infiltrating pancreatic islets in contact with ABO-compatible blood. Clin Exp Immunol 2005; 142:125-31.
25. Mercalli A, Sordi V, Ponzoni M, Maffi P, De Taddeo F, Gatti G, et al. Rapamycin induces a caspase-independent cell death in human monocytes. Am J Transplant 2006; 6:1331-41.
26. Larsen JL, Bennett RG, Burkman T, Ramirez AL, Yamamoto S, Gulizia J, et al. Tacrolimus and sirolimus cause insulin resistance in normal sprague dawley rats. Transplantation 2006; 82:466-70.
27. Sehgal SN. Rapamune (sirolimus, rapamycin): An overview and mechanism of action. Ther Drug Monit 1995; 17:660-5.
28. Raught B, Gingras AC, Sonenberg N. The target of rapamycin (TOR) proteins. Proc Natl Acad Sci USA 2001; 98:7037-44.
29. Venturini M, Angeli E, Maffi P, Fiorina P, Bertuzzi F, Salvioni M, et al. Technique, complications, and therapeutic efficacy of percutaneous transplantation of human pancreatic islet cells in type 1 diabetes: The role of US. Radiology 2005; 234:617-24.
30. Yin D, Ding JW, Shen J, Ma L, Hara M, Chong AS. Liver ischemia contributes to early islet failure following intraportal transplantation: Benefits of liver ischemic-preconditioning. Am J Transplant 2006; 6:60-8.
31. Sudo T, Hiyama E, Murakami Y, Yokoyama Y, Takesue Y, Sueda T. Hepatic regeneration promotes engraftment of intraportally transplanted islet cells. Surgery 2005; 137:612-9.
32. Bhargava R, Senior PA, Ackerman TE, Ryan EA, Paty BW, Lakey JR, et al. Prevalence of hepatic steatosis after islet transplantation and its relation to graft function. Diabetes 2004; 53:1311-7.
33. Eckhard M, Lommel D, Hackstein N, Winter D, Ziegler A, Rau W, et al. Disseminated periportal fatty degeneration after allogeneic intraportal islet transplantation in a patient with type 1 diabetes mellitus: A case report. Transplant Proc 2004; 36:1111-6.
34. Barshes NR, Lee TC, Goodpastor SE, Balkrishnan R, Schock AP, Mote A, et al. Transaminitis after pancreatic islet transplantation. J Am Coll Surg 2005; 200:353-61.
35. Shapiro AM, Gallant HL, Hao EG, Lakey JR, McCready T, Rajotte RV, et al. The portal immunosuppressive storm: relevance to islet transplantation? Ther Drug Monit 2005; 27:35-7.
36. Contreras JL, Eckstein C, Smyth CA, Bilbao G, Vilatoba M, Ringland SE, et al. Activated protein C preserves functional islet mass after intraportal transplantation: a novel link between endothelial cell activation, thrombosis, inflammation, and islet cell death. Diabetes 2004; 53:2804-14.
37. Johansson H, Lukinius A, Moberg L, Lundgren T, Berne C, Foss A, et al. Tissue factor produced by the endocrine cells of the islets of langerhans is associated with a negative outcome of clinical islet transplantation. Diabetes 2005; 54:1755-62.
38. Moberg L, Johansson H, Lukinius A, Berne C, Foss A, Kallen R, et al. Production of tissue factor by pancreatic islet cells as a trigger of detrimental thrombotic reactions in clinical islet transplantation. Lancet 2002; 360:2039-45.
39. Bertuzzi F, Marzorati S, Maffi P, Piemonti L, Melzi R, de Taddeo F, et al. Tissue factor and CCL2/monocyte chemoattractant protein-1 released by human islets affect islet engraftment in type 1 diabetic recipients. J Clin Endocrinol Metab 2004; 89:5724-8.
40. Rafael E, Ryan EA, Paty BW, Oberholzer J, Imes S, Senior P, et al. Changes in liver enzymes after clinical islet transplantation. Transplantation 2003; 76:1280-4.
41. Hyon SH, Ceballos MC, Barbich M, Groppa R, Grosembacher L, Vieiro MM, et al. Effect of the embolization of completely unpurified islets on portal vein pressure and hepatic biochemistry in clinical practice. Cell Transplant 2004; 13:61-5.
42. Rollins BJ, Sunday ME. Suppression of tumor formation in vivo by expression of the JE gene in malignant cells. Mol Cell Biol 1991; 11:3125-31.
43. Lewis EC, Shapiro L, Bowers OJ, Dinarello CA. Alpha1-antitrypsin monotherapy prolongs islet allograft survival in mice. Proc Natl Acad Sci USA 2005; 102:12153-8.
44. Solomon MF, Kuziel WA, Simeonovic CJ. The contribution of chemokines and chemokine receptors to the rejection of fetal proislet allografts. Cell Transplant 2004; 13:503-14.
45. Maurer M, von Stebut E. Macrophage inflammatory protein-1. Int J Biochem Cell Biol 2004; 36:1882-6.
46. Pacini G, Thomaseth K, Ahren B. Contribution to glucose tolerance of insulin-independent vs. insulin-dependent mechanisms in mice. Am J Physiol Endocrinol Metab 2001; 281:E693-703.
47. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985; 28:412-9.
48. Melzi R, Sanvito F, Mercalli A, Andralojc K, Bonifacio E, Piemonti L. Intrahepatic islet transplant in the mouse: functional and morphological characterization. Cell Transplant 2008; 17:1361-70.
49. Battaglia M, Stabilini A, Draghici E, Gregori S, Mocchetti C, Bonifacio E, et al. Rapamycin and interleukin-10 treatment induces T regulatory type 1 cells that mediate antigen-specific transplantation tolerance. Diabetes 2006; 55:40-9.